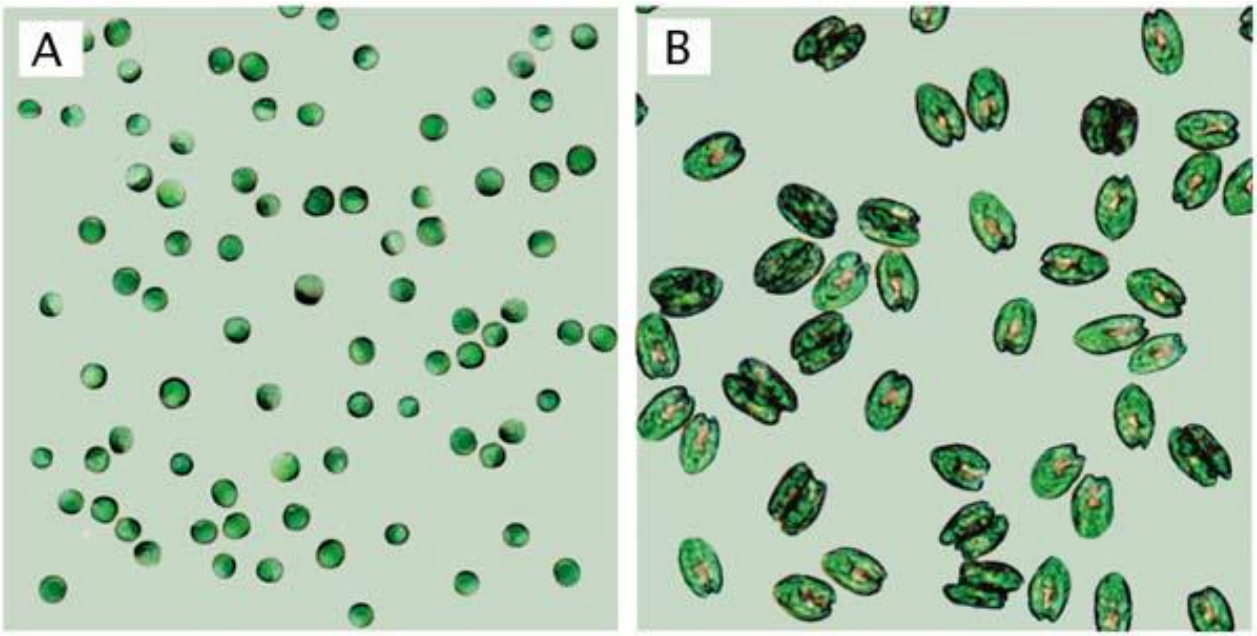
A comparative study of the growth rate and fatty acid content of *I. Galbana*, *T. Suecica* and a mix of these under different light intensities



Photomicrographs of *Isochrysis* sp. (A) and *Tetraselmis* sp. (B) showing the relative difference in cell size. (FAO)

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1. Abstract

Monocultures of marine microalgae *Isochrysis galbana* and *Tetraselmis suecica* are often used as fodder for copepods, which are then fed to fish in recirculating aquatic systems. It has been shown that by feeding copepods on a mixed algal diet growth is often greater than when each of the species of the diet is fed individually. In our study the effects of different light intensities (10-200 µmol photons m^{-2} s^{-1}) on growth performance and FA profile (with emphasis on polyunsaturated FAs) of the mixed culture of *I. galbana* and *T. suecica*, grown in batch cultures under laboratory conditions were compared with those in monoculture conditions. The maximum specific growth rates reached for *I. galbana*, *T. suecica* and mixed culture were 0,576 day^{-1} (80 µmol photons m^{-2} s^{-1}), 1.008 (60/100 µmol photons m^{-2} s^{-1}) and 0,216 day^{-1} (5 different light irradiances), respectively. The increase in growth rate of *T. suecica* along the increasing irradiances was more significant than that of *I. galbana* and the mixed culture which had the lowest growth rate of all three. Growth became light saturated at 60 µmol photons m^{-2} s^{-1} for *I. galbana* and *T. suecica*, whereas growth rate of mixed culture was almost stable after 20 µmol photons m^{-2} s^{-1}. *T. suecica* grew more efficiently and seemed to suppress the natural growth of *I. galbana* in the mixed experiment with increasing light irradiance. *T. suecica* can be therefore considered as the dominant species. FA productivity was lowest in *I. galbana* at all light intensities. *T. suecica* yielded the highest FA productivity among the three cultures at 10, 20, 80 and 100 µmol photons m^{-2} s^{-1} and mixed culture at all other light irradiances. The greater docosahexaenoic acid (DHA) content was found in *I. galbana*, whereas *T. suecica* contained greater concentrations of α-Linolenic acid (ALA), linoleic acid (LA) and eicosapentaenoic acid (EPA). The content of polyunsaturated FAs (PUFAs) essential to copepods were increasing with increasing light intensity for *I. galbana* and *T. suecica* (P-value<0,05). In the mixed culture DHA was abundant at low light intensities, whereas greater concentrations of ALA, LA and EPA were achieved above 60 µmol photons m^{-2} s^{-1} and simultaneously DHA was reduced to insignificant amounts. All in all, our conclusion is that it is better to grow monocultures of *I. galbana* and *T. suecica* at their most optimal growth conditions separately and use a mix of both as ideal feed for copepods instead of growing them in a mixed culture.

2. Hypotheses and objectives

Our main hypotheses are:

1. We expect that the growth rate of the mixed culture will be higher than the mean growth rate of the two different algal cultures grown as monocultures.

2. We surmise that the growth rate will be the highest in medium irradiance (aprox. 60 - 100 μmol photons m^{-2} s^{-1}) for all cultures.

3. We think that Tetraselmis will be dominating in terms of cell density at the end of each growth period for the mixed culture compared to the initial cell count.

4. We predict that the PUFA concentration of the mixed culture would be higher and more varied than in the least rich and diverse monoculture and that it would follow the trend of the most abundant (in biomass) algae.

5. We hypothesise that the percentage of PUFAs in total fatty acids will be highest in medium irradiances (aprox. 60-100 μmol photons m^{-2} s^{-1}) for all cultures.

Although the growth and fatty acid (FA) profiles of both species used in this work (*T. suecica* and *I. galbana*) have already been characterized, intra-specific variability is common in microalgae, resulting in different growth rates and FA profiles. This might also be explained by exposure to diverse abiotic factors (e.g., temperature, salinity, pH, irradiance) that are known to influence the growth and FA content of microalgae (Vu et al., 2016; H. Pereira et al., 2012).

Against this background, the objective of this work is to assess the performance of a mixed culture of *T. suecica* and *I. galbana* compared with their monocultures to evaluate whether a mixed culture of these species (and possibly other microalgae species) is a resource- and cost-effective production method for microalgae in commercial aquaculture. The results are presented in terms of growth, cell density and FA content and discussed particularly from an aquaculture viewpoint.

3. Introduction

3.1 The need for Aquaculture

Taking world population growth, rising fish consumption and overfishing into account the need for aquaculture becomes obvious. Figure 3.1 shows the state of world marine stocks since 1974 and in 2013 only 10% of the worlds marine fish stocks are underfished and more than 30% is today already overfished. UN predicts that world population will grow from 7.3 billion today (2016) to 9.7 billion in 2050. With no way of expanding our wild fish catches we only have one option if we want to continue eating fish and that is aquaculture. One way of putting it is that fish production has to undergo the same intensification as our production of livestock in agriculture has gone through in the 20th century.

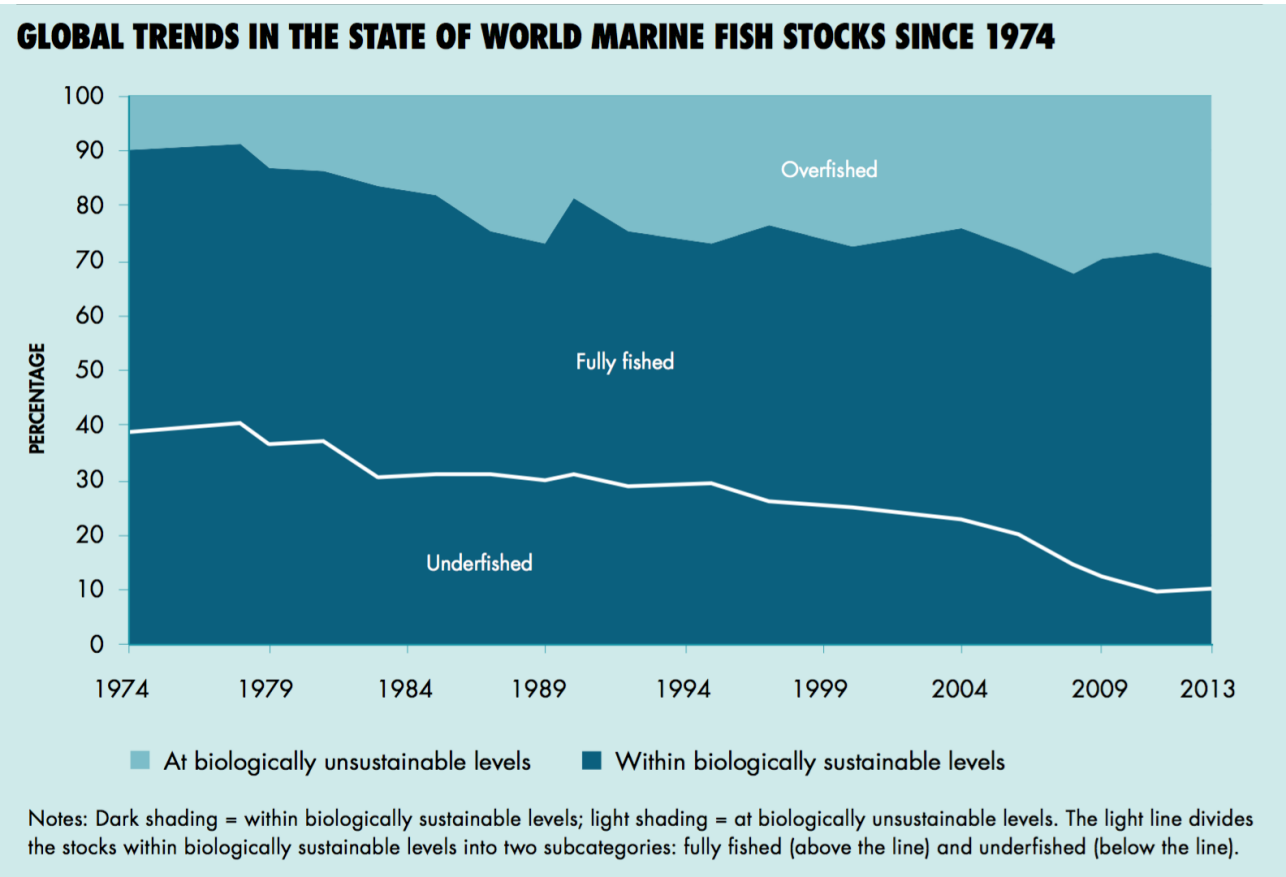


Figure 3.1 The state of world marine fish stocks (FAO, 2016).

Aquaculture is already playing a mayor and still growing role in world fish production at present, while wild captures have been stable since the mid 80'ies at around 90 million tonnes, aquaculture has been growing to almost 45% of total world fish production (Figure 3.2). As the industry is growing it is becoming more and more clear that a sustainable solution is needed.

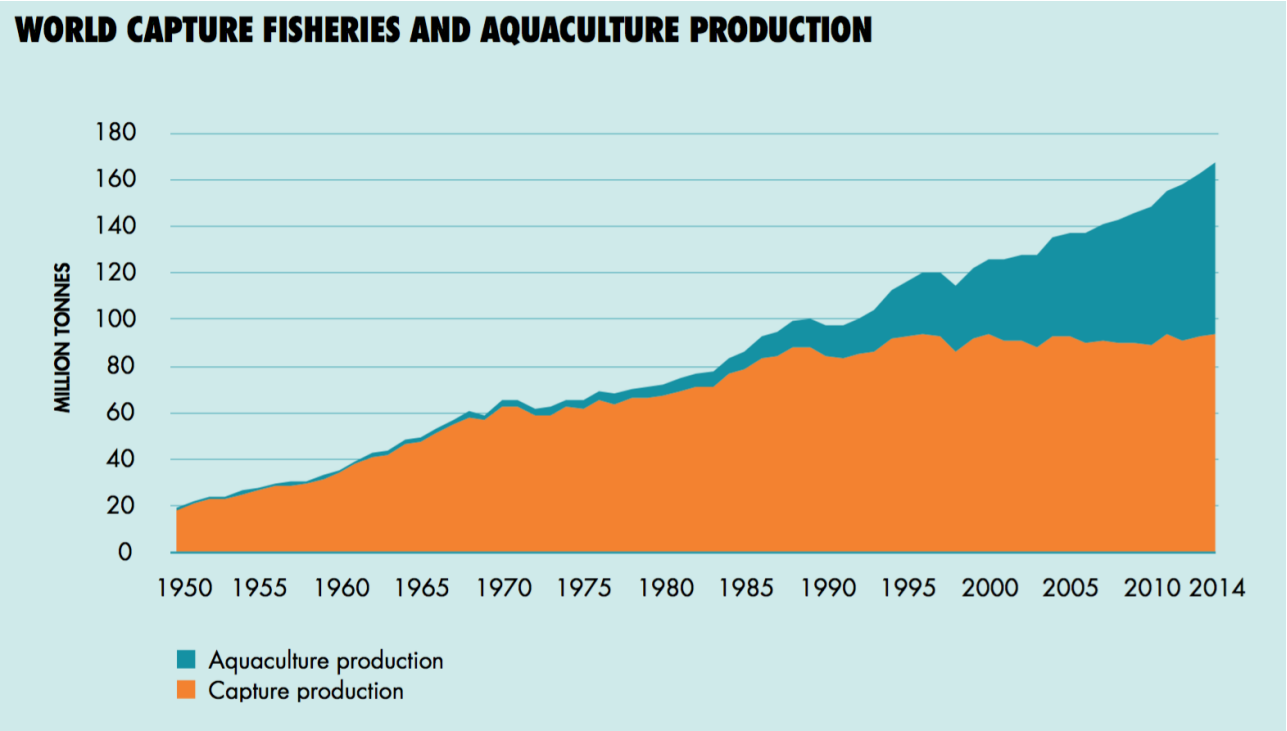


Figure 3.2 World capture fisheries and aquaculture production (FAO, 2016).

3.1.1 Sustainable Aquaculture

Aquaculture poses many problems for the environment and local communities' food security. Figure 3.3, summarizes the main environmental threats in open ocean aquacultural systems. One is the great amounts of dissolved effluents that are produced which contaminate the water and sea floor, also on larger scale due to dilution. Fish excrete mainly contain compounds of nitrogen and phosphorus. The amount of excreted N, P and faecal matter in one year from one fish farm can be similar to untreated wastewater from tens of thousands of humans but on a much smaller area. What is more, farm waste is rarely controlled or treated but enters directly into the sea. Due to increase in organic nutrient-rich material, microbial processes are enhanced which can lead to deficiency of oxygen (Holmer & Kristensen, 1992).

This nutrient rich environment often results in eutrophication, which promotes growth of aquatic plants and algae. Such algal blooms lead to reduction of dissolved oxygen and poisoning which in the end can kill the farmed fish. Other effects of blooms are foaming, dissipation of light ending in retarded growth of seaweeds and sea grasses and thus creation of disturbance in the balance of organisms (Hjorth, Dahllof, & Forbes, 2006; Maraldo & Dahllöf, 2004).

Other effects are release of medicines, pesticides, herbicides and chemicals that are used to treat fish to make them grow faster or prevent diseases and unwanted sea organisms from growing on cages. These compounds can then be accumulated through the food chain. Top predators can end up bioaccumulating very high levels of these substances and pass it on to humans when they are consumed.

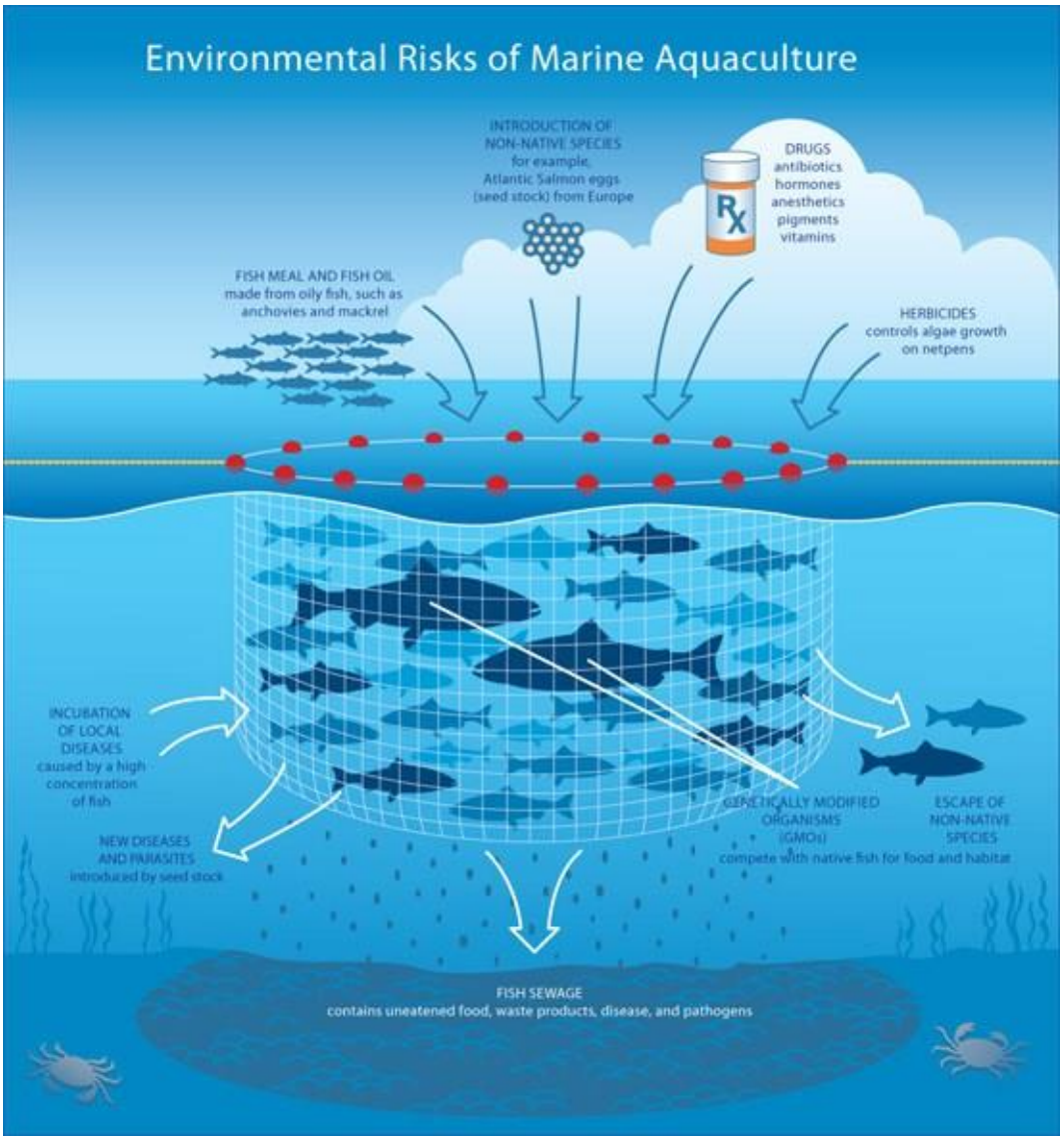


Figure 3.3 Summarises the main problems in most aquaculture production today (FAO, 2016) .

One of the more promising sustainable solutions in fish farming is land based recirculating aquaculture systems (RAS). When moving aquaculture from open ocean to land based systems it becomes possible to control the many negative impacts on the ecosystem. In RAS it is possible to control all inputs and outputs in fish farms and recirculate and clean the wastewater, reduce the need for antibiotics, pesticides and herbicides considerably and since it is land based there is no risk of fish escaping and mixing with a wild population. The biggest disadvantage of RAS is initially the cost compared to open systems. Furthermore, RAS need to be controlled and monitored continuously since even a small failure in the system can have a catastrophic outcome for the production (Nazar, Jayakumar, & Tamilmani, 2013). One example of a RAS already in production from Akvagroup$_{TM}$ is shown in figure 3.4.

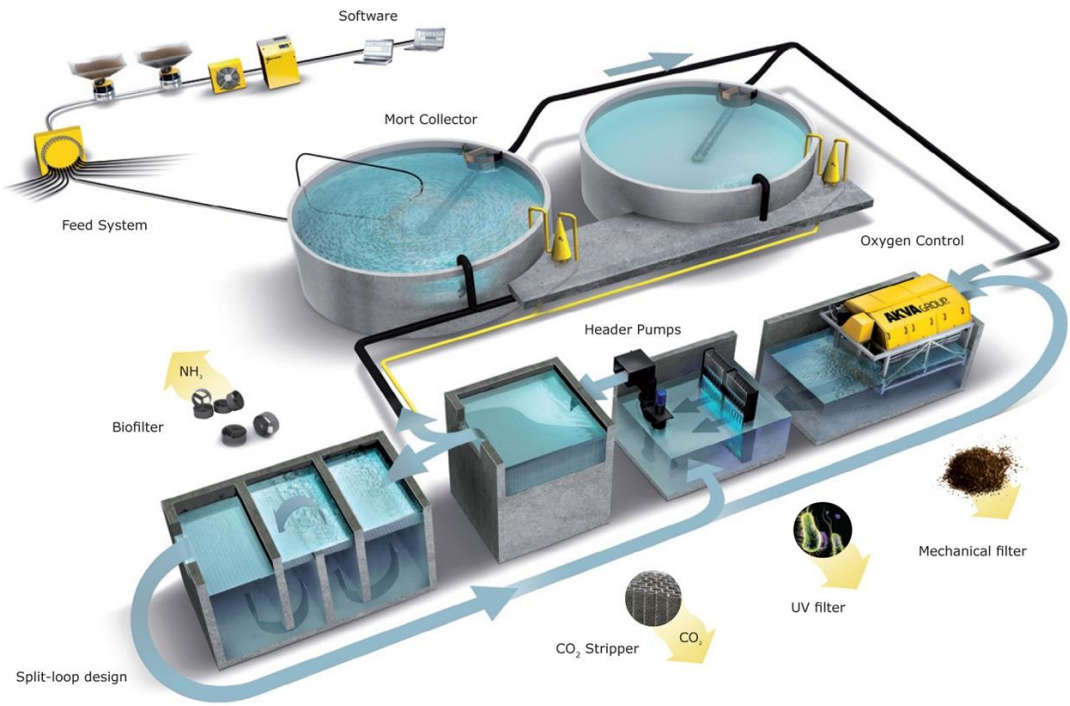


Figure 3.4 Recirculating Aquaculture System (RAS) from Akvagroup$_{TM}$ (http://www.akvagroup.com/products/land-based-aquaculture/recirculation-systems)

However the focus of this report is not to improve fish farming systems but is concentrated on finding a more sustainable production of fish feed that could be coupled with RAS. With the development of the RAS it has become possible to feed aqua cultural fish with live feeds such as copepods, which for obvious reasons is impossible in an open ocean system, since there is now way of keeping the live feed inside the nets. RAS with live feed has also made it possible to extend the amount of fish species raised in aquaculture because that many fish larvae demand live feed as copepods, *Artemia* or rotifers to survive and grow. Mimicking the natural food chain by producing algae, feeding them to small crustaceans, like copepods, which are then fed to fish larvae and juvenile fish is a way of ensuring the essential PUFAs in aquaculture-fish and at the same time reducing the negative impacts of fodder production. Many different parameters affect the growth and biochemical composition of microalgae, therefore light conditions, temperature, nutrients, pH and salinity can be adjusted to reach the optimal growing conditions for algae production.

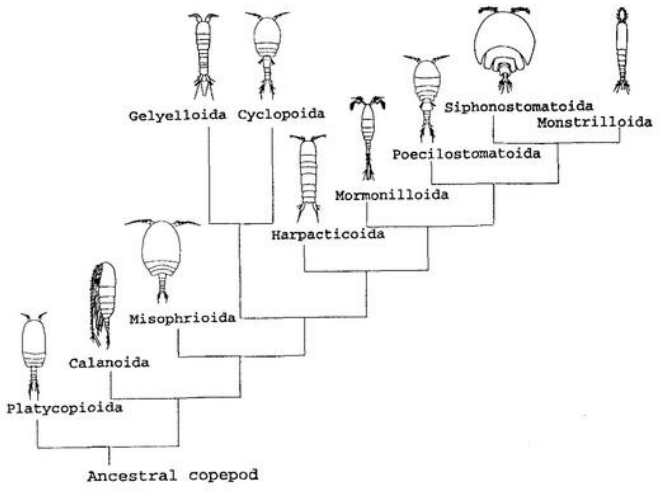
3.1.2 Fish feed in aquaculture

Fishmeal and fish oil are still the most common contributors of essential nutrients (especially PUFAs) to aquaculture feeds. Though, fishmeal and fish oil are becoming a limiting factor because of overfishing and weather oscillations caused by events like El Niño (FAO 2016: 70). Many marine fish species and crustaceans require PUFAs, especially DHA and EPA, for early larval stage development and these FAs are very common in zooplanktonic animals. Rotifers, brine shrimp (*Artemia)* and copepods are therefore used as live feed in hatchery rearing of almost all marine fin fish (Dhont et. al. 2013).

Rotifers (especially *Brachionus spp*.) have been use since the 1970's hence their many attributes. They are suitable in size for many marine fish, because these are very small when they hatch, they are easy to culture in high densities, have a high reproductive rate and are very tolerant to fluctuations in temperature and salinity. In addition, they are filter feeders, meaning that they can be fed on microalgae and yeast (combinations also), and are susceptible to 'enrichment', which means that their biochemical profiles/content can change relatively fast after food intake i.e. that this can be done just before harvest to insure a good nutritional value. But a drawback for the enrichment of rotifers is that the effect will not last for long. Other disadvantages for Rotifer cultures are that these are prone to collapses caused by pathogenic bacteria, rotifer birnarvirus and fungus attacks. A consequence of this is that cultures have to be backed up in different sizes and kept cultured year-round, which leads to extra costs (Dhont et al., 2013).

Artemia is a primitive arthropod of the phylum Crustacea. It has been used in an aquacultural context since the 1930's, but its popularity is decreasing. As live feed, it is quite easy to use because it is adapted to quite extreme environments. From resting cysts, it can be hatched within 24 hours, and then distributed or stored for a long time. Though, *Artemia* is not natural fish larvae prey and its nutritional value is not optimal for all fish species. Therefore, enrichment, as previously described, is also quite common for *Artemia* (Dhont et al., 2013).

Copepods are small, usually between 1-2 mm in length, crustaceans (phylum Crustacea) that constitute the biggest part of metazoans in the water communities around the world. The name copepod is derived from greek, where it is a contraction of the words kope, meaning oar, and podos, meaning foot, alluding to their flat swimming legs. They make up a very diverse subclass that comprises 10



Figur 3.5 Phylogenetic relationship of the subclass copepoda (Mauchline 1998).

orders (though this is still up for scientific debate) and currently 14710 accepted species. In the figure 3.5 below, the phylogenetic relationship between the 10 orders is shown. Their habitats can be in salt and fresh waters, ranging from oceans, rivers, lakes to caves, moist soils and on leaves, but also stretches to places with extreme conditions such as high altitudes, in the cold waters near the polar ice and near hot active hydrothermal vents. Copepods can be free-living, mutualistic and parasitic (Water & Boxhall 2016; Mauchline 1998). In the aquatic food chain, planktonic copepods have an important ecological role by being a connection between microscopic algae and fish larvae (Walter & Boxshall 2016) and copepods are found to be the preferred prey by fish larvae in nature, compared to

Artemia and Rotifers (Last 1980). In addition, copepods are seen as superior live feed in terms of size, behaviour and biochemical composition (Dhont et al., 2013).

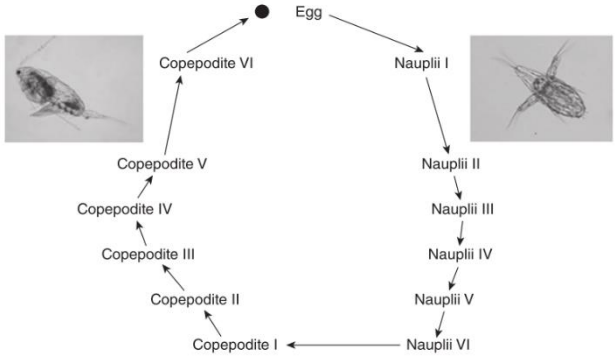


Figure 3.6 Life stages of a copepod.

Many marine fish larvae need live feed in an appropriate size for the first feeding, because their mouth is not large enough and their digestive system still undeveloped at this point. The relation between size and nutritional value must be correct, so the energy used in obtaining the prey is minimal. The mentioned preys are all oblongate and swallowed head first by fish larvae, meaning that the width of the prey is essential in comparison to the mouth width of fish larvae. In this context, copepods have an advantage over rotifers and *Artemia* by being smaller, and therefore more suitable for the first feeding. Many different copepods and nauplii stages within each species can then be chosen to fit different fish larvae (Jepsen 2014).

The behaviour of the prey versus the predator is also of crucial importance, when determining an ideal live feed. Marine fish larvae are stimulated by movement. Both copepods and *Artemia* nauplii have shown to perform 'zig-zag' or swarming movement, the first can trigger more attacks from the larvae, but the latter can make an encounter more rare. Copepods are also faster than rotifers and *Artemia.* This can be a disadvantage because they more easily escape from the fish larvae, but this can also stimulate the fish. The first nauplii stages of some copepods are so small and elusive because they have not fed yet. This can be a problem for visual predators to discover them. However, the problem caused by a swarming movement can be solved by adding a modest disturbance and the less visible copepod nauplii stages can be solved by applying a heterogenous culture of copepods in different nauplii stages (Jepsen 2014).

Nonetheless, the biochemical content of copepods is also superior to that of *Artemia* and rotifers. They do not need to be enriched liked these and if they are insured with nutritious algae in the right sizes, they will keep their biochemical profile. In respect to FAs, Linolenic acids and especially the highly unsaturated FAs (HUFAs) Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential for copepods, just as they are for fish larvae, because these cannot synthesize EPA and DHA themselves. Without the right amount and composition of PUFAs fish larvae are exposed to health problems, low growth and various malformations etc. (Jepsen 2014).

3.2 Micro Algae

Today micro algae are produced commercially worldwide for many different products. The industry is trying to produce biofuels at competitive prices and is focusing on other products to make revenue, while still investing in the biofuel production. Table 3.1 gives an overview of the most important algae based products. (Oilgae, 2016)

High-value	Medium-High value	Low to Medium value
Nutraceuticals a) Astaxanthin b) Beta carotene c) Omega-3 fatty acid (DHA and EPA) d) CoenzymeQ10 **Cosmetics** a) Anti-cellulite b) Skin Anti-ageing and sensitive skin treatment – Alguronic acid ***Pharmaceuticals***	**Nutraceuticals** - Spirulina and Chlorella **Hydrocolloids** - Agar, Alginate, Carrageenan **Chemicals** - Paints, Dyes and Colourants	**Fertilizer and Animal Feed** **a)** Aquaculture feed **(** Shrimp feed, Shellfish Feed, Marine Fish Larvae cultivation) b) Animal Feed c) Fertilizer **Substitutes for Synthetics** a) Biopolymers and Bioplastics b) Lubricants **Bioremediation** a) Wastewater treatment and nutrient credits b) CO2 capture and carbon credits

Table 3.1 Overview of the prominent products from algae, classified on the basis of their market values (Oilgae, 2016).

Algae in general have a high ecological importance because they create a constant flow of organic matter to higher trophic levels (Falkowski et al. 2004). They are primary producers, meaning they generate oxygen and nutrients through photosynthesis and secrete atmospheric CO_2 in seawater. Thus, they are providing food (FAs, polysaccharides etc.) for other organisms such as bacteria and animals (Seckbach, 2015).

Practical applications of green algae include usage as biofertilizers, growing them as fish feed in marine culture and as food and dietary supplements for humans. This is because some green algae (for example *Tetraselmis suecica*) synthesize omega-3 (ω-3) long chain polyunsaturated FAs (PUFAs) and some essential amino acids required for human and animal nutrition. In aquaculture they are used as fodder for juvenile molluscs, shrimp larvae, brine shrimp, rotifers and copepods, which are then fed to fish. They also liberate oxygen and remove CO_2 in seawater, sustaining fish life and creating a recycling cycle of nutrients. Fish waste and effluents contain nitrates and phosphates, which are biofiltered by algae in fish tanks and thus enabling reuse of water. Therefore green algae are indispensably valuable due to treatment of seawater and sewage disposal where they photosynthesize oxygen which breaks down waste into simple and soluble inorganic compounds (Seckbach, 2015).

Green algae also fixate atmospheric CO_2 and therefore reduce greenhouse gas emissions, which have already been used by some power plants. One of the novel usages is as a source of renewable energy because of their fast growth and relatively high lipid and carbohydrate content. Algal biofuel which causes almost no pollution is one of the substitutes to fossil fuel (Seckbach, 2015).

This paper will focus on improving the methods for growing the microalgae, *Tetraselmis Suecica* and *Isochrysis Galbana*, as feed for copepods used as aquaculture feed for fish larvae. These two species of algae is already widely used commercially (Patil et al. 2007).

3.2.1 Logistic growth in Algae cultures

Any given population will in theory show an exponential growth if the access to necessary resources is unlimited. In nature this is rarely the case and most populations will at some point reach a limit for their growth that is determined by their access to resources. This is called the carrying capacity for the population and it will result in logistic growth rather than an exponential. Figure 3.7 shows a population with exponential growth and one with logistic growth. The carrying capacity is the upper boundary for the population (Stiling, 2012).

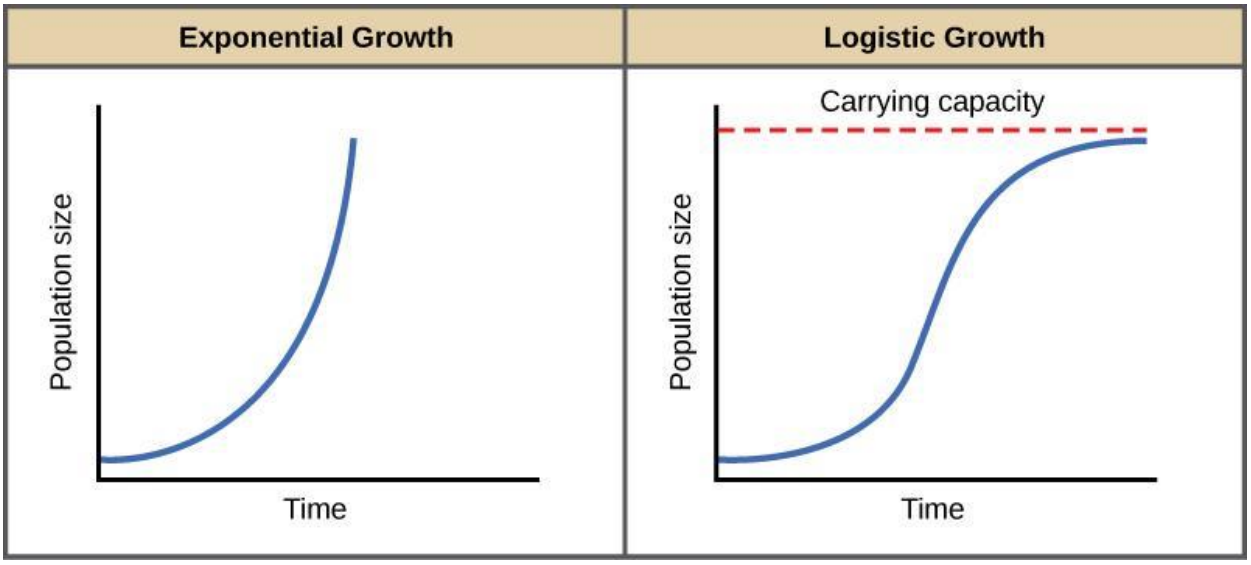


Figure 3.7 Exponential growth vs. Logistic growth (Stiling, 2012).

A population that is growing exponentially can be described mathematically by the equation:

$$\frac{dN}{dt} = r * N$$

Where dN is the change in the number of individuals in the population and dt the change in time. r is the growth rate and N the number of individuals in the population. Logistic growth can be described mathematically in the logistic equation, discovered by the Belgian mathematician P. F. Verhulst in 1838:

$$\frac{dN}{dt} = r * N(\frac{K - N}{K})$$

Where K is the carrying capacity of the population that represent the limit of the populations size determined by the access of resources (Stiling, 2012).

Algae are cultured in many different ways, from open ponds or raceways where nutrients are added often as fertilizer or wastewater, to monocultures grown in controlled indoor environments. An algae culture is expected to have a logistic growth curve where growth rates will mainly depend on light intensity, available nutrients, temperature, salinity (for marine species) and available CO_2. A big issue when culturing algae for commercial use is reducing the cost of production and much research is focused on that. Figure 3.8 shows the different phases of growth in algal cultures. In the lag phase the alga is getting used to their new environment and typically have no or very limited growth, after that it goes into the exponential phase with very high growth rates. It continuous to the stationary phase and end by crashing in the senescent phase. When growing alga it makes sense to harvest in the exponential phase to minimise time and cost of production (Sun & Wang, 2009; Creswell, 2010).

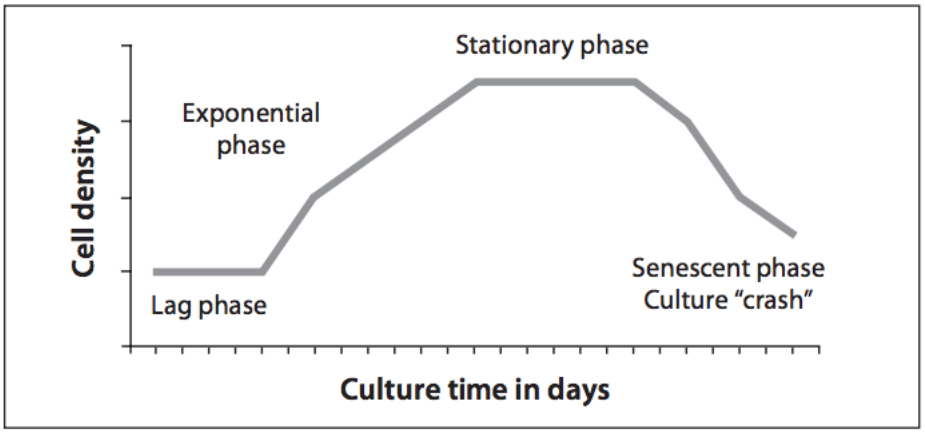


Figure 3.8 Phases in the growth of algal cultures (Creswell, 2010).

3.2.2 Fatty acids in aquaculture

Marine fish's limited capacity to synthesize LC-PUFA, long-chain polyunsaturated fatty acids, de novo from some essential acids presents the requirement of fish oils for the enrichment of live feed and fish feed in aquaculture farming. But as the fish oil implies an environmental impact new sources have been studied. One of these new alternatives are microalgae, the primary natural producers of LC-PUFA, which offer a non-polluted resource for biotechnology and bioengineering of LC-PUFA production that is transferred trough food webs.

To use a specie of microalgae it must be easy to culture and have a lack of toxicity, high nutritional value (determinated by protein-, vitamin- and polyunsaturated FA content), correct cell size and shape and a digestible cell wall to make nutrients available for the prey (Raja et al. 2004b; Patil et al., 2007). There are different ways to use microalgae to feed the next trophic level in the food web. Some studies focus on dried microalgae, but with the oxidation produced by drying them resulted in a low level or absence of FAs (Muller-Feuga et al. 2003). Because of this, these dried algae are of no interest as aquacultural feed. Freeze-dried products have also been fabricated, but these have not presented a nutritional difference from live algae (Lubzens E, Gibson; Yamasaki et.al. 1989). Some of the FAs derived from microalgae are known to be essential for various organisms (Sargent et al. 1997).

FAs are straight carbon chains with a carboxylic end (COOH) and a methyl (CH3) or omega (ω) end. These aliphatic chains vary in length, degree of saturation and structure. They can be classified as

saturated FAs, that do not have double bonds, and unsaturated FAs that have (D.R. & J.H., 2009). PUFA refers to the polyunsaturated FAs, which means that they have more than two methylene-interrupted double bonds. The two main families of PUFA are distinguished by the distance of the last double bond from the methyl end of the acyl chain: ω-3 (or alternatively n-3) and ω-6 (Figure 3.9). Thus, the first one designates a PUFA whose last double bond is located three carbon atoms from the ω-end of the carbon chain (Khozin-Goldberg, Iskandarov, & Cohen, 2011).

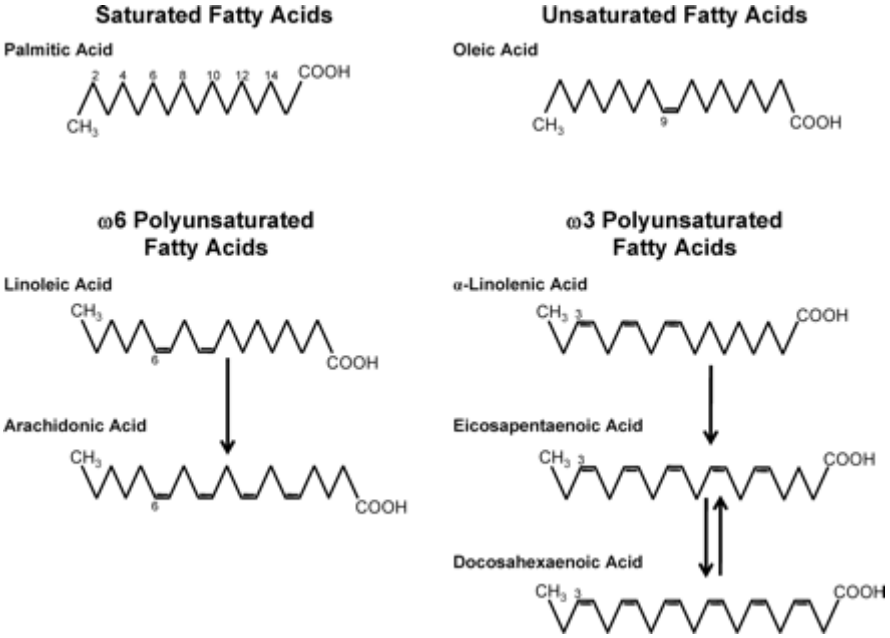


Figure 3.9. More common FAs present in microalgae (D.R. & J.H., 2009).

The main LC-PUFAs present in microalgae are the ω-6 group arachidonic acid (ARA, 20:4ω-6) and the ω-3 group, eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) (figure 3.10). All of them have linoleic acid (LA, 18:2 ω-6) and α-linolenic acid (ALA, 18:3 ω-3) as precursors (Khozin-Goldberg et al., 2011). It is important to maintain a proper balance between n-3 and n-6 PUFAs because of the competition for metabolic enzymes.

Class	Genus species	Major LC-PUFA	References
Bacillariophyta (diatoms)			
Bacillariophyceae	*Phaeodactylum tricornutum*	EPA	Arao et al. 1994 Molina-Grima et al. 1999
	Nitzschia laevis		Tan and Johns 1996
Mediophyceae	*Odontella aurita*		Guihéneuf et al. 2010 Pulz and Gross 2004
Chlorophyta (green algae)			
Prasinophyceae	*Ostreococcus tauri*	EPA, DHA	Wagner et al. 2010
	Micromonas pusilla *Pyramimonas cordata*		Dunstan et al. 1992
Trebouxiophyceae	*Parietochloris incisa*	ARA	Bigogno et al. 2002a
	P. incisa (Δ5 desaturase mutant)	DGLA	Iskandarov et al. 2011
Cryptophyta			
Cryptophyceae	*Chroomonas salina*	DHA	Henderson and Mackinlay 1992
Dinoflagellata			
Dinophyceae	*Pyrocystis fusiformis*, *P. lunula*, *P. noctiluca*	EPA, OPA, SDA	Leblond et al. 2010
Eustigmatophyta			
Eustigmatophyceae	*Nannochloropsis* sp. *N. salina, N. oculata*	EPA	Volkman et al. 1993; Sukenik 1999
	Monodus subterraneus	EPA	Cohen 1994 Khozin-Goldberg et al. 2002a
Haptophyta			
Prymnesiophyceae	*Isochrysis galbana*	SDA, EPA, DHA	Molina Grima et al. 1992 Qi et al. 2002
	Emiliania huxleyi	DHA, OPA	Sayanova et al. 2011
Pavlovophyceae	*Pavlova lutheri*	EPA, DHA, SDA	Volkman et al. 1991 Kato et al. 1995 Meireles et al. 2003

Figure 3.10. Examples of long chain polyunsaturated FA presence in different microalgae classes (Khozin-Goldberg et al., 2011).

ω-3 LC-PUFAs as EPA and DHA are abundant in many organisms living in marine environments. Most microalgae have moderate to high percentages of EPA (7-34%), however ω-6 LC-PUFAs are relatively rare, appearing mainly as precursors in the biosynthesis of EPA; moreover, in most marine species, they do not account for more than a few per cent of total FAs.

There is more than one route to biosynthesise LC-PUFAs from C18 PUFAs alternating desaturation and elongation steps. When FA desaturates it catalyzes the introduction of cis double bonds at specific positions in the FA chain. PUFA-specific elongation complex mediates a two-carbon extension of the acyl chain by condensation of malonyl-CoA to an existing acyl-CoA moiety (Figure 3.11). Generally, these routes happen in the endoplasmic reticulum. One of the microalgae used in the experiment, *I. galbana*, could have alternative pathways that initiate with the elongation of LA or ALA to finish with the desaturations (Khozin-Goldberg et al., 2011).

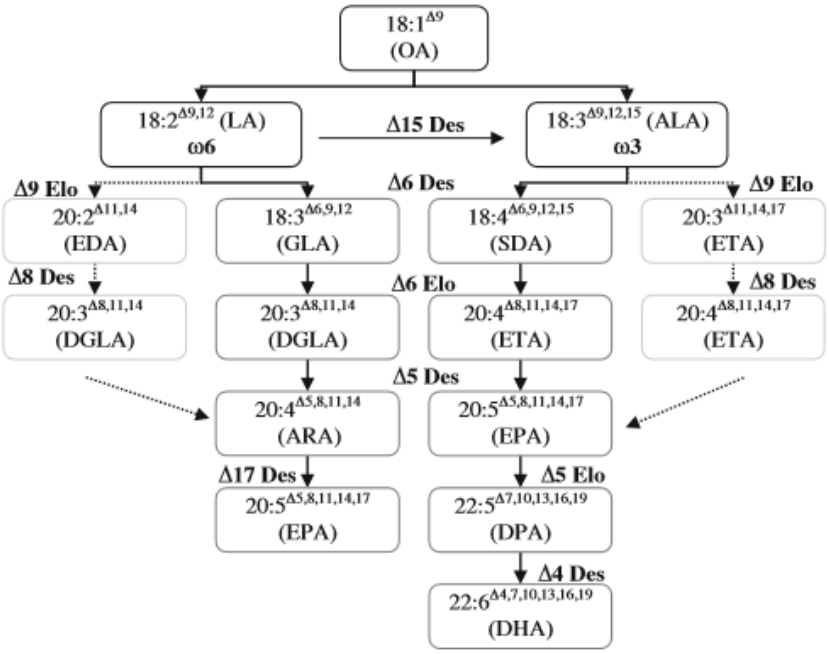


Figure 3.11 Pathway for the biosynthesis of LC-PUFA in microalgae. Where Des means desaturations and Elo elongation. (Khozin-Goldberg et al., 2011).

3.2.3 Mixed culture

One of the directions of future and present research is to cultivate the most optimal feed by mixing different species of algae. It was shown in previous studies that cellular concentrations of chlorophyll a, protein, carbohydrate, lipid, particulate organic carbon and calculated dietary energy content were significantly higher in mixed cultures (D. C. Pereira, Trigueiro, Colepicolo, & Marinho-Soriano, 2012).

It has also been reported that diets consisting of more than one algal species generally promote faster growth of fish, bivalve larvae and copepods than diets consisting of single species of the mixed diet (Romberger & Epifanio, 1981; Helm, 1977; Enright, Newkirk, Craigie, & Castell, 1986; Fidalgo, Cid, Lopezmunoz, Abalde, & Herrero, 1994; Oconnor, Nell, & Diemar, 1992; Whyte, Bourne, & Hodgson, 1990). A few studies even showed that some algal species do not support growth when fed alone, but when they are combined with another alga they can be digested easier and provide a wider variety of nutrients, resulting in growth of the larvae (Epifanio, 1979; Laing & Gil Verdugo, 1991). Helm et al. determined that yield of bivalve larvae was twice as much than when fed separately with only *T. suecica* or *I. galbana*. Their experiment suggested that mixed culture provided a more balanced diet, which may be because nutrients lacking in one-alga diet were supplemented by the other species from the mixture. The usual method in aquatic farms is to culture each species separately and then feed them to larvae together. One of the reasons to grow mixed cultures would be to save money because of the amount of algae needed, as feed would be smaller due to higher PUFA content. There is not a lot of research done on growing two algal species together, therefore comparison with their monocultures and research on what the most optimal and cost efficient growth conditions are is still a blank spot. *T. suecica* and *I. galbana* are often used as feed separately, but not a lot of experiments were conducted to study growing them together in mixed culture.

Moreover, a mixed culture composed of various similarly productive strains with various optimal growing conditions would be less susceptible to environmental shifts than a monoculture (Johnson & Admassu, 2013). When growing a monoculture, optimal growth conditions have to be controlled and for this we need specific and expensive equipment (coolers, heaters etc.). On the other hand mixed culture with different algae strains could adapt easier to changing environmental conditions, which is a cheaper option in these kind of systems (for example in wastewater remediation system). Mixed cultures can reduce risks associated with unknown conditions such as light and temperature. Ultimately if environmental conditions can be controlled perfectly then there is no need to grow a mixed culture. But all conditions can never really be controlled when algae are grown in outside ponds (Johnson & Admassu, 2013).

Mixed algae cultures may grow faster than the average of two or more monocultures, leading to conclusion that there may be some mutually beneficial interactions between algae. It could also indicate that algae are secreting advantageous substances that will increase their growth rate, or perhaps that both algae can use available resources more efficiently when grown together (Johnson & Admassu, 2013).

The results from Johnsons & Admassus study showed that growing a mixed culture of 4 different freshwater algae strains was beneficial regarding to both biomass and lipid production. It appeared that the strains *Scenedesmus sp.* and *Chlorella sp.* had the highest growth rates compared to the two other monocultures, but the mixed culture seemed to follow the growth trend of best growing algae even though it was made up of 25% of each algae strain (Johnson & Admassu, 2013). In the same study, they found that the lipid production followed more or less the same pattern. *Scenedesmus sp.* showed the highest lipid production at all light intensities and *Chlorella sp.* showed high lipid production at low light intensities, but much lower at high light intensities. Again the mixed culture seemed to follow the high lipid production of *Scenedesmus sp.* through all light intensities. Johnson & Admassu concluded:

"This indicates that there may be some effect which pushes mixed cultures to grow faster than the sum of their parts and that there may be some mutually beneficial interactions between algae, or perhaps a more efficient use of available resources. However, this effect was too small to be statistically verified based only on the results of these tests." (Johnson & Admassu, 2013)

With all of this said, it is important to note that if a stable growth environment fit to a certain algal strain could be ensured, a monoculture will produce a higher yield than any mixed culture (Johnson & Admassu, 2013). On the other hand, the equipment used to control the conditions has costs in terms of money and resources. Therefore, the costs and benefits should be weighted to conclude if it is more beneficial to grow algae separate or mixed under the given specific circumstances.

One issue the study does not take into consideration is interspecific competition. One explanation for the mixed cultures positive growth rates and lipid production could be that the *Scenedesmus sp* simply outcompeted the other strains and therefore what we are seeing in the mixed culture could be only one or two strains.

Interspecific competition can be defined as two/or more populations fighting for the same limited resources thus affecting both populations negative regarding to survival, growth and/or reproduction.

Alfred J. Lotka and Vito Volterra both developed a mathematical model in the 1920s, based on logistic growth to describe the outcomes of competition, now referred to as the Lotka-Volterra competition model. Figure 3.12 shows the four different outcomes of interspecific competition based on the Lotka-Volterra competition equations. Situation a, b and c all result in one of the species driven to extinction and only situation d will result in a stable equilibrium though still resulting in both species being reduced in population size and not reaching their carrying capacity (Stiling, 2012).

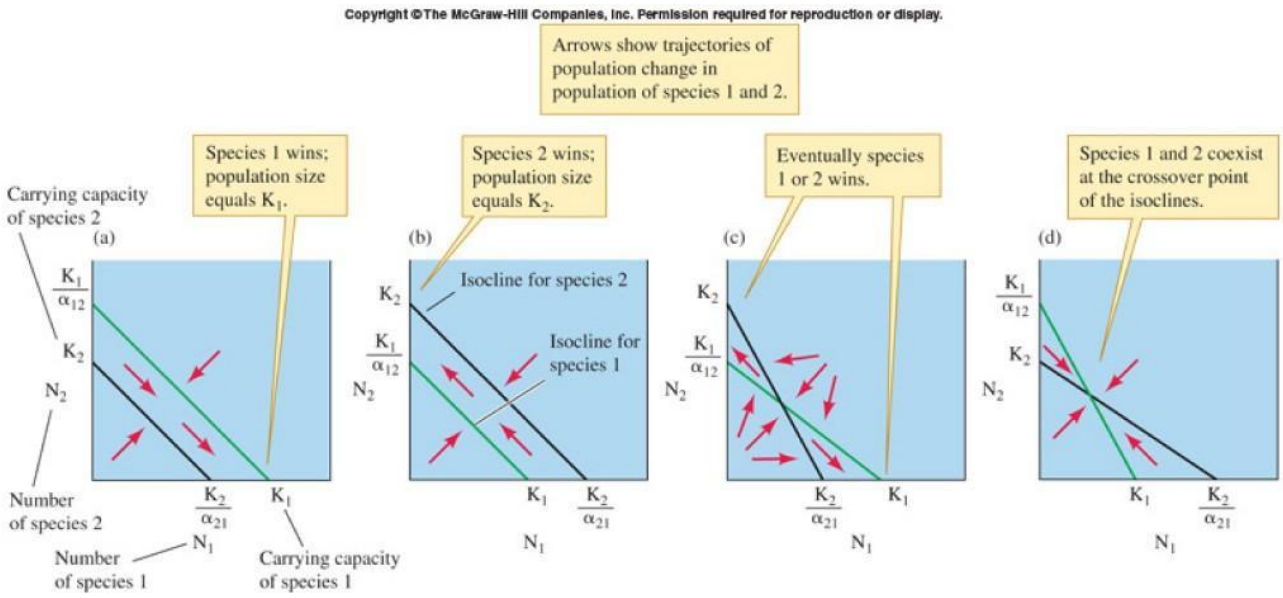


Figure 3.12 The four consequences of the Lotka-Volterra competition equations (Stiling, 2012).

Mathematically the Lotka-Volterra competition equation for each species can be described as:

Species 1:

$$\frac{dN1}{dt} = r1 * N1(\frac{K1 - N1 - \alpha N2}{K1})$$

Species 2:

$$\frac{dN2}{dt} = r2 * N2(\frac{K2 - N2 - \beta N1}{K2})$$

Where α and β are the competition coefficients where α is defined by the per capita competitive effect on species 1 of species 2 and β the per capita competitive effect on species 2 of species 1. (Stiling, 2012).

All in all, very few studies deal with the mixed cultures and provide data about mixed cultures. In addition, comparisons of mixed cultures with monocultures are scarce, so there should be more research done in this field. This is our main motivation to do a research of mixed species in different irradiances and compare them with monoculture experiments under laboratory conditions.

3.2.4 Tetraselmis suecica

Empire	Eukaryota
Kingdom	Plantae
Subkingdom	Viridiplantae
Phylum	Chlorophyta
Subphylum	Chlorophytina
Class	Chlorodendrophyceae
Order	Chlorodendrales
Family	Chlorodendraceae
Genus	Tetraselmis
Species	Suecica

Tetraselmis suecica is an unicellular green alga living in marine habitats. It is widely used as source for feeding (e.g. for rotifers, shrimps, molluscs) in marine aquaculture due to its high lipid and vitamin E content. At present, the main method of production is with artificial light in plastic bubble-columns (Michels, Slegers, Vermuë, & Wijffels, 2014). Cells of this phytoplanktonic alga are photosynthetic and motile with 4 flagella. Cell sizes vary between 8 to 14 µm, whereas biovolume is on average 450 μm^3 (Patil et al., 2007). It was discovered that this alga has antimicrobial and probiotic characteristics (Abiusi et al., 2014). *T. suecica* is considered as a cosmopolitan alga due to its relation to polluted areas. It has a wide tolerance for salinity and pH change and has been extensively tested for wastewater treatments. This quality allows the usage of *T. suecica* in recycling aquaculture systems, where nutrient-rich wastes of fish are used as feed for algae. This enables re-use of the water for aquaculture. It is easily grown in laboratory conditions (Weiss, Gromet-Elhanan, & Halmann, 1985), which is why many studies have been conducted, trying to change growth conditions to find the most optimal and least expensive way to produce it in large quantities.

Grima et al. determined that growth of *Tetraselmis sp.* became saturated between 80 and 100 W/ m^2 with the maximum specific growth rate 0,059 h^{-1}. It was demonstrated that lipid content of Tetraselmis sp. decreased slightly with increasing light intensity, thus concluding that PUFA percentage was highest at lower light intensities (figure 3.15). This points out that under conditions that limit growth rate, lipid biosynthesis is enhanced (Grima, Camacho, Pérez, & Sánchez, 1994). Another research showed that *Tetraselmis sp.* can endure a wide range of salinities from 9 to 70 PSU (practical salinity unit). Lipid productivity increased at high salinity of 70 PSU but overall lipid content was lower because growth rate decreased. Therefore salinities below 35 PSU resulted in increased growth rates and lipid productivities compared to high salinities above 45 PSU. This was shown under conditions scarce and rich with nitrogen. On the contrary there was no significant change in PUFA content at low salinities under 35 PSU (Kim et al., 2016). Another study determined that optimal salinity for *T. suecica* was between 25 and 35‰ and nutrient concentrations of 2, 4 and 8 (m · mol)/L of $NaNO_3$. The total protein of the culture increased when the salinity increased and the total protein of the cultures decreased when the nutrient concentration increased (Fabregas, Abalde, & Veiga, 1984). Regarding temperature one study indicated that the optimal growth temperature for *T. suecica* was between 25 and 27°C. With enough nutrients populations can be sustained in a wide range of 15-32°C (Weiss et al., 1985). It was demonstrated that the temperature 25°C is suitable for most optimal protein and chlorophyll concentration, while for lipids it would be 20°C (Molina, Martinez, Sanchez, Garcia, & Contreras, 1991). Highest biomass and lipid productivities were observed at pH 7.5 and 7 (Moheimani, 2013).

Kim, Lee and Lee wrote about *Tetraselmis sp*. after doing an experiment with it. It is seen that the most common FAs in this species are palmitic acid (C16:0), oleic acid (C18:1) and the polyunsaturated linolenic acids (C18:2 and C18:3) (Kim, Lee, & Lee, 2016). Even though, Patil et al. showed that C18:2 is not that common and stearidonic acid (C18:4) and eicosapentaenoic acid (C20:5) are more present (Patil et al., 2007) (Figure 3.13).

	Isochrysis galbana	*Tetraselmis* sp.
Saturated		
C12:0	–	–
C14:0	8.9	0.5
C16:0	11.5	6.3
C 18:0	–	1.2
C 20:0	–	–
C 24:0	–	–
Sum	20.4	8.0.
Monosaturated		
C 16:1	3.3	1.3
C18:1[c]	13.1	10.7
C 20:1[c]	–	0.9
C 22:1[c]	0.6	–
Sum	17.0	12.9
Polyunsaturated		
C 18:2[b]	7.0	2.5
C 18:3[a]	3.8	6.4
C 18:4[a]	12.5	4.1
C 20:2[b]	–	–
C 20:4[b]	–	0.6
C 20:5[a]	0.8	4.8
C22:5[b]	–	–
C22:6[a]	15.8	0.2
Sum	39.9	18.6

Dashes indicate FA not detected
[a]ω-3 fatty acids
[b]ω-6 fatty acids
[c]ω-9 fatty acids

Figure 3.13. FA profile of the microalgae tested. Data are given as mg/g of dry weight (Patil et al., 2007).

3.2.5 Isochrysis galbana

Empire	Eukaryota
Kingdom	Chromista
Phylum	Haptophyta
Class	Coccolithophyceae
Order	Isochrysidales
Family	Isochrysidaceae
Genus	Isochrysis
Species	Galbana

Isochrysis galbana is a marine alga with an unialgal structure and is widely used in aquaculture as feed, mainly because of its robustness, abundance and high content of LC-PUFAs. *I. galbana* is a haptohyte, whose most distinctive feature is their threadlike structure. The most known member of the haptophytas is probably the coccoliths, which have an outer shell of calcium carbonate (Brown, Jeffrey, Volkman, & Dunstan, 1997) and famous for the creation of the massive fossil rich chalk cliffs in Moen, Denmark, and Dover, England. The family Isochrysidaceae consist of two different microalgae, *I. galbana Parke* and the clone T-ISO strain (Whyte, 1987). Both are Haptophyceae but the FA production is not the same. The clone T-ISO lacks EPA while both have presence of DHA (Liu & Lin, 2001).

I.galbana can be grown in very different environments and still perform well when it comes to growth rates and lipid production, which is part of the explanation for the algaes popularity in aquaculture and algal production in general. Going through previous research does not give a clear answer for the optimal growth conditions for *I. galbana*, however there seems to be a coherence in positive results regarding growth rates and lipid production at temperatures between 20-30 C°, pH 8 and salinity around 20-30 PSU and light intensities from 50-300 µmol photons m^{-2} s^{-1}(Kurpan Nogueira, Silva, Araújo, & Chaloub, 2015);(H. Pereira et al., 2012);(Liu & Lin, 2001);(Sun & Wang, 2009).

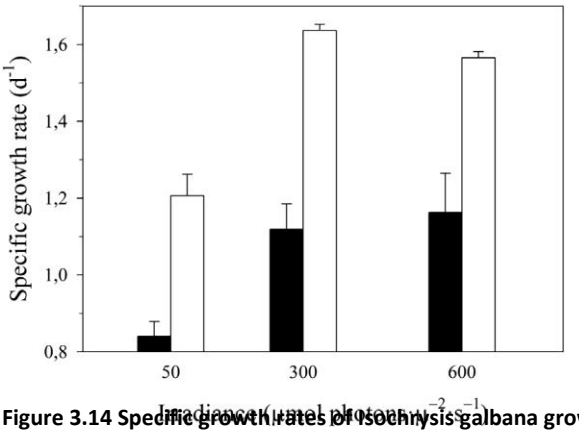


Figure 3.14 Specific growth rates of Isochrysis galbana grown in f/2 medium at 20 (black columns) and 30 C (white columns) and 50, 300 and 600 µmol photons m^{-2} s^{-1} (Kurpan Nogueira et al., 2015).

Kurpan et al. reached specific growth rates at 0.84 μ d^{-1} with light intensities at 50 μmol photons m^{-2} s^{-1} at 20 °C and showed that *I. galbana* was completely light saturated at 300 μmol photons m^{-2} s^{-1} reaching a specific growth rate of 1,1 μ d^{-1} (figure 3.14). Furthermore, Kurpan et al. showed that *I. galbana* increased its specific growth rate significantly at 30°C especially at low light intensities. Grima et al. 1994 investigated the FA content of *I. galbana* and *Tetraselmis sp.* at different light intensities (figure 3.15) and found that there seemed to be a correlation between unfavourable growth conditions (low light intensities) and high energetic content like PUFA for both species.

The other algae used in this project, *I. galbana,* was tested by Zhu, Lee and Chao and they found that the FA composition of each lipid class changed with the temperature, but not the total content of lipids. Patil et al. also tested this specie. Both experiment indicate that the most common FAs are palmitic acid (C16:0), oleic acid (C18:1) and the polyunsaturated stearidonic acid (C18:4), the docosahexaenoic acid (C22:6) and the linoleic acid (C18:2) (Patil et al., 2007) (Figure 3.13).

Fatty acid	I_0 ($W m^{-2}$)												
	15·8	*22·2*	*27·2*	*40·2*	*50·0*	*50·6*	*75·4*	*107·0*	*107·6*	*132·0*	*137·2*	*159·4*	*198·4*	*218·4*
14:0	—	0·33	0·36	0·26	0·69	0·42	0·43	0·39	1·01	—	0·42	0·37	0·42	0·38
16:0	17·04	20·00	21·78	22·73	24·97	24·45	24·76	23·93	25·33	25·30	26·76	26·00	27·33	25·47
16:1*n*9,*n*7	2·76	2·21	2·05	1·88	1·78	1·82	1·62	1·58	1·85	1·17	1·79	1·29	1·88	1·57
16:2	1·89	2·11	1·84	1·53	1·53	1·62	1·46	1·52	1·60	1·13	1·42	1·11	1·56	1·67
16:3	—	0·36	0·68	0·64	1·14	0·78	0·98	0·73	2·78	2·19	0·74	1·80	0·96	0·72
18:0	0·72	0·75	0·78	0·75	0·67	0·69	0·69	0·80	0·42	—	0·65	0·40	0·55	0·46
18:1*n*9,*n*7	23·02	23·48	24·52	28·10	25·88	25·39	24·89	25·98	24·40	25·76	25·54	24·55	26·22	24·30
18:2*n*6	5·03	7·02	6·37	6·03	6·28	6·05	6·53	6·20	5·79	7·56	6·18	6·81	6·22	6·57
18:3*n*3	18·52	16·29	15·78	14·96	13·58	13·56	14·63	14·65	11·13	13·39	13·26	13·80	12·71	13·99
18:4*n*3	11·12	6·18	5·58	4·51	3·45	3·27	3·63	3·56	2·32	3·25	2·94	3·27	2·67	3·64
20:1*n*9	0·46	0·62	0·72	1·07	0·94	0·93	1·06	1·13	1·07	2·05	1·34	1·70	1·45	1·18
20:4*n*6	0·81	1·24	1·31	1·32	1·55	2·11	1·68	1·68	1·83	2·06	1·64	2·58	1·61	2·02
20:5*n*3	4·75	5·85	6·06	6·69	5·98	5·91	7·51	8·03	6·73	10·21	7·57	9·69	7·76	10·27
22:1	0·68	0·80	0·80	0·88	0·76	0·63	0·81	0·96	0·76	1·48	0·92	1·26	0·86	1·06
PUFA	35·20	29·92	29·41	28·12	25·70	25·63	28·43	28·65	24·79	31·10	26·15	31·14	25·71	30·64

Fatty acid	I_0 ($W m^{-2}$)									
	42·4	*55·8*	*64·7*	*81·4*	*105·8*	*108·5*	*124·2*	*137·2*	*198·1*	*218·4*
14:0	6·59	8·43	8·50	9·83	9·44	10·92	11·28	12·56	12·68	15·51
16:0	18·45	30·17	20·24	28·17	20·71	25·67	22·68	20·67	23·68	25·75
16:1*n*9,*n*7	9·54	24·96	18·98	26·08	24·09	25·75	28·00	27·88	25·17	27·65
16:2	1·45	0·25	0·00	0·25	0·24	0·31	0·00	0·00	0·28	0·29
16:3	1·80	0·27	0·00	0·31	0·47	0·39	0·53	0·61	0·49	0·47
18:0	3·29	0·97	0·64	0·73	0·58	0·56	0·54	0·50	0·64	0·64
18:1*n*9,*n*7	9·03	4·06	3·12	3·27	2·68	2·75	2·63	2·26	3·27	2·84
18:2*n*6	0·00	2·18	1·69	2·79	2·49	3·07	2·56	2·58	2·61	2·44
18:4*n*3	0·26	0·44	0·00	0·52	0·31	0·45	0·39	0·00	0·30	0·25
20:0	1·96	0·55	0·93	0·66	0·73	0·86	0·70	0·80	0·82	0·67
20:1	1·49	3·40	5·43	1·85	1·76	2·91	2·41	3·59	2·95	2·67
20:4*n*6	0·00	2·18	5·05	1·96	1·33	1·68	1·81	1·08	1·52	0·72
20:5*n*3	23·08	15·55	19·18	12·78	12·54	12·35	12·68	14·36	12·43	10·46
22:0	0·00	0·00	0·88	0·00	0·77	0·48	0·00	0·00	0·00	0·00
22:3	0·00	2·01	2·32	1·32	1·97	2·26	1·97	2·92	1·88	1·72
22:5*n*3	0·00	0·73	0·00	0·64	0·79	0·93	0·78	0·71	1·07	0·62
22:6*n*3	7·51	7·45	6·69	6·09	5·78	6·13	6·26	7·94	8·13	6·65
24:0	0·00	0·00	0·00	0·33	0·00	1·17	0·00	0·00	0·32	0·00
PUFA	40·65	28·63	33·24	23·62	23·19	24·18	24·42	27·62	25·82	20·89

Figure 3.15 Variation in FA composition (% of total FAs) of total lipids with L_o for *I. galbana* at different light intensities (Grima et al., 1994).

Grima et al. (1994) also investigated the correlation between light intensities and specific growth rate of *I. galbana* and *Tetraselmis sp.* and found that both species become light saturated at around 100 W

m^{-2} (228 µmol photons m^{-2} s^{-1}) and reaching a specific growth of 0,03 µ h^{-1} (0,72 µ day^{-1}) and 0,055 µ h^{-1} (1,32 µ day^{-1}).

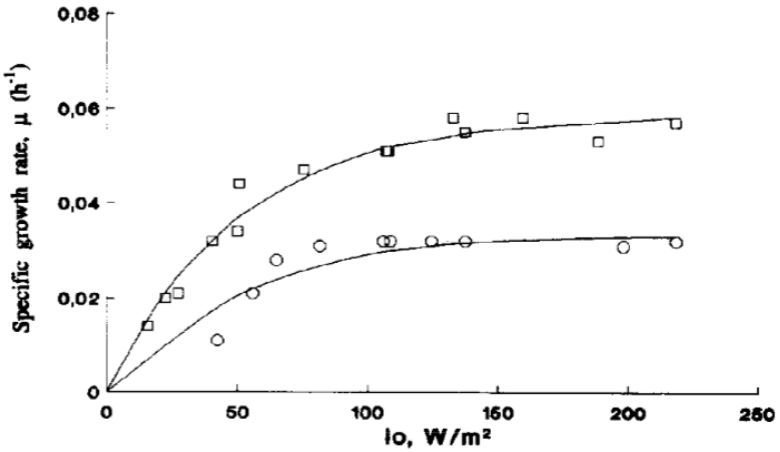


Fig. 3.16. Relationship between the specific growth rate and incident light intensity. ▯*I. gulbana*; ☐Tetraselmis sp (Grima et al 1994).

4. Material and Methods

4.1 Culture

For the culture, the algae used were *Isochrysis galbana* K-1355 Parke, coming from the Isle of Man in England and *Tetraselmis suecica* coming from DTU Aqua. We kept the base cultures in a temperature controlled room at 18°C, and the temperature in the algae water was measured to 17,6°C. The light intensity measured in and outside the glass tank was 54 µmol photons m^{-2} s^{-1}.

3L of F2 medium was mixed with 300ml of algae for each species. Every time a sample of algae was collected the same amount of medium was replaced into the culture. *T. suecica* has a vibrant greenish colour, unlike *I. galbana*, which is yellowish. This difference of colour makes these two species easy to differentiate.

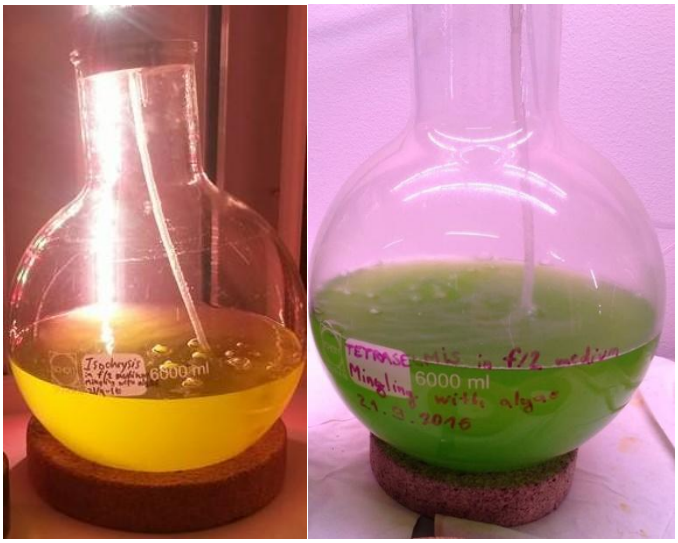


Figure 4.1 Stock pile of *Isochrysis galbana* (left) and *Tetraselmis suecica (*right).

4.2 F2 medium

F2 medium and seawater (salinity 30 ‰) were used as growth medium and produced by using a 4 stock solution:

Stock 1: Trace elements (without cobalt).

Stock 2: Vitamin mix stock solution (Vitamin B12, vitamin B1, biotin).

NaNO3: It increases the amount of nitrogen contained in the algae, which is crucial to grow thicker and stronger as a consequence of a higher production of carbon.

NaH2PO4**:** It is also used to control pH as a buffer.

To make 1 litre of medium 1 ml of each stock solution is required with 1 litre of filtered natural seawater, which has been sterilized by autoclaving and then cooled to room temperature.

f/2 Medium

Stocks	**per litre**
(1) Trace elements (chelated)	
Na_2 EDTA	4.16 g
$FeCl_3.6H_2O$	3.15 g
$CuSO_4.5H_2O$	0.01 g
$ZnSO_4.7H_2O$	0.022 g
$CoCl_2.6H_2O$	0.01 g
$MnCl_2.4H_2O$	0.18 g
$Na_2MoO_4.2H_2O$	0.006 g
(2) Vitamin mix	
Cyanocobalamin (Vitamin B_{12})	0.0005 g
Thiamine HCl (Vitamin B_1)	0.1 g
Biotin	0.0005 g

Medium	**per litre**
$NaNo_3$	0.075 g
$NaH_2PO_4.2H_2O$	0.00565 g
Trace elements stock solution (1)	1. 0 ml
Vitamin mix stock solution (2)	1. 0 ml

Figure 4.2: f/2 medium recipe (Ryther & Guillard, 2009).

4.3 Experimental Design

4.3.1 Organization of the experiment

Each experiment is divided into 3 steps: Cultivation of algae in the Multi-Cultivator, counting of cells and filtering. 3 experiments were conducted: *Tetraselmis suecica* monoculture, *Isochrysis galbana* monoculture and *a* mixed culture of both. Each experiment was grown in 8 tubes with different lights intensity for 3 days and 3 replications each.

4.3.2 Multi-cultivation

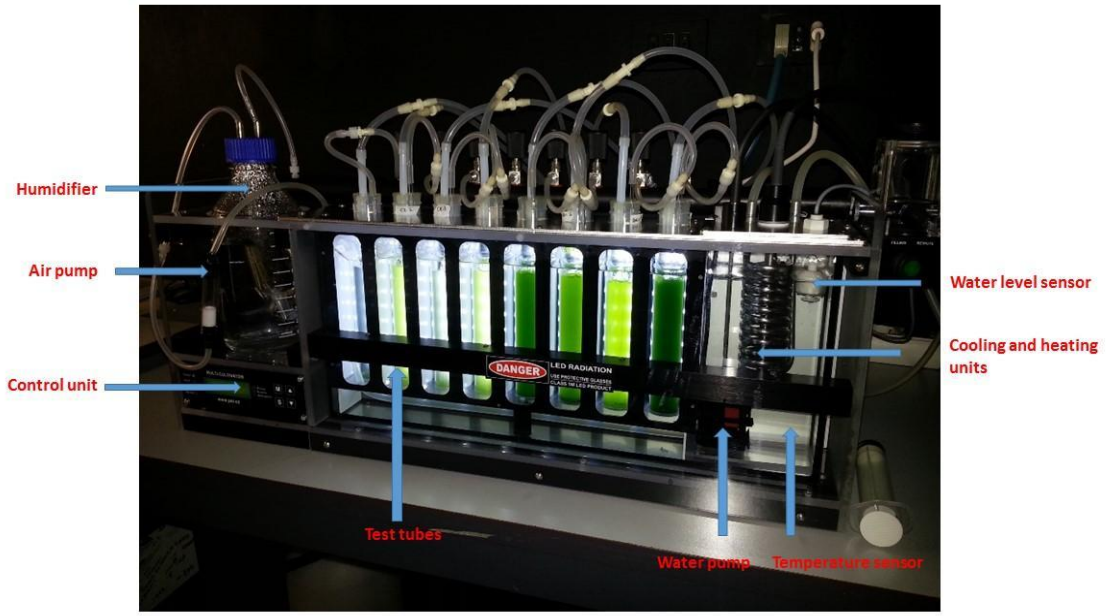


Figure 4.3: The Multi-Cultivator MC 1000.

The Multi-Cultivator MC 1000 can be used to grow both algae and bacteria. The Multi-Cultivator MC1000 is a rectangular glass container with the capacity of 5 litres. This container includes 8 cultivations glass tubes, which are immersed into a thermo stated water bath with the same temperature for all tubes. Each tube can independently be illuminated by white LEDs, which can generate a desired light intensity from 0 to 500 µmol photons m^{-2} s^{-1}. In our experiment the several light intensities used were 10, 20, 40, 60, 80, 100, 150 and 200 µmol photons m^{-2} s^{-1}, respectively.

In addition, each tube is bubbled with atmospheric air in order to provide enough CO_2 for the algal photosynthesis. The maximum amount of culture that can be cultivated in a test tube is 100 ml. Nevertheless, in our experiment the test-tubes were filled to 80 ml in order to minimize the risk of overflow during the experiment (Mc, n.d.).

During the cultivation, several parameters can be controlled. The light intensities are controlled by the internal software. The temperature is controlled (20°C) by a temperature sensor and cooled with an external device. During the last experiments, the cooling system broke down and the temperature for the last two *T. suecica* and for two of the mixed cultures rise to approx. 23°C, with minor fluctuations.

Tetraselmis suecica monoculture experiment:

First step: 200 ml of *T. suecica* base culture was collected from the basement. We diluted the algae five times with 800 ml of F2 medium and mixed in a 1 L beaker.

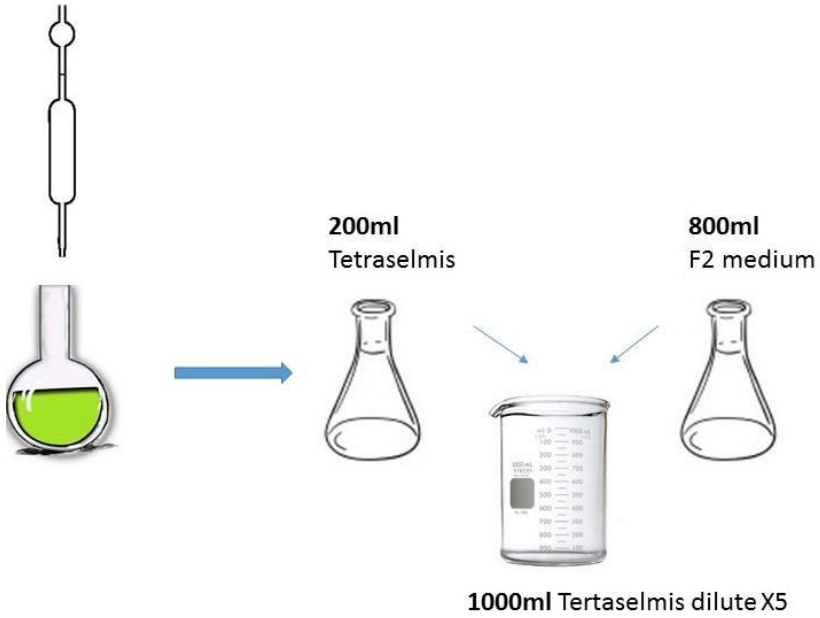


Figure 4.4: Dilution of *T. suecica*

Second step: Each test tube were then filled with 80 ml of diluted algae-solution.

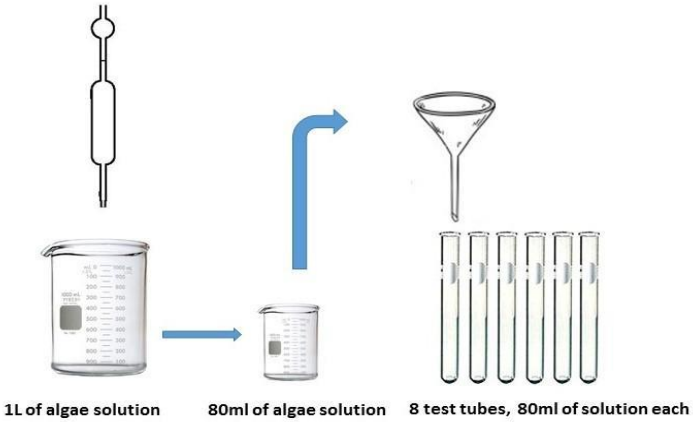


Figure 4.5 Filling tubes step

Figure 4.6 Multicultivator after 3 days of experiment

Step 3: Then the eight test tubes were placed in the multi-cultivator and temperature, irradiance and bubbling were set. Same rate of bubbles were used for all test tubes to keep the algae in movement so they would not rest on the tube's wall but not too much to avoid that the solution overflowed.

Isochrysis galbana monoculture

This experiment followed exactly the same procedure as for *T. suecica*. Only the dilution factor was has changed because the concentration of *I. galbana* basement culture was higher. Therefore we decided to dilute the algae 10 times with F2 medium. Thus, 100 ml of *T. suecica* and 900 ml of F2 medium have been mixed in a 1 L beaker.

Mixed culture

Cells were counted for each species to try to obtain a 1:1 ratio.

A sample of 25 ml of *I. galbana* and 350 ml of *T. suecica* was collected. The concentration of microalgae in the basement culture for *I. galbana* was very high, so 325 ml of medium had been added to the 25 ml of *I. galbana.* Then 350 ml of *I. galbana* with medium and 350 ml of *T. suecica* was mixed in an Erlenmeyer flask. It was very hard to obtain the same number of cells because of the lack of precision of the counter machine.

	Count algea from the basement	Ratio	Count after dilution + mix	Final ratio
Tetraselmis suecica	1046456	1	523225	1
Isochrysis galbana	12857006	12,23	605500	1,157245927

Table 4.1 Example of the cell count of each algae species before culturing

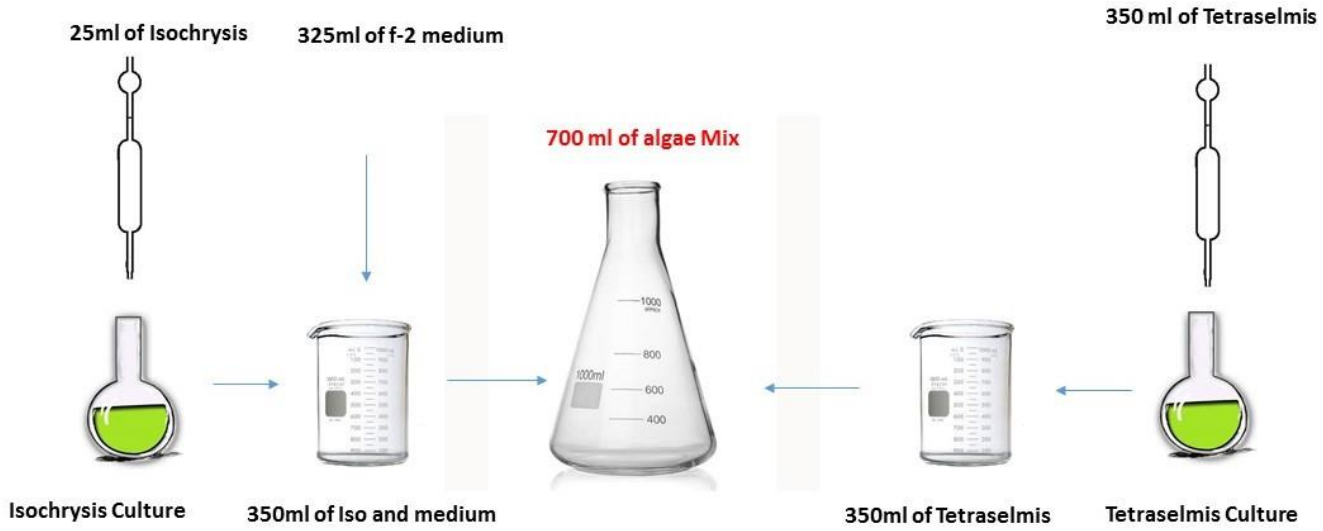


Figure 4.7 Dilution step of the mix culture.

4.3.3 Filter

The filtering device is composed of a vacuum pump, an air dryer (bottle with silica crystals) and a filter. The pump creates a vacuum that filters the water from the algae.

Figure 4.8.a: Pump

Figure 4.8.b: Air dryer

Figure 4.8.c: The filtering cylinders

The bottle with silica crystals, which is between the filter part and the pump, dries the air before it goes in the pump (figure 4.8). At the bottom of each metallic cylinder we placed filters to catch the algae.

4.3.4 Cell counter

To count the cells we used a Beckman Multisizer 4 coulter counter. This multisizer, using the coulter principle or the electrical sensing zone, that gives the possibility to know the size distribution in number, volume and surface area in a small flask of 10 ml. The results are given in number of particles of each size per ml.

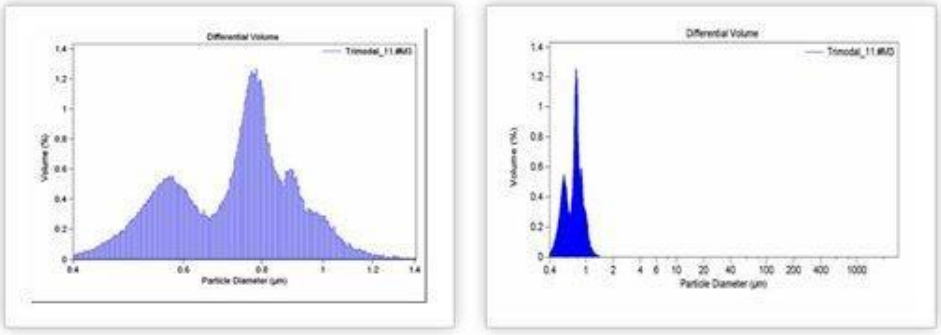


Figure 4.9: Example of results from the multisizer.

The multisizer uses the coulter principle: each particle is going through an electric current that produces a change in impedance proportional to the volume or size of the particle. The machine works on a simple principle: particles suspended in a weak electrolyte solution (we used sea water) are drawn through a small aperture separating two electrodes through which an electric current flows. According to that, the machine sends this difference of signal to the controller (the computer with the controlling program) and it calculate the size of each particle corresponding to the signal (Analysis, n.d.).

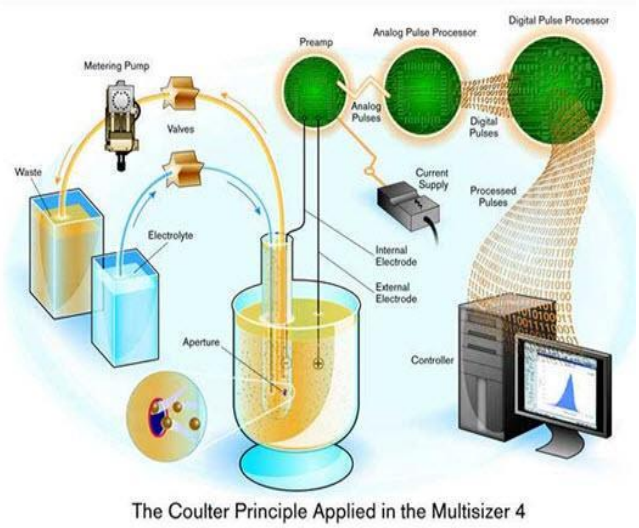


Figure 4.10: Schematic of a Coulter Counter (Analysis, n.d.).

4.4 Measurement

4.4.1 Absorbance

The measuring of absorbance was automatized by the multi-cultivator, but had to be configured before cultivation began. After doing the calibration only with medium on the first experiment the machine was set to measure the absorbance of the solution every hour for two different wave lengths: 680 nm and 720 nm. Finally, during the experiment, a black cover was put on the machine to avoid the sunlight interfering with the experiment.

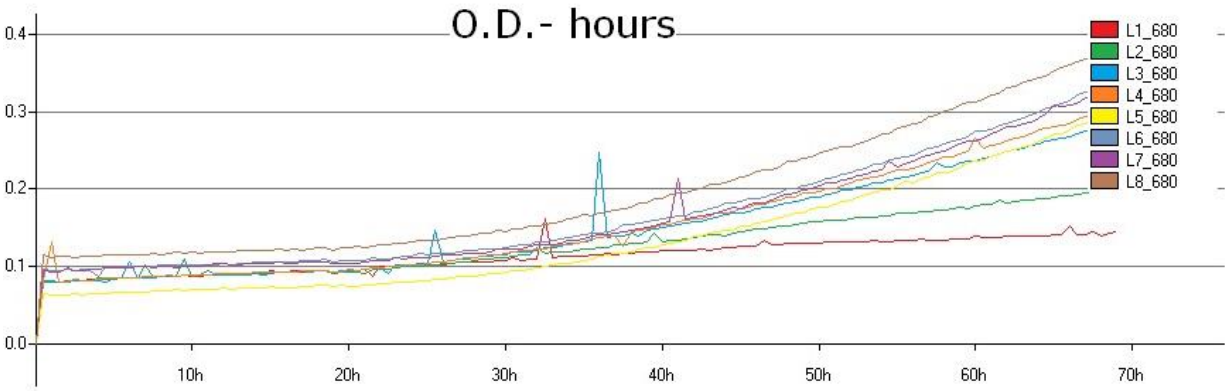


Figure 4.11 Example of the absorbance per hours of Isochrysis culture.

4.4.2 Data collection

The temperature and OD data were collected on a computer and analysed, whereas a sample from each tube where diluted with seawater sufficiently for the coulter counter (0,1 ml for *T. suecica* and 0,01ml for *I. galbana*).

Gloves were used when handling the algae. 10 to 15 ml of the solution, depending on the cells count realized before (at least 10 million cells are needed to have good results), were used to the filtering. When the filtering was finished each filter was fold and placed in Eppendorf tubes. The samples were kept in a freezer at -80°C until the start of the PUFAs extraction.

4.4.3 PUFA profile

Before the PUFA analysis, the samples needed to be prepared for the lipids extraction. They were processed in the following steps: First, the filter with the sample was placed in a tube and freeze- dried for 24h. Then, 3ml of Chloroform: Methanol ((v/v) 2:1) and 20µl of internal standard C23 methyl ester were added to the tubes and stored at -20°C for 24h before the actual extraction of lipids. Next, as much liquid as possible was transferred from these tubes to GC vials.

Then the GC tubes were placed in an aluminium block set at 60°C in order to dry out the chloroform and methanol with the help of needles applying nitrogen in their opening space. When completely dry, 1 ml of AcOMe/HCl reagent of Toluene was added in the vials. Next they were put for 2 hours on an aluminium block set at 95°C. At the same time, it is important to start bubbling nitrogen on a solution of $NaHCO_3$.

When the vials were cooled down 500µl of $NaHCO_3$ were added and each tube was shaken before next step were the upper phase that appeared was withdrawn and put in a clean GC vial. 500µl of Heptane containing BHT were added in the first vials and removed from the upper phases to put it in the other vial two times. When this was done, the content of the final GC Vials (containing the upper phases) were dried by applying nitrogen in the same way it was done before. When they were perfectly dry the vials were removed from the aluminium block and 500µl of Chloroform were added on it. Finally the samples were closed and freezed at -80°C if they were not being used immediately in the PUFA analysing machine.

The machine used to analyse the PUFA is an Agilent 5973 inert Gas Chromatograph. Each sample was processed in the machine for half an hour and the result was displayed on the control computer (Agilent, Chromatograph, Spectrometer, Rtl, & Gc, n.d.).

4.5 Data analysis

For the data analysis, the absorbance data measured at 680 nm were used. Each of the 8 tubes gave a OD vs. time graph. As shown in Figure 4.11, each experiment were run for 3 days with the intention to observe only the logistic growth of the algae when the biovolume was greatest. Therefore, we were not interested in the growth after the point of saturation.

After each experiment the growth rates of the 8 irradiances in each culture was determined. There are different step to calculate the growth rate.

4.5.1 Growth rate

The growth rate of algae can be calculated by determining the slope of the linear regression curve fit to the graphs obtained after plotting the change in OD (on a logarithmic scale) against time during the exponential phase. The time represents the X-axis and the logarithm of the absorbance represent the Y-axis.

With Excel we used a linear regression model to fit a trend curve to our graphs and then displayed the equation of the line. The equation of a linear curve is y= a*x+b, where a represents the growth rate of the algae. The slope of the straight line represents the growth rate of the algae per day, which is the number of cells division per day. Therefore, we obtained 8 curves, one for each light intensity.

Lastly, after the determination of the growth rate of each alga for each light intensity, we created graphs representing the light intensities on the X-axis and the growth rate on the Y-axis. This graph showed the relation between the irradiance and the growth rate.

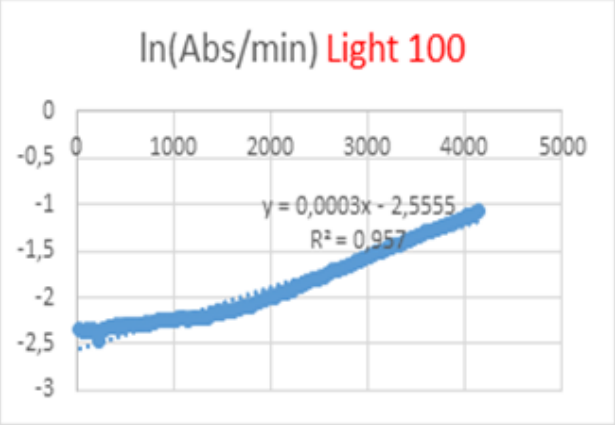


Figure 4.12 Example curve ln (Abs/min curve)

4.5.2 Mean

The arithmetic mean consists of the sum of a set of numbers divided by the total of numbers in the data set. In our case the set of results correspond to the different growth rate or FA content for each light intensity.

I. galbana and *T. suecica* monocultures and the mixed culture have been replicate 3 times in order to perform more consistency in the results. Therefore, we decided to use the means of the data regarding both growth rate and FAs.

4.5.3 Standard deviation

The standard deviation measures the dispersion of a set of data from its mean. For example, if the data points are far from each other it results in a high deviation, and opposite if they are close. Thus, to analysis the data the standard deviation can give us a measurement of how consistent our replicated experiments are. When experimenting with living cells it is difficult to obtain constant results, so the standard deviation tends to be really high.

4.5.4 Statistical Analysis

The growth rates of the cultures were analysed by two-way ANOVA-test using the software Past 3.14 (HAMMER, Harper, & Ryan, 2001). Although, since there were not enough replicates for all the light intensities for *T. suecica*, the PUFA content was analysed by two – way ANOVA without replication-test using the same software. The significance level for both tests were $P<0,05$.

5. Results

5.1 Growth rate

The specific growth rates for *Isochrysis galbana, Tetraselmis suecica* and the mixed culture were calculated from the Optical Density data obtained from the multicultivator.

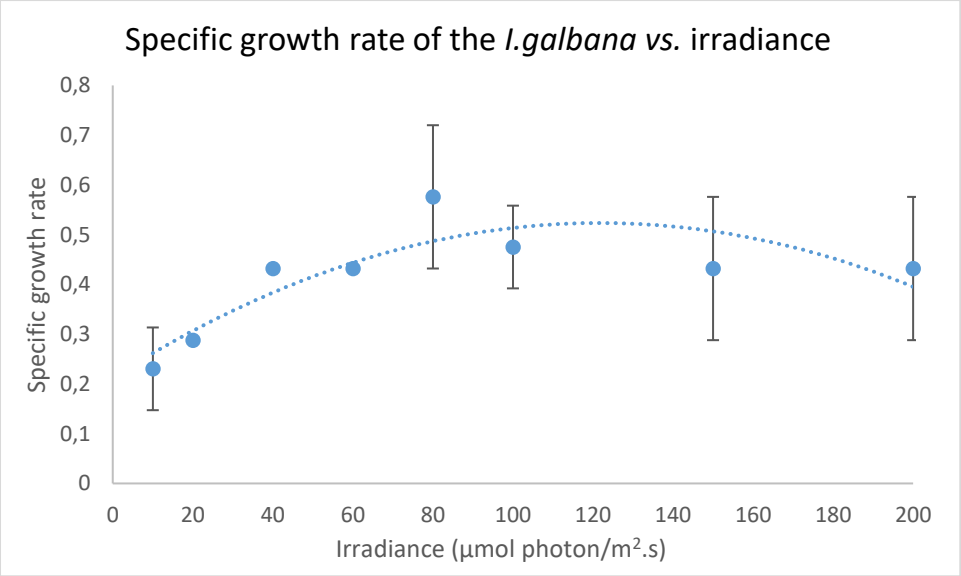


Figure 5.1: Mean specific growth rate and light intensity of *Isochrysis galbana* and standard deviations.

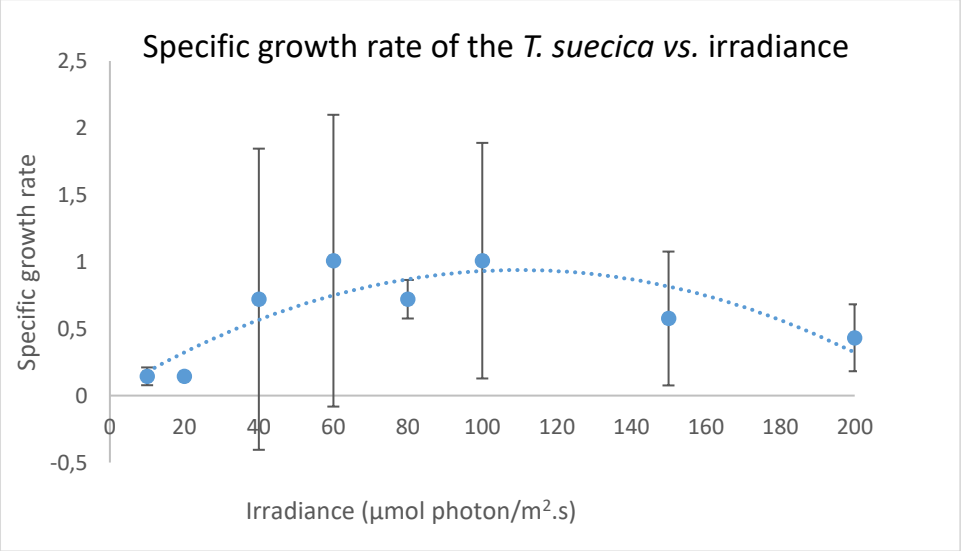


Figure 5.2: Mean specific growth rate and light intensity of *Tetraselmis suecica* and standard deviations.

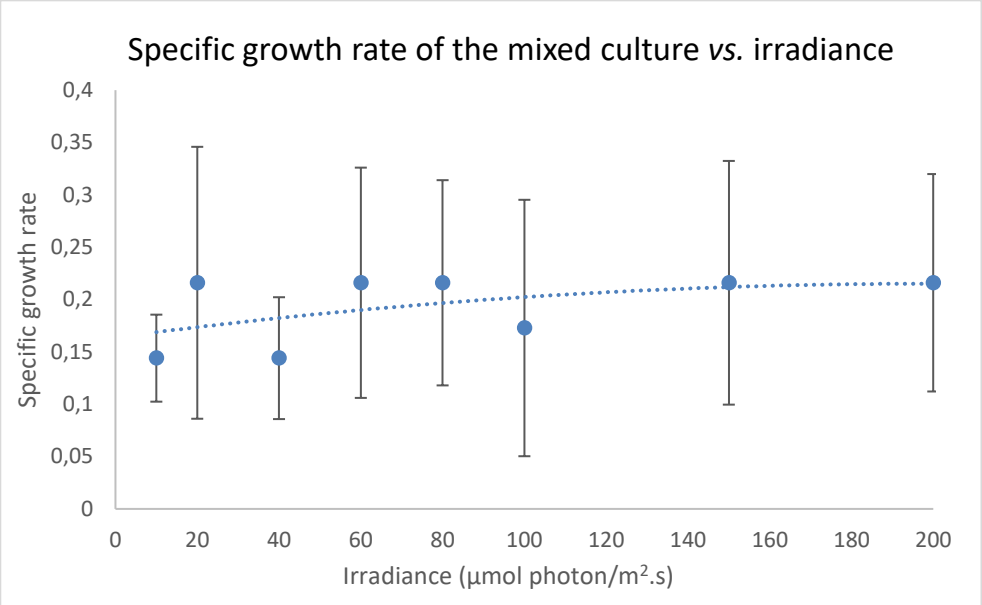


Figure 5.3: Mean specific growth rate and light intensity of *Isochrysis Galbana* and *Tetraselmis Suecica grown in a mixed culture* and standard deviations.

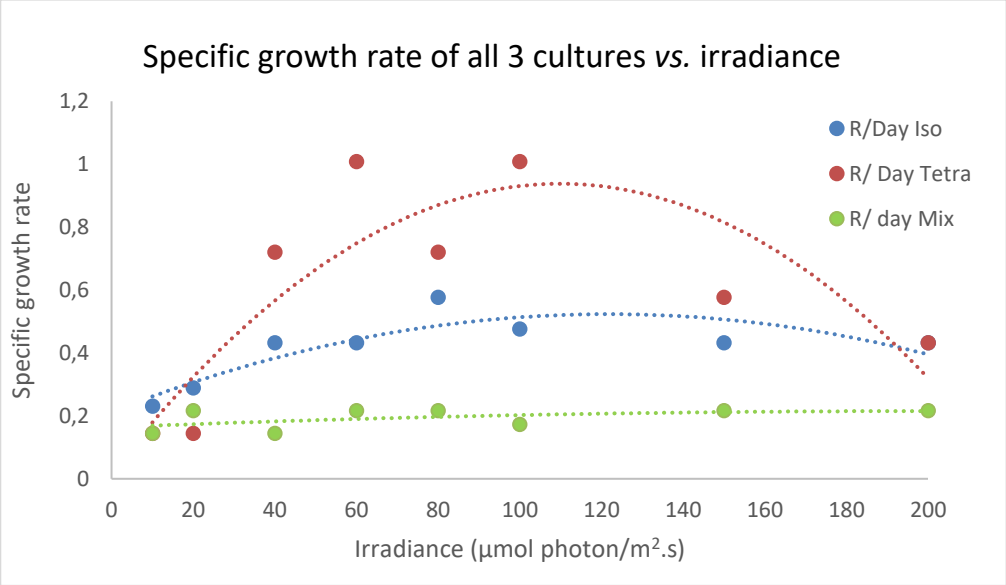


Figure 5.4: Mean specific growth rate d^{-1} and light intensity µmol photons m^{-2} s^{-1} of *Isochrysis galbana, Tetraselmis suecica* and mixed, without standard deviations.

Figure 5.1-5.3 shows the specific growth rate (day^{-1}) and the light intensity in µmol photons m^{-2} s^{-1} with the standard deviations for the 3x3 experiments with *I. galbana, T. suecica* and mixed culture.

The specific growth rate for *I. galbana* (Figure 5.1) is at 10 and 20 µmol photons m^{-2} s^{-1} the lowest (0,23 ± 0.08 and 0,28 d^{-1}). As soon as the light intensity rises above 40 µmol photons m^{-2} s^{-1} the growth rate per day is no longer increasing when the standard deviations is taken into account, though it does seem that *I. galbana* reaches the highest specific growth rate 0,58 ± 0,14 d^{-1} at 80 µmol photons m^{-2} s^{-1} and then decrease slowly with higher light intensities.

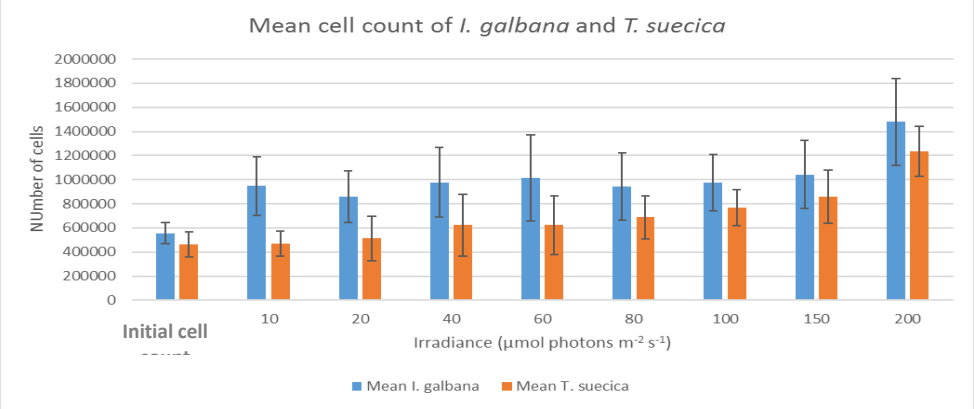
T. suecica (Figure 5.2) showed a similar pattern with an even lower growth rate (0,14 ± 0.07 d^{-1}) at 10 µmol photons m^{-2} s^{-1}. The specific growth rate for *T. suecica* peaks at 60 (1,008±1,09 d^{-1}) and 100 (1,008±0,88 d^{-1}) µmol photons m^{-2} s^{-1}, but the large standard deviations makes it difficult to precise.

Figure 5.3 shows the growth rates from the mixed culture where all the light intensities except 10 and 40 µmol photons m^{-2} s^{-1} result in a specific growth rate of 0,216 d^{-1} but with very high standard deviations from ±0,1 to ±0,13. Therefore, a maximum specific growth rate can not be determined to a single irradiance in this experiment. The minimum growth rate is 0.144 ± 0.06 d^{-1} at light intensity of 40 µmol photons m^{-2} s^{-1}.

The results of two-way ANOVA showed that with a P-value of 0,002 the difference in growth between the three cultures was significant. On the other hand, there was no significant difference between the different light intensities (P-value = 0.1703).

Figure 5.5.a shows the cell number ratio of *I. galbana* per *T. suecica* for each light intensity. The ratio reveals that there is more *I. galbana* than *T. suecica* for all irradiances. The ratio is higher for lower light intensities but is decreasing with increasing irradiances and approaches the initial ratio at 100 µmol photons m^{-2} s^{-1} and above.

The data demonstrate that after an initial doubling of the cell count there was no substantial change in cell number of *I. galbana* over the different light intensities, with the exception of 200 µmol photons m^{-2} s^{-1} where the cell count almost tripled compared to the initial count. On the other hand, *T. suecica* was increasing in number of cells with increasing irradiances. Even though the number of cells of *T. suecica* was less than *I. galbana* for all light intensities, the ratio between the two changes in favour of *T. suecica* the higher the irradiance becomes.



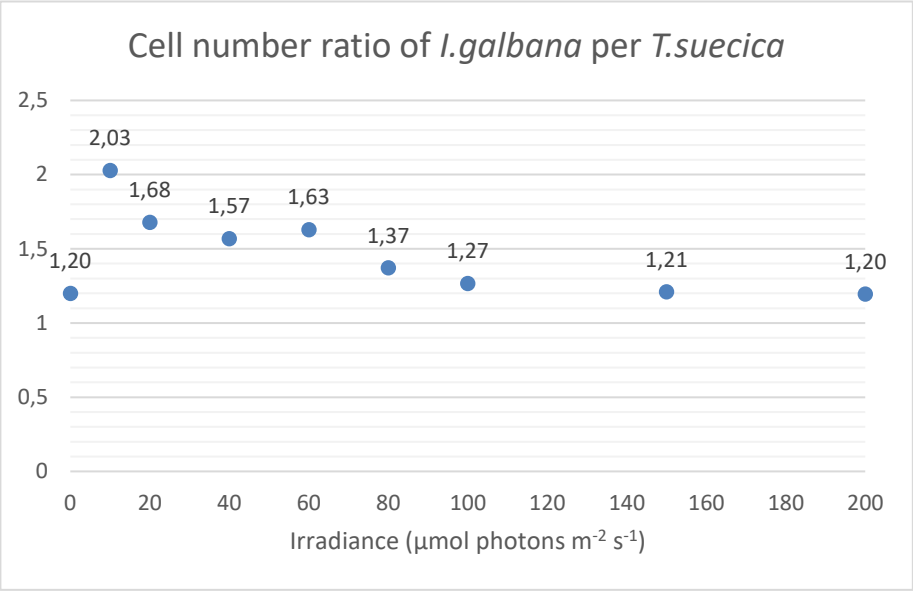
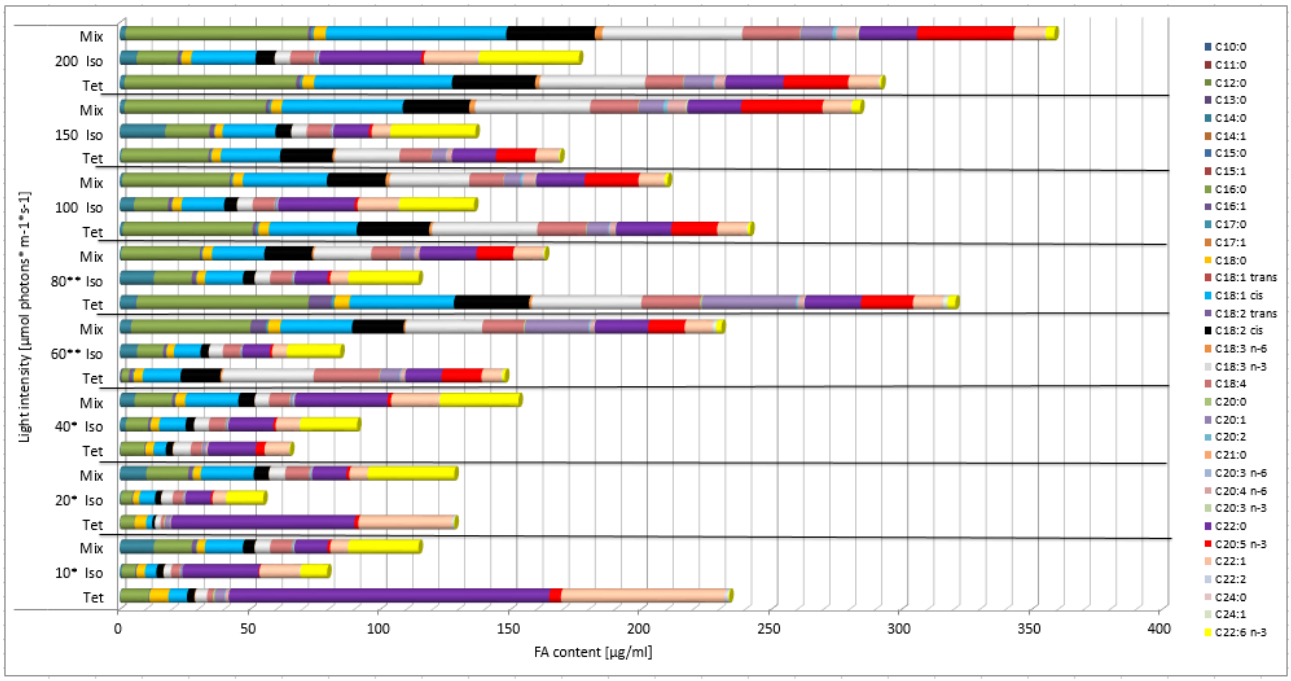


Figure 5.5: a. Mean number of cells for *T. suecica* and *I. galbana* in the mixed culture dependent on the light intensity. Initial cell count refers to the beginning of the experiment where the algae were diluted to achieve the same ratio between them. Standard deviations represented. b. Graph of the cell number ratio of *I. galbana* per *T. suecica* in the mix culture dependent on the light intensity where the zero value represents the start ratio before the experiments were run.

5.3. PUFA



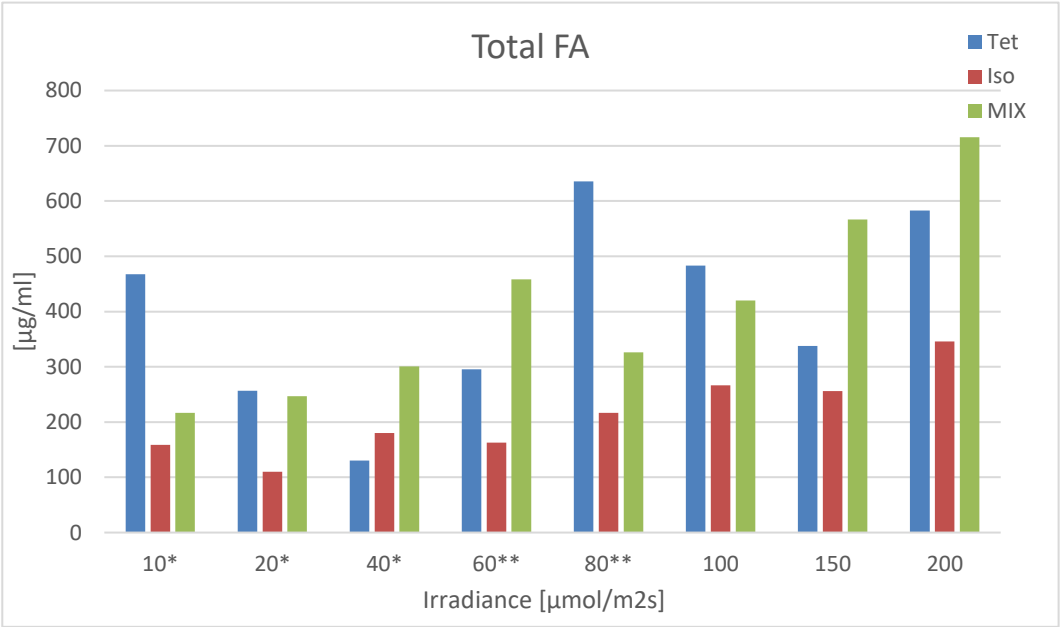


Figure 5.6: a. Bar chart of means of FA content for all three cultures at different light irradiances. b. Bar chart of total FA in µg/ml for each culture and light intensity. * number of replicates for *T. suecica* = 1 **number of replicates for *T. suecica* = 2

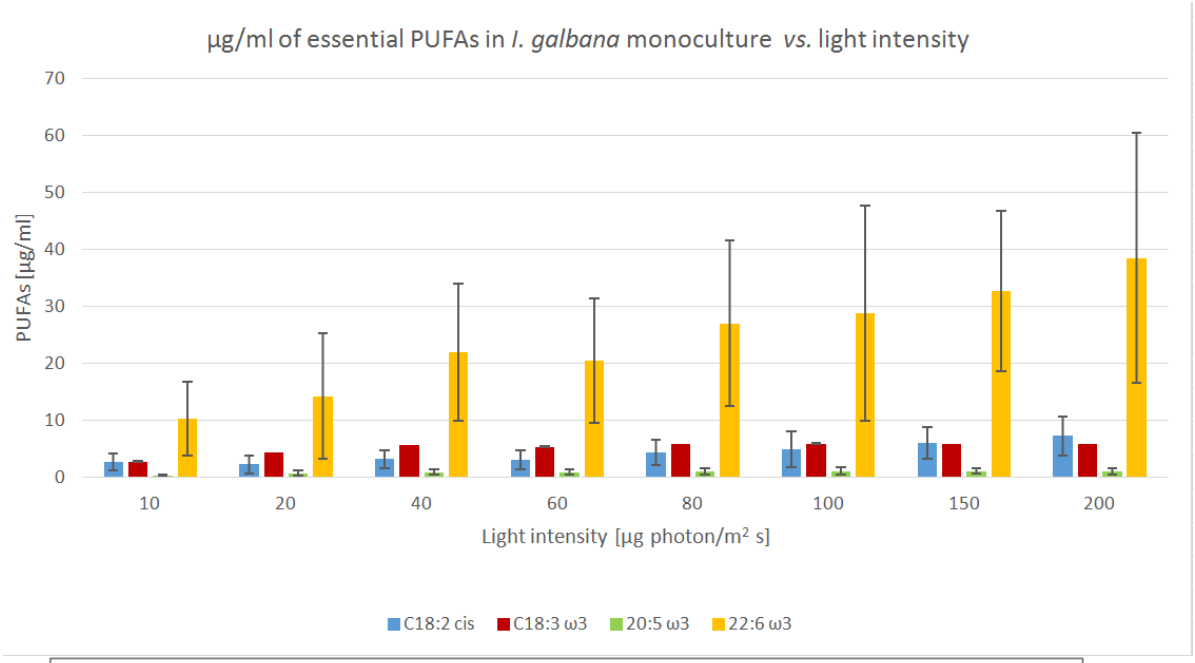
The global trend of this bar chart is that the mixed culture has a greater amount of FAs (FA) at light irradiances 40, 60, 150 and 200 µmol photons m^{-2} s^{-1}, while the total FA concentration of *T. suecica* is higher at the others.

For *I. galbana* in the light range of 10- 60 µmol photons m^{-2} s^{-1} the sum amount of FA was oscillating around 80 µg ml^{-1}, whereas after 60 µmol photons m^{-2} s^{-1} it started increasing gradually. The maximum total amount of FA was 176 µg ml^{-1} at the highest light intensity (200 µmol photons m^{-2} s^{-1}), whereas the total minimum amount was 55 µg ml^{-1} at 20 µmol photons m^{-2} s^{-1}. The four most represented PUFAs at all light intensities were C22:6 n-3 (Docosahexaenoic acid or DHA), C22:0 (Behenic acid), C16:0 (Palmitic acid) and C18:1 cis

(Oleic acid). C22:6 n-3 (DHA) increased slightly with increasing light intensity, reaching the maximum at 200 µmol photons m^{-2} s^{-1}. Behenic acid reached its maximum amount at 100 and 200 µmol photons m^{-2} s^{-1}. The content of Palmitic acid was growing at low light irradiances and became constant at 80 µmol photons m^{-2} s^{-1}. Oleic acid was increasing steadily with raising light intensity.

Concerning the most important FA in the experiment the focus should lie on the polyunsaturated FAs.

Figure 5.7a illustrates the amount of essential PUFAs in the monoculture of *I. galbana* for each light intensity. From that, it can be seen that the concentration of DHA increased gradually from 10,32 µg ml^{-1} at 10 µmol photons m^{-2} s^{-1} to 38,54 µg ml^{-1} at 200 µmol photons m^{-2} s^{-1} where it peaks. A small increase of C18:2 cis (Linoleic acid or LA), C18:3 n-3 (α-Linolenic acid or ALA) and C20:5 n-3 (Eicosapentaenoic acid or EPA) with increasing light intensity is visible, though they all remain under 10 µg ml^{-1} at 200 µmol photons m^{-2} s^{-1}, with the greatest being LA at 7,30 µg ml^{-1}.



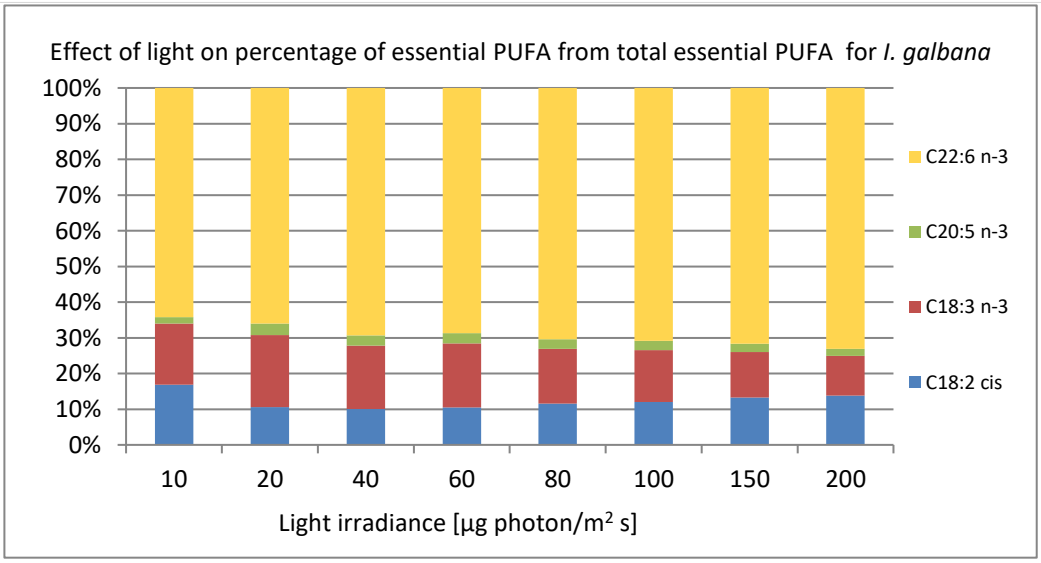


Figure 5.7: a. Absolut content of essential PUFAs vs irradiance in *I.galbana* with standard deviations represented. b. Percentage of each essential PUFAs of the total amount of essential PUFAs.

For *T. suecica* there is no general trend for the sum amount of FA because it was fluctuating wildly. The maximum total amount was reached at 80 µmol photons m^{-2} s^{-1} with 320 µg ml^{-1} and the minimum was at 10 µmol photons m^{-2} s^{-1}. In *T. suecica* C16:0, C18:1 cis, C18:3 n-3 (ALA), C18:2 cis (LA), C22:0, C20:5 n-3 (EPA) stood out from the others, being the most abundant. There were greatest quantities of EPA at the highest light intensity, whereas ALA was most abundant at 80 µmol photons m^{-2} s^{-1}. C22:0 represented 55,2% of the total FA in the lowest light and decreased until the highest light (7.67%).

In the monoculture of T. *suecica* DHA was measured as 2,94 µg ml^{-1} at the irradiance of 80 µmol photons m^{-2} s^{-1} though almost not present (<1 µg ml^{-1}) in the irradiances of 10, 20, 40, 150 and 200 µmol photons m^{-2} s^{-1}. The concentrations of LA, ALA and EPA all follow the same trend with increasing irradiances, being most apparent from the irradiance of 60 µmol photons m^{-2} s^{-1}, from where both LA and EPA reaches a concentration of 15,30 µg ml^{-1} while ALA rises to just around 35 µg ml^{-1}. EPA peaks with a concentration of 24,85 µg ml^{-1} at the irradiance of 200 µmol photons m^{-2} s^{-1}.

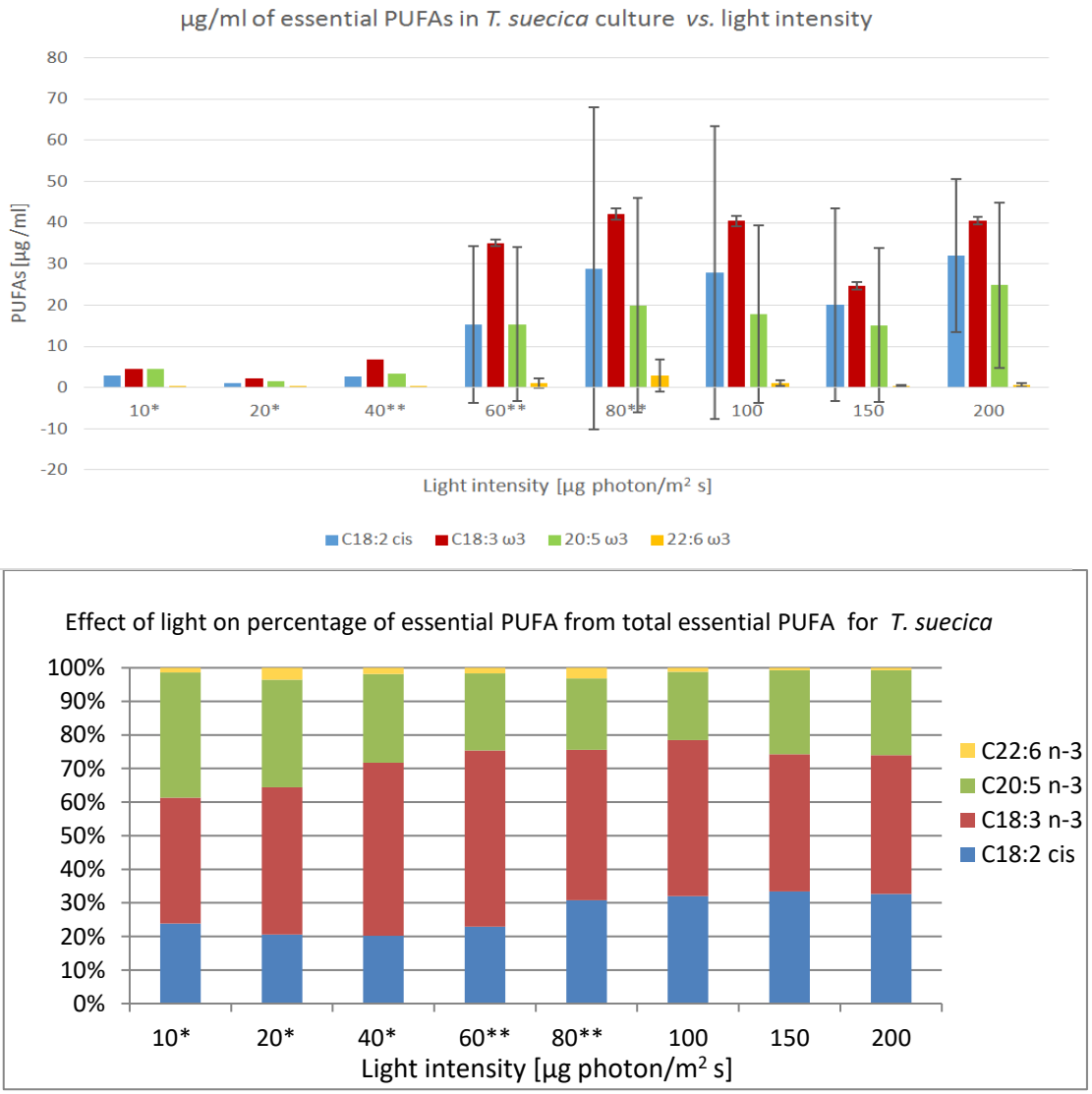


Figure 5.8: a. Absolut content of essential PUFAs vs irradiance in *T. suecica* with standard deviations represented. b. Percentage of each essential PUFAs of the total amount of essential PUFAs. * number of replicates for *T. suecica* = 1 **number of replicates for *T. suecica* = 2

In the mixed culture there was a tendency of increasing FA concentration with increasing irradiance even though the content decreased suddenly at light 80 µmol photons m^{-2} s^{-1}. The maximum was achieved at irradiance 200 µmol photons m^{-2} s^{-1} with 358 µg ml^{-1} of FA and the minimum at the lowest light. The most represented FA were C16:0, C18:1 cis, LA, ALA and EPA all of which started increasing after 60 µmol photons m^{-2} s^{-1} and had the maximum content at the highest light. On the other hand, DHA ceased to increase after 40 µmol photons m^{-2} s^{-1}.

Figure 5.8 shows a bar chart for the mixed culture, where DHA fluctuated around 30 µg ml^{-1} for light intensities from 10-40 µmol photons m^{-2} s^{-1}. Nevertheless at 60 µmol photons m^{-2} s^{-1} DHA content suddenly decreased to 2,07 µg ml^{-1}, unlike LA, ALA and EPA which suddenly reached 20, 30 and 13 µg ml^{-1}, respectively. EPA content is highest (33,37 µg ml^{-1}) under the irradiance of 20 µmol photons m^{-2} s^{-1}, while lowest at 80 µmol photons m^{-2} s^{-1} with a concentration of 0,43 µg ml^{-1}. DHA content reaches it highest concentration of 37,20 µg ml^{-1} at the irradiance of 200 µmol photons m^{-2} s^{-1} and is lowest at the light intensity of 10 with a concentration of 1,06 µg ml^{-1}.

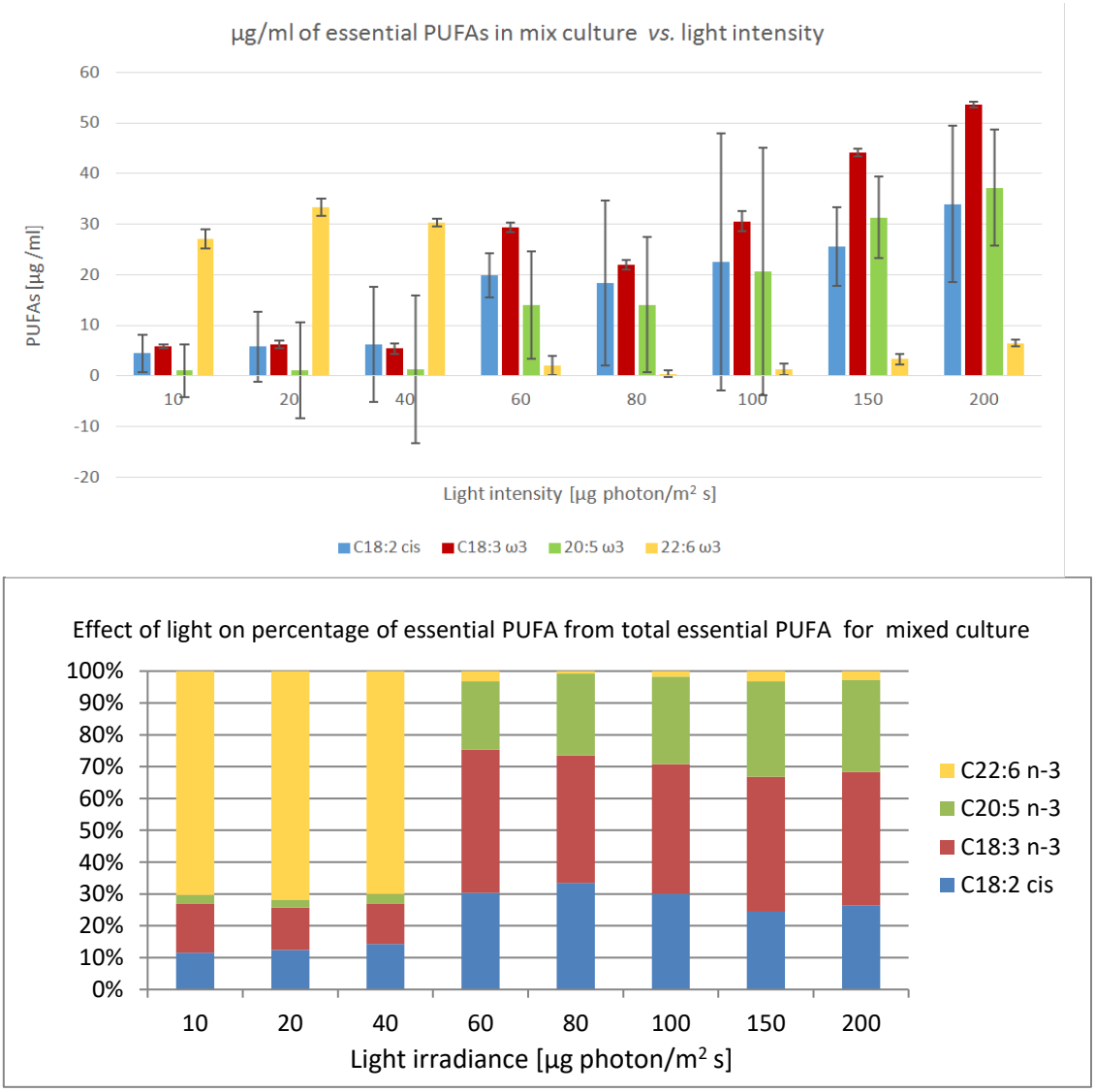


Figure 5.8: a. Absolut content of essential PUFAs vs irradiance in the mixed culture with standard deviations represented. b. Percentage of each essential PUFAs of the total amount of essential PUFAs.

From Figure 5.9, the relationship between LA, ALA, EPA and DHA in the tree cultures are shown. DHA was only present in quantities above 10 µg/ml in the monoculture of *I. galbana* and in the three lowest light intensities of the mixed culture. LA and ALA showed a similar pattern for all of the cultures, although LA was the most abundant. EPA had the same tendency in the monoculture of *T. suecica* and in the mixed culture for the first 6 irradiances, after which it became relatively more abundant in the mixed culture. Figure 5.10 show how the percentage of PUFAs of FAs fluctuated mildly around 40% from the light intensity of 20 µmol photons m^{-2} s^{-1}. The exception is *T. suecica* at the irradiances of 20, 40 and 60 µmol photons m^{-2} s^{-1}, where it showed larger variations.

There is a significant difference in the total FA content between different cultures and different light intensities (p-value= 4,10E-07 and 0.012 respectively). With p-value= 0,004 there is also a significant difference in the total amount of essential PUFAs between the eight irradiances. But analysing the four essential PUFA separately only LA and ALA have a significant difference between light intensity (P-value= 0,01 and 0,042 respectively). Differently, between the three cultures there is a significant difference for both the total amount of essential PUFA (p-value= 1,57E-07) and the amount of each essential PUFA. Note that we have not conducted one-way ANOVA of each separate cultures.

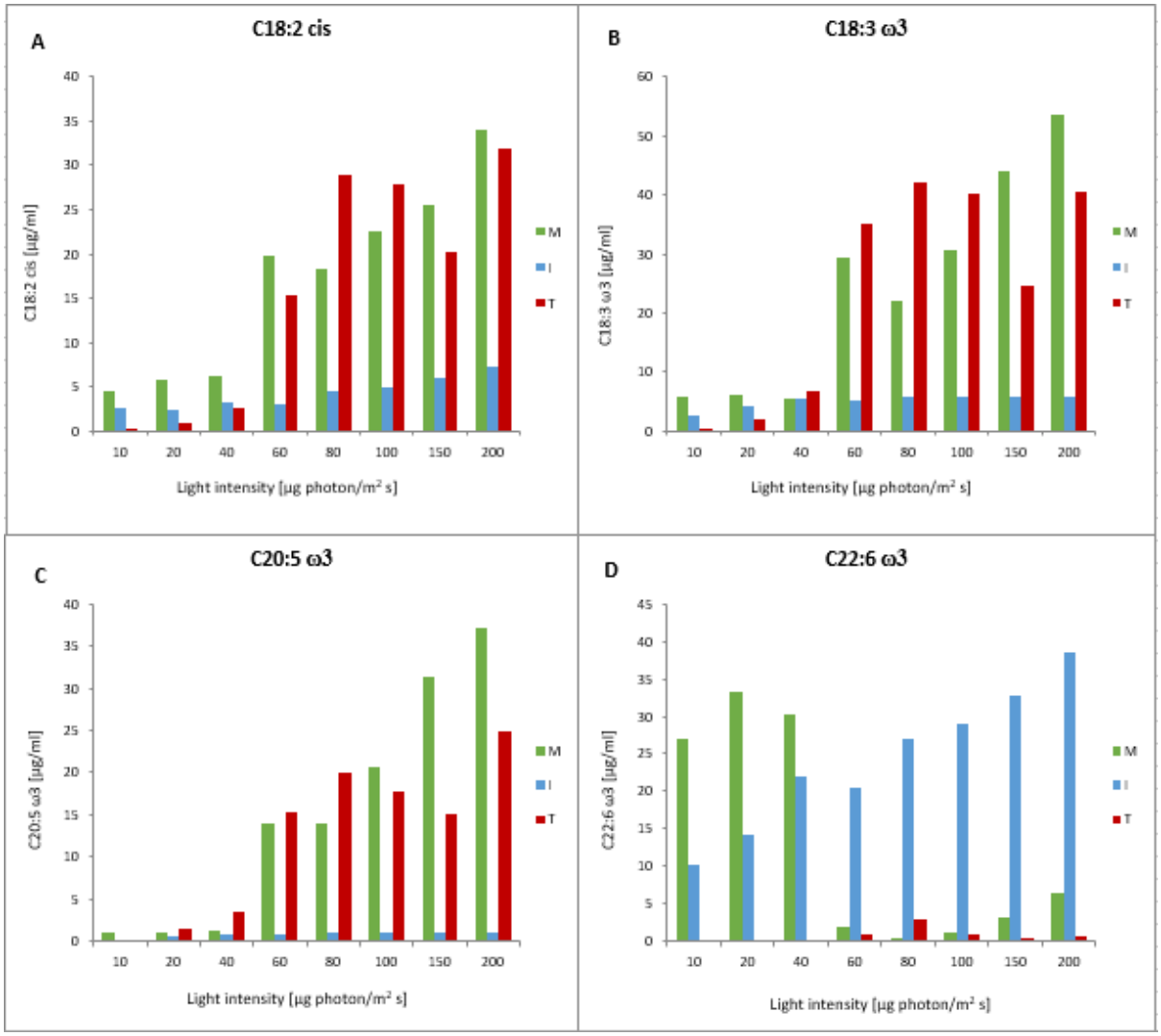


Figure 5.9: Absolut concentration of 4 essential PUFAs in the different cultures at all the light intensities.

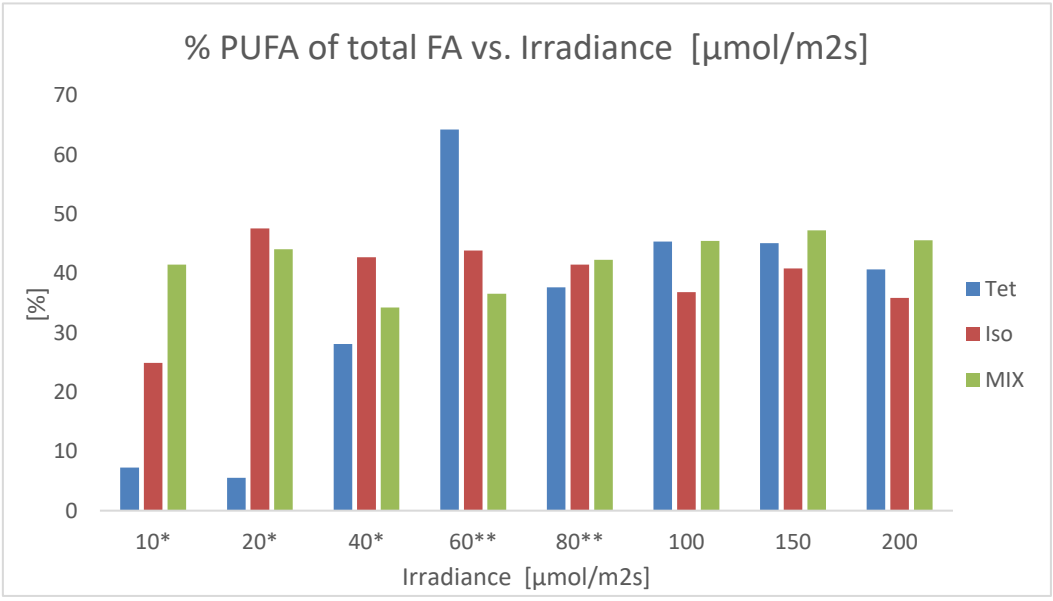


Figure 5.10: PUFA content in percentage of total FA in each culture and light intensity. * number of replicates for *T. suecica* = 1 **number of replicates for *T. suecica* = 2

6. Discussion

6.1 Growth rate

The maximum specific growth rates for the monocultures of *Isochrysis galbana* and *Tetraselmis suecica* were observed in our experiment 0,576 ± 0,144 day^{-1} at 80 µmol photons m^{-2} s^{-1} and 1.008 ± 0,88 day^{-1} at 60 µmol photons m^{-2} s^{-1} were slightly lower than the literature cites (0.768 and 1.416 day^{-1}) (Grima et al., 1994). *T. suecica*'s maximum growth rate was close to two times higher than the growth rate of *I. galbana*. Moreover, the specific growth rate of *T. suecica* was higher than of *I. galbana* at almost all irradiances, showing that *T. suecica* was more adept at storing aliment in general. However, *I. galbana* had a higher growth rate at lower light intensities (10-20 µmol photons m^{-2} s^{-1}) in monoculture.

Our findings were that growth already became light saturated at 60 µmol photons m^{-2} s^{-1} for *I. galbana* and *T. suecica*, whereas Grima et al. demonstrated that growth for both species stabilized between 80 and 100 W*m^{-2} (360 – 450 µmol photons m^{-2} s^{-1}) (Grima et al., 1994). In contradiction with this study, our findings were that the growth rate started decreasing after reaching its maximum. The decrease in growth rate of both species after 100 µmol photons m^{-2} s^{-1} may be because they could not digest nutrients at that light intensity due to saturation.

Our results show that the most optimal light intensity for growing *I. galbana* and *T. suecica* were 80 and 60/100 µmol photons m^{-2} s^{-1}, respectively. This data support our hypothesis (2) with which we predicted that the growth rate for both monocultures would be the highest in medium irradiance (approx. 60 - 100 µmol photons m^{-2} s^{-1}). Our findings on the mixed culture do not support the hypothesis because the maximum growth rate was reached at 5 different light intensities. Our data on the monocultures also seem to be in alignment with other findings, where the most optimal light intensity for growth of *I. galbana* and *T. suecica* in monoculture is between 80 and 100 µmol photons m^{-2} s^{-1} (Grima et al., 1994). These results would suggest that the monocultures of our experiments needed a similar light intensity to grow most efficiently as was reported for the *Rhodomonas salina* (60-100 µmol photons m^{-2} s^{-1}) which is also used as feed for copepods (Vu et al., 2016).

Our hypothesis (1) was that the growth rate in the mixed culture would be higher than the average growth rate of the two different algal cultures grown as monocultures. This prediction was based on a study that showed that biomass production of a mixed culture with four different algae followed the trend of the biomass production of the most productive algae, being just under this, but above the three other algae biomasses (Johnson & Admassu, 2013). This assumption turned out to be wrong, as the average growth rate of both monocultures was higher than the united growth rate of the mixed culture for all light intensities, except for the starting point at 10 µmol photons m^{-2} s^{-1} where the growth rate of *T. suecica* and mixed culture were the same. The lower growth rate in mixed culture could be explained by greater competition for the same limited resources between the two species. A research conducted by Phatarpekar et al. shows that the growth rate of mixed culture was lower than that of both monocultures *I. galbana* and *Chaetoceros calcitrans* (Phatarpekar, Sreepada, Pednekar, & Achuthankutty, 2000). It should be noted that the behaviour of mixed culture is hard to predict by studying the behaviour of monocultures of the species separately. This is why experiments with mixed culture are essential (Fogg & Thake 1987).

It has been reported that species that are smaller grow faster than larger ones. This can be due to smaller species having greater surface/ volume ratio, meaning that in comparison with the volume they have bigger surface through which they can assimilate nutrients more efficiently than larger sized species (Fogg & Thake 1987; Phatarpekar et al. 2000 and references whithin). This did not coincide with our results, which showed that the bigger species *T. suecica* had a higher growth rate. Despite this, the results of *T. suecica* are less precise than the others because of the large standard deviations at 40, 60 and 150 µmol photons m^{-2} s^{-1} that were ± 1,125; ±1,09 and ±0,5 respectively. This deviation could probably be explained by a failure in the temperature control in the multi-cultivator during some of the experiments with *T. suecica* where the temperature fluctuated between 17°C and 23°C.

To sum up, the increase in growth rate of *T. suecica* along the increasing irradiances was more significant than that of *I. galbana*. The growth rate of the mixed culture also continued to increased gradually with increasing irradiances though still being lower than both of the monocultures. The growth rate of *T. suecica* was raised from 0,144 ± 0,083 to 1,008 ± 1,09 day^{-1} between light intensities 10 and 60 µmol photons m^{-2} s^{-1}. With almost the same pattern, the growth rate of *I. galbana* changed from 0,23 ± 0,08 to 0,43 day^{-1}. On the other hand, the specific growth rate of the mixed culture reached its maximum growth rate at 20 µmol photons m^{-2} s^{-1} and was almost stable through the other irradiances.

6.2 Cell count

We tried to achieve the same initial cell number of both species at the beginning of each experiment for the mixed culture because we wanted to have an equal start for both. The cell number of *T. suecica* was increasing steadily as the light intensity increased. On the other hand, the cell number of *I. galbana* almost doubled already at 10 µmol photons m^{-2} s^{-1}, then fluctuated insignificantly between 20-150 µmol photons m^{-2} s^{-1} to almost triple from the initial count at the highest light intensity.

The ratio between the cell numbers of both species became smaller at higher irradiances. Thus, we can argue that *T. suecica* grew more efficiently that *I. galbana* in the mixed experiment when the lights increased. In the light of this evidence (not statistically significant), we may suggest that *T. suecica* suppressed the natural growth of *I. galbana* and can therefore be considered as the dominant species. This claim could probably be pursued in depth by prolonging the retention period.

The reason for slow growth of *I. galbana* in the mixed culture may be due to toxic or growth inhibiting substances excreted by *T. suecica*. Past research has showed that some species of algae can secrete such substances and suppress the growth of other species fighting for the same limiting resources (Regan & Ivancic, 1984). However, no such evidence was found for *T. suecica* in research for this project. The highly increased cell number of *I. galbana* at the light intensity of 200 µmol photons m^{-2} s^{-1} could suggest that *I. galbana* assimilates nutrients better at higher irradiances when cultured in combination with *T. suecica* compared to its monocultural growth pattern. *T. suecica* was still lower in cell number than *I. galbana* at high light intensities in mixed culture; therefore, the possibility of secretion of growth inhibiting substances by both species at the same time could also be a possibility.

Our third hypothesis was that *T. suecica* would dominate and outgrow *I. galbana* in the mixed culture which was based on a study from Grima et al. showing that *T. suecica* had a higher growth rate at all light intensities

(Figure 6.1). Our hypothesis was partly confirmed by our experiment. At 10 µmol photons m^{-2} s^{-1} *I. galbana* doubled by cell count and fluctuated there for the continuing 6 light intensities, whereas *T. suecica* slowly but continuously increased its cell count, catching up on *I. galbana* with increasing irradiance. Therefore the ratio between the two algae cell count fell and an assumption would be that *T. suecica* would outgrow *I. galbana* had the experiment lasted for a longer period. The standard deviations for the cell count are overlapping; therefore it makes the difference in the increasing growth of both algae in the mixed culture difficult to interpret.

Even though the pattern of the ratio of *I. galbana* per *T. suecica* in the mix suggest that *I. galbana* is growing faster than *T. suecica* at low light intensities (below 100 µmol photons m^{-2} s^{-1}), it is important to mention that the initial ratio was 1.2, which might have had an influence on the rest of the cell counts. Both in the monoculture of *T. suecica* and in the mixed culture some cells of this species were deposited and stuck on the bottom of the tubes and were hard to extract, which could also effect the analysis of number of cells.

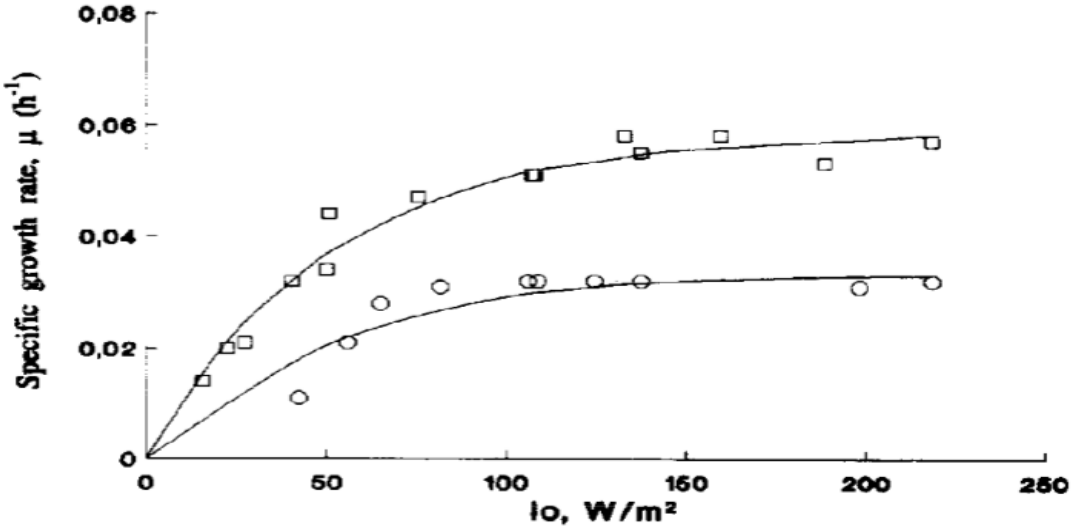


Figure 6.1. Relationship between the specific growth rate and light intensity. ▲: I. galbana □: T. suecica. (Grima et al., 1994)

6.3 PUFA

In general, FAs with long chains (18, 20 and 22 carbons) are important for the development of copepods. More specifically, Linolenic acids (18:3), Docosahexaenoic acid (22:6 n-3) and Eicosapentaenoic acids (20:5) are thought to be essential acids for copepods (Vu et al. 2015 and references within; Jepsen 2014).

One of our hypothesis (5) was that the percentage of PUFAs of total FAs would be highest in medium irradiances (60-100 µmol photons m^{-2} s^{-1}). This was mainly based on the results of a study by Grima et. al. (1992) that suggested a connection between unfavorably growth conditions (in their case low light intensities, which correspond to our medium-highest light intensities) and high content of PUFA. Our results did not show the same connection, but this difference in results can be attributed to our different choices of irradiance. The monoculture of *I. galbana* did not show any substantial changes of the essential PUFAs ALA, LA and EPA at any irradiance, whereas it seems that there was a correlation with high light intensities and

high content of DHA. This correlation with high light intensities and a high content of DHA was also found in another study (Renaud et al., 1991) for *Isochrysis sp.* T.ISO whereas all other FAs showed a decrease with high light intensities.

In the monoculture of *T. suecica* we found that at very low light intensities 10-40 µmol photons m^{-2} s^{-1} there were hardly any production of essential PUFAs. Where LA, DHA and EPA did not show a difference at any light intensities (though it does seem that LA and EPA increases at 200 µmol photons m^{-2} s^{-1}), ALA increases to 35 µg/ml at 60 µmol photons m^{-2} s^{-1} and then to 40 µg/ml at 80, 100 and 200 µmol photons m^{-2} s^{-1}, which indicates that these light intensities does not differ much when it comes to production of ALA. Grima et al. (1992) found the highest production of ALA at 70 µmol photons m^{-2} s^{-1} and then a decrease with higher light intensities.

The results of the mixed culture are more similar to the profile of *T. suecica* than that of *I. galbana*. Although, we were surprised to find that DHA almost decreased to insignificant amounts from the irradiance of 60 µmol photons m^{-2} s^{-1}, whereas the ratio between the two algae were almost similar to the three low light intensities, which suggests that *T. suecica* dominates and somehow provoke *I. galbana* not to synthesize DHA, as it would under its monoculture conditions. However, the mixed culture shows a correlation between high light intensities and high production of ALA reaching approx. 53 µg/ml at 200 µmol photons m^{-2} s^{-1} (which is higher than the ALA production of both monocultures put together). This again suggest that *T. suecica* is dominating and furthermore producing even more ALA in the mixed culture than in a monoculture, or that *I. galbana* is synthesizing more ALA instead of DHA.

Our study shows a significant difference between the three cultures in regard of both their FA content and its composition ($P<0,05$). This difference between the FA profiles of the two monocultures is in line with previous studies (Grima et al. 1992; Patil et al. 2007). We hypothesized that the PUFA concentration of the mixed culture would be higher and more varied than in the least rich and diverse monoculture and that it would follow the trend of the most abundant (in biomass) algae. We assumed this out from the simple rationale that the FA profile of the mixed culture would simply make up an approximate average of the two separate species' monoculture FA profiles, if the biomass of the two algae were equal. Other studies have shown that the total lipid amount of mixed cultures tends to follow the largest and most productive algae (Johnson & Admassu 2012; Phatarpekar et al. 2000), and that the composition of lipids may vary a lot with different abiotic conditions (Vu et al. 2015; Renaud et al. 1995; Reunaud et al. 1990). In our study, the mixed culture had a greater FA content at 40, 60, 150, 200 µmol photons m^{-2} s^{-1}, together with a higher percentage of total PUFAs at 10, 80, 150 and 200 µmol photons m^{-2} s^{-1}, compared to the monocultures.

Although the number of cells of *I.galbana* were higher at all lights in the mixed culture, the literature cites that *T. suecica* has a biovolume 10 times greater than *I. galbana* (mean cell size 450 vs. 45 μm^3 $cell^{-1}$, (from Patil et al. 2007), indicating that *T. suecica* is actually dominating in terms of biomass from the beginning of every experiment of the mixed culture, and becoming more and more abundant with increasing light intensities. This could be the main reason why most of the FA profile of the mixed culture, follows the same trend as the FA profile of *T. suecica.*

Comparing the FA profile of *T. suecica* and *I. galbana*, we found that *T. suecica* contains three essential PUFAs that were scarcely present in *I. galbana* at higher irradiances. These are EPA, LA and ALA. On the other hand,

I. galbana contained FA C14:0 (Myristic acid) and DHA, which were only present in inconsiderable quantities in *T. suecica* (table 6.1).

Light intensity	Species	C14:0	C16:0	C18:1 cis	C18:2 cis (LA)	C18:3 n-3 (ALA)	C22:0	C20:5 (EPA)	C22:1	C22:6 (DHA)
	T. suecica	0,08	4,06	1,80	0,78	1,66	54,79	1,21	27,84	0,13
20	***I. galbana***	0,84	7,57	10,95	4,17	7,90	17,78	1,27	9,12	25,86
	Mixed culture	7,79	12,51	15,83	4,50	4,79	10,27	0,90	5,27	25,98
	T. suecica	1,79	20,58	12,51	9,00	13,11	6,76	6,22	3,50	0,92
80	***I. galbana***	11,20	12,69	12,71	3,88	5,13	11,04	0,93	5,79	23,60
	Mixed culture	0,29	18,49	12,31	11,23	13,45	13,42	8,62	6,87	0,26
	T. suecica	0,56	22,55	18,09	10,93	13,85	7,67	8,50	3,93	0,23
200	***I. galbana***	3,59	8,89	14,01	4,14	3,31	22,24	0,60	11,61	21,87
	Mixed culture	0,54	19,53	19,31	9,47	14,96	6,22	10,37	3,19	0,96

Table 6.1. Comparison between mixed and monocultures in percentage of total FAs for the most abundant or essential FA.

If we compare the maximum growth rates with the optimal FA profiles (i.e. highest concentration of essential PUFAs) the most optimal irradiance spectra for the three cultures can be found. So in synthesis of our results, we determine optimal irradiances of 200, 80 and 2009 µmol photons m^{-2} s^{-1} the *I. galbana, T. suecica* and the mixed culture, respectively. However, because of the insignificant results of the effect of irradiance on growth rate between each culture, these should only be regarded as approximations.

I. galbana and *T. suecica* are already utilized in aquaculture as a mixed feed, and this studied was carried out whether to find out if the growth, PUFA content and resource use (costs) could be maximized by growing the two algae together. Our results cannot support this production method, because the combined growth rate of the mixed culture was too low and because the amount of EPA and DHA correlate negatively, though the total amount of FA and the content of Linolenic acids were almost constant in the mixed culture. Instead we recommend that these two cultures should be grown as separate monocultures and subsequently mixed before feeding.

What our study cannot tell is whether a mixed culture grown for a longer retention period and with higher irradiances would have a positive effect on the combined growth rate and/or on biosynthesis of both EPA and DHA. Like previously stated, a mixed culture can also have advantages, if grown under conditions where the abiotic factors are not fully controlled, though we have not gained any information of the possible robustness of *I. galbana* and *T. suecica* in combination from this study.

8. Conclusions and future perspective

This study shed some light on how *I. galbana* and *T. suecica* react to each other when grown in a mixed culture. It was shown which is the most optimal light intensity to achieve the maximum growth rate and the greatest PUFA concentrations.

The maximum specific growth rates reached for *I. galbana*, *T. suecica* and mix culture were 0,576 ± 0,144 day^{-1} (80 µmol photons m^{-2} s^{-1}), 1.008 ± 0,88 day^{-1} (60/100 µmol photons m^{-2} s^{-1}) and 0,216 day^{-1} (20, 60, 80, 150 and 200 µmol photons m^{-2} s^{-1}), respectively. The specific growth rate of the mixed culture was almost stable after 20 µmol photons m^{-2} s^{-1}. We can conclude that 80 µmol photons m^{-2} s^{-1} is the most optimal light intensity for growing *I. galbana*, 60 or 100 µmol photons m^{-2} s^{-1} for *T. suecica* and above 60 µmol photons m^{-2} s^{-1} for mixed culture if highest growth rate is to be achieved.

In general the increase in growth rate of *T. suecica* along the increasing irradiances was more substantial than that of *I. galbana.* However, *I. galbana* had a higher growth rate at lower light intensities (10-20 µmol photons m^{-2} s^{-1}) in monoculture. The mixed culture had the lowest growth rate at all light irradiances, except for the starting point at 10 µmol photons m^{-2} s^{-1} where the growth rate of *T. suecica* and mixed culture were the same. Growth became light saturated at 60 µmol photons m^{-2} s^{-1} for *I. galbana* and *T. suecica.*

T. suecica grew more efficiently that *I. galbana* in the mixed experiment, when the light intensities increased. The ratio of *I. galbana* per *T. suecica* in the mix suggests that *I. galbana* is growing faster than *T. suecica* at low light intensities (below 100 µmol photons m^{-2} s^{-1}). We found indications, that *T. suecica* suppressed the natural growth of *I. galbana*, why we consider it as the dominant species. The reason for the slow growth of *I. galbana* in the mixed culture may be due to toxic or growth inhibiting substances excreted by *T. suecica*, which is very common among microalgae. Although the number of cells of *I. galbana* did not seem to reduce with growing lights (and increasing concentration of *T. suecica*).

The results of the PUFA analysis show that the sum of the PUFA content in monocultures was higher than the mixed PUFA content (except at 10 µmol photons m^{-2} s^{-1}). We can conclude that it is more reasonable to grow monocultures and mix them after to feed copepods rather than growing them as a mixed culture. Secondly, the total fatty acid content was the highest at a light intensity of 80 µmol photons m^{-2} s^{-1} for *T. suecica* (320 µg/ml), and at a light intensity of 200 µmol photons m^{-2} s^{-1} for the mix (358 µg/ml) and *I. galbana* (175 µg/ml).

The mixed culture had a greater FA content at 40, 60, 150, 200 µmol photons m^{-2} s^{-1} than the monocultures. Our findings reveal that C16:0 was the major saturated fatty acid in all the algae.

T. suecica contained three essential PUFA's (EPA, LA, ALA) which were scarcely present in *I. galbana* at higher irradiances, whereas *I. galbana* contained much more considerable amounts of FA C14:0 and DHA than the other alga.

I. galbana does not show any significant changes of the copepod essential PUFAs C18:2 cis, C18:3 ω3 and 20:5 ω3 at any light irradiances, whereas with high light intensities content of 22:6 ω3 is increasing substantially.

In the monoculture of *T. suecica* we found that at very low light intensities (10-40 µmol photons m^{-2} s^{-1}) there was hardly any production of essential PUFAs. All essential PUFA contents were increasing gradually but

decreased at a light range of 100-150 µmol photons m^{-2} s^{-1}. Lipid productivity was highest for C18:2 cis and C:18 ω3.

Mixed culture showed highest DHA content (*I. galbana* specific FA) at low lights and closely followed the increasing trend in C:18 ω3, C18:2 cis and C20:5 ω3 (PUFAs abundant in *T. suecica*) after 60 µmol photons m^{-2} s^{-1}. This could support our theory that *T. suecica* is dominating *I. galbana* at already 60 µmol photons m^{-2} s^{-1} and above. At light intensities above 100 µmol photons m^{-2} s^{-1} mixed culture even had greater amounts of C:18 ω3 and C20:5 ω3 than both monocultures put together. This could again suggest that *T. suecica* is dominating and furthermore producing even more C:18 ω3 in the mixed culture than in a monoculture.

Although the maximum growth rates achieved in a given time with the mixed culture were relatively lower than those of monocultures, it is, however, compensated in terms of higher PUFA content per cell at harvest. The conclusions drawn for most optimal growth conditions have to be interpreted with some caution, depending on what is the desired result. There are four possible diets to feed copepods with; monocultures of each, mixed culture of both, or both grown separately and mixed at the end. We conclude that for the most optimal copepod fodder it is better to grow monocultures of *I. galbana* and *T. suecica* at their most optimal growth conditions and use a mix of both as feed, instead of growing them in a mixed culture. The reason for this is that the specific growth rate of both monocultures is higher and the combined FA composition of both is more diverse than in the mixed culture. Furthermore, growth conditions can be controlled much more efficiently for each separate monoculture to achieve the highest growth rate and PUFA content as superfeed for copepods. All in all, the most optimal light intensities to cultivate monocultures of *I. galbana, T. suecica* and a mixed culture of these are 200, 80 and 200 µmol photons m^{-2} s^{-1} to obtain algae with high production and a balanced biochemical composition as feed for copepods.

8.1 Future perspectives

Another microalga, *Rhodomonas salina*, is also commonly used as feed for copepods. Therefore, a comparison between the optimal growth conditions of this alga with our findings would be interesting to do in future studies. It has been reported that the optimal range of irradiance for growth and highest total FA content of *R. salina* is between 60-100 µmol photons m^{-2} s^{-1}. Highest growth rate obtained was 0.752 day^{-1} at 100 µmol photons m^{-2} s^{-1} and total FA content 0,000066 µg $cell^{-1}$ at 80 µmol photons m^{-2} s^{-1} (Vu et al. 2016). Our findings were that TFA for *I. galbana* was 0.000017 µg $cell^{-1}$ (200 µmol photons m^{-2} s^{-1}), for *T. suecica* 0.00038 µg $cell^{-1}$ (80 µmol photons m^{-2} s^{-1}) and for mixed culture 0.000029 µg $cell^{-1}$ (200 µmol photons m^{-2} s^{-1}).

Taking into account that the TFA content and the growth rate of our mixed culture was lower than in *R. salina* at its optimal light intensity, production should not be switched to grow mixed culture of *T. suecica* and *I. galbana*. However, it would be reasonable to switch production from *R. salina* to separate monocultures of *I. galbana* and *T. suecica* that would be mixed at the end, because these have a better FA profile in combination.

Although the optimal irradiance for *I. galbana* and *T. suecica* monoculture was found, it was not possible to determinate the optimal light intensity for the mix culture because the general trend of growth rate was quite stable and maximum growth rate value was reached at five different light intensities. Consequently, further studies are necessary to determinate the light intensity which probably would provide a more clear maximum growth rate for the mixed culture. According to our data, it seems that a higher irradiance (more than 200 µmol photons m^{-2} s^{-1}) and a longer retention period could be much better for

cultivating a mixed culture. Our mean cell count bar charts for *I. galbana* and *T. suecica* confirm this argument. The highest number of *I. galbana* and *T. suecica* cells are observed at 200 µmol photons m^{-2} s^{-1}, therefore it could be relevant that our experiment is repeated at a higher irradiance.

It could also be significant to focus on other culture parameters than the light intensity in order to provide a better growth rate and FA profile in future studies. As for example the pH, salinity, temperature, aeration and the composition of the medium can be interesting parameters to study.

Another future perspective could be to grow *I. galbana* or *T. suecica* in combination with other species of algae which does not have any dominant effect on the cohabitant species in the mix in order to perform an equal growth of each alga.

Concerning the polyunsaturated fatty acids composition, it could be interesting to cultivate other species of algae which would provide a bigger amount or a wider array of the essential PUFAs than *I. galbana* and *T. suecica.* For example, it would be reasonable to grow an alga species with a higher concentration of copepod essential PUFAs, such as *Pavlova sp*., *Phaeodactylum tricornutum* or *Rhodomonas baltica* (Patil et al. 2006).

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10. References

Abiusi, F., Sampietro, G., Marturano, G., Biondi, N., Rodolfi, L., D'Ottavio, M., & Tredici, M. R. (2014). Growth, photosynthetic efficiency, and biochemical composition of Tetraselmis suecica F&M-M33 grown with LEDs of different colors. *Biotechnology and Bioengineering*, *111*(5), 956–964. https://doi.org/10.1002/bit.25014

Agilent, T., Chromatograph, G., Spectrometer, M., Rtl, U., & Gc, S. (n.d.). Agilent 5973 inert GC / MS System Data Sheet GC / MS Agilent 5973 inert GC / MS System Data Sheet.

Analysis, C. (n.d.). The higher resolutin for particle sizing and counting-Multisizer 4 COULTER COUNTER.

Brown, M. R., Jeffrey, S. W., Volkman, J. K., & Dunstan, G. A. (1997). Nutritional properties of microalgae for mariculture. *Aquaculture*, *151*(1–4), 315–331. https://doi.org/10.1016/S0044-8486(96)01501-3

Fogg, G. E. & Thake, B., (1987). Algal Cultures and Phytoplankton Ecology. The University of Wisconsin Press. pp. 269. ISBN: 0-299-10560-1

Creswell, L. (2010). Phytoplankton Culture for Aquaculture Feed. *SRAC Publication*, (5004), 1–16. https://doi.org/10.1039/b914312b

D.R., V. W., & J.H., R. (2009). Impact of dietary fatty acids on cardiac arrhythmogenesis. *Circulation: Arrhythmia and Electrophysiology*, *2*(4), 460–469. https://doi.org/10.1161/CIRCEP.109.880773

Dhont, J., Dierckens, K., Stottrup, J., Stappen, M., Wille, M., & Sorgeloos, P. (2013). *Rotifers, Artemia and copepods as live feeds for fish larvae in aquaculture*. *Advances in Aquaculture Hatchery Technology*. Woodhead Publishing Limited. https://doi.org/10.1533/9780857097460.2.404

Enright, C. T., Newkirk, G. F., Craigie, J. S., & Castell, J. D. (1986). Evaluation of phytoplankton as diets for juvenile Ostrea edulis L. *Journal of Experimental Marine Biology and Ecology*, *96*(1), 1–13.

Epifanio, C. E. (1979). Growth in Bivalve Mollusks Nutritional Effects of 2 or More Species of Algae in Diets Fed to the American Oyster Crassostrea-Virginica and the Hard Clam Mercenaria-Mercenaria. *Aquaculture*, *18*(1), 1–12.

Fabregas, J., Abalde, J., & Veiga, M. (1984). Growth of the marine microalga. *Aquaculture*, *42*, 207–215.

FAO. (2016). *The State of World Fisheries and Aquaculture.*

Fidalgo, J. P., Cid, A., Lopezmunoz, I., Abalde, J., & Herrero, C. (1994). Growth and biochemical profile of

juvenile mussels (mytilus-galloprovincialis lmk) fed on different algal diets. *J.Shell.Res.*\r*Journal of Shellfish Research*, *13*(1), 67–75.

Grima, E. M., Camacho, F. G., Pérez, J. A. S., & Sánchez, J. L. G. (1994). Biochemical productivity and fatty acid profiles of Isochrysis galbana Parke and Tetraselmis sp. as a function of incident light intensity. *Process Biochemistry*, *29*(2), 119–126. https://doi.org/10.1016/0032-9592(94)80004-9

HAMMER, Ø., Harper, D. A. T. a. T., & Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica*, *4(1)*(1), 1–9. https://doi.org/10.1016/j.bcp.2008.05.025

Hjorth, M., Dahllof, I., & Forbes, V. (2006). Effects on the function of three trophic levels in marine plankton communities under stress from the antifouling compound zinc pyrithione. *Aquatic Toxicology*, *77*, 105–115.

Holmer, M., & Kristensen, E. (1992). Impact of marine fish cage farming on sediment metabolism and sulfate reduction of underlying sediments. *Marine Ecology Progress Series*, *80*, 191–201.

Johnson, K. R., & Admassu, W. (2013). Mixed algae cultures for low cost environmental compensation in cultures grown for lipid production and wastewater remediation. *Journal of Chemical Technology and Biotechnology*, *88*(6), 992–998. https://doi.org/10.1002/jctb.3943

Khozin-Goldberg, I., Iskandarov, U., & Cohen, Z. (2011). LC-PUFA from photosynthetic microalgae: Occurrence, biosynthesis, and prospects in biotechnology. *Applied Microbiology and Biotechnology*, *91*(4), 905–915. https://doi.org/10.1007/s00253-011-3441-x

Kim, G., Lee, C. H., & Lee, K. (2016). Enhancement of lipid production in marine microalga Tetraselmis sp. through salinity variation. *Korean Journal of Chemical Engineering*, *33*(1), 230–237. https://doi.org/10.1007/s11814-015-0089-8

Kurpan Nogueira, D. P., Silva, A. F., Araújo, O. Q. F., & Chaloub, R. M. (2015). Impact of temperature and light intensity on triacylglycerol accumulation in marine microalgae. *Biomass and Bioenergy*, *72*, 280–287. https://doi.org/10.1016/j.biombioe.2014.10.017

Laing, I., & Gil Verdugo, C. (1991). Nutritional value of spray-dried Tetraselmis suecica for juvenile bivalves. *Aquaculture*, *92*(C), 207–218.

Last.J.M . (1980) Th e foo d o f twenty specie s o f fish in th e west-centra l North Sea . Fish. Res. Tech. Rep., 60 , 4 4 pp .

Liu, C., & Lin, L. (2001). Ultrastructural study and lipid formation of Isochrysis sp. CCMP1324. *Botanical Bulletin of Academia Sinica*, *42*, 207–214. Retrieved from http://ejournal.sinica.edu.tw/bbas/content/2001/3/bot423-08.pdf%5Cnhttp://ejournal.sinica.edu.tw/bbas/content/2001/3/bot423-08-info.html

Maraldo, K., & Dahllöf, I. (2004). Seasonal variations in the effect of zinc pyrithione and copper pyrithione on pelagic phytoplankton communities. *Aquatic Toxicology*, *69*(2), 189–198.

Mauchline, J., Douglas, B., Blaxter, J.H.S., John Mauchline, and Tyler, P.A. (1998). The Biology of Calanoid Copepods. Elsevier Science, 1998. ISBN 9780080579566

Mc, M. (n.d.). MC 1000-OD Instruction Manual and User ´ s Guide for cultivation.

Michels, M. H. A., Slegers, P. M., Vermuë, M. H., & Wijffels, R. H. (2014). Effect of biomass concentration on the productivity of Tetraselmis suecica in a pilot-scale tubular photobioreactor using natural sunlight. *Algal Research*, *4*(1), 12–18. https://doi.org/10.1016/j.algal.2013.11.011

Moheimani, N. R. (2013). Inorganic carbon and pH effect on growth and lipid productivity of Tetraselmis suecica and Chlorella sp (Chlorophyta) grown outdoors in bag photobioreactors. *Journal of Applied Phycology*, *25*(2), 387–398. https://doi.org/10.1007/s10811-012-9873-6

Molina, E., Martinez, M. E., Sanchez, S., Garcia, F., & Contreras, A. (1991). Growth and Biochemical-Composition With Emphasis on the Fatty-Acids of Tetraselmis Sp. *Applied Microbiology and Biotechnology*, *36*(1), 21–25.

Nazar, A. K. A., Jayakumar, R., & Tamilmani, G. (2013). Recirculating aquaculture systems. *Regional Center of CMFRI*, 77–82.

Oconnor, W. A., Nell, J. A., & Diemar, J. A. (1992). The evaluation of 12 algal species as food for juvenile sydney rock oysters Saccostrea-commercialis (Iredale and Roughley). *Aquaculture*, *108*(3–4), 277–283.

Oilgae. (2016). *Comprehensive Report on Attractive Algae Product Opportunities*.

Patil, V., Källqvist, T., Olsen, E., Vogt, G., & Gislerød, H. R. (2007). Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquaculture International*, *15*(1), 1–9. https://doi.org/10.1007/s10499-006-9060-3

Pereira, D. C., Trigueiro, T. G., Colepicolo, P., & Marinho-Soriano, E. (2012). Seasonal changes in the pigment composition of natural population of Gracilaria domingensis (Gracilariales, Rhodophyta). *Brazilian Journal of Pharmacognosy*, *22*(4), 874–880. https://doi.org/10.1590/S0102-695X2012005000075

Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., … Varela, J. (2012). Polyunsaturated fatty acids of marine macroalgae: Potential for nutritional and pharmaceutical applications. *Marine Drugs*, *10*(9), 1920–1935. https://doi.org/10.3390/md10091920

Phatarpekar, P. V., Sreepada, R. A., Pednekar, C., & Achuthankutty, C. T. (2000). A comparative study on growth performance and biochemical composition of mixed culture of Isochrysis galbana and Chaetoceros calcitrans with monocultures. *Aquaculture*. https://doi.org/10.1016/S0044-8486(99)00227-6

Regan, D. L., & Ivancic, N. (1984). Mixed populations of marine microalgae in continuous culture: Factors affecting species dominance and biomass productivity. *Biotechnology and Bioengineering*, *26*(11), 1265–71.

Renaud, S. M., Parry, D. L., Thinh, L. Van, Kuo, C., Padovan, A., & Sammy, N. (1991). Effect of light intensity on the proximate biochemical and fatty acid composition of Isochrysis sp. and Nannochloropsis oculata for use in tropical aquaculture. *Journal of Applied Phycology*, *3*(1), 43–53. https://doi.org/10.1007/BF00003918

Romberger, H. P., & Epifanio, C. E. (1981). Comparative effects of diets consisting of one or two algal species upon assimilation efficiencies an growth of juvenile oysters, Crassostrea Ĺirginica. *Aquaculture*, *25*, 77–87.

Ryther, & Guillard. (2009). 藻类培养基- f/2 Medium. *Физика Земли*, *44*(1), 64–75. https://doi.org/10.1016/B978-0-444-53363-0.10034-9

Seckbach, J. (2015). *The Algae World* (Vol. 26). https://doi.org/10.1007/978-94-017-7321-8

Stiling, P. D. (2012). *Ecology: Global Insights & Investigations* (1st ed.). New York: McGraw-Hill.

Sun, Y., & Wang, C. (2009). The optimal growth conditions for the biomass production of Isochrysis galbana and the effects that phosphorus, Zn2+, CO2, and light intensity have on the biochemical composition of Isochrysis galbana and the activity of extracellular CA. *Biotechnology and Bioprocess Engineering*, *14*(2), 225–231. https://doi.org/10.1007/s12257-008-0013-8

Vu, M. T. T., Dou??tte, C., Rayner, T. A., Thoisen, C., Nielsen, S. L., & Hansen, B. W. (2016). Optimization of photosynthesis, growth, and biochemical composition of the microalga Rhodomonas salina???an established diet for live feed copepods in aquaculture. *Journal of Applied Phycology*, *28*(3), 1485–1500. https://doi.org/10.1007/s10811-015-0722-2

Walter, T.C. & Boxshall, G. (2016). World of Copepods database. Accessed at http://www.marinespecies.org/copepoda on 2016-12-20

Weiss, V., Gromet-Elhanan, Z., & Halmann, M. (1985). Batch and continuous culture experiments on nutrient limitations and temperature effects in the marine alga Tetraselmis suecica. *Water Research*, *19*(2), 185–190. https://doi.org/10.1016/0043-1354(85)90197-6

Whyte, J. N. C., Bourne, N., & Hodgson, C. A. (1990). Nutritional condition of rock scallop, Crassadoma gigantea (Gray), larvae fed mixed algal diets. *Aquaculture*, *86*(1), 25–40.

Appendix

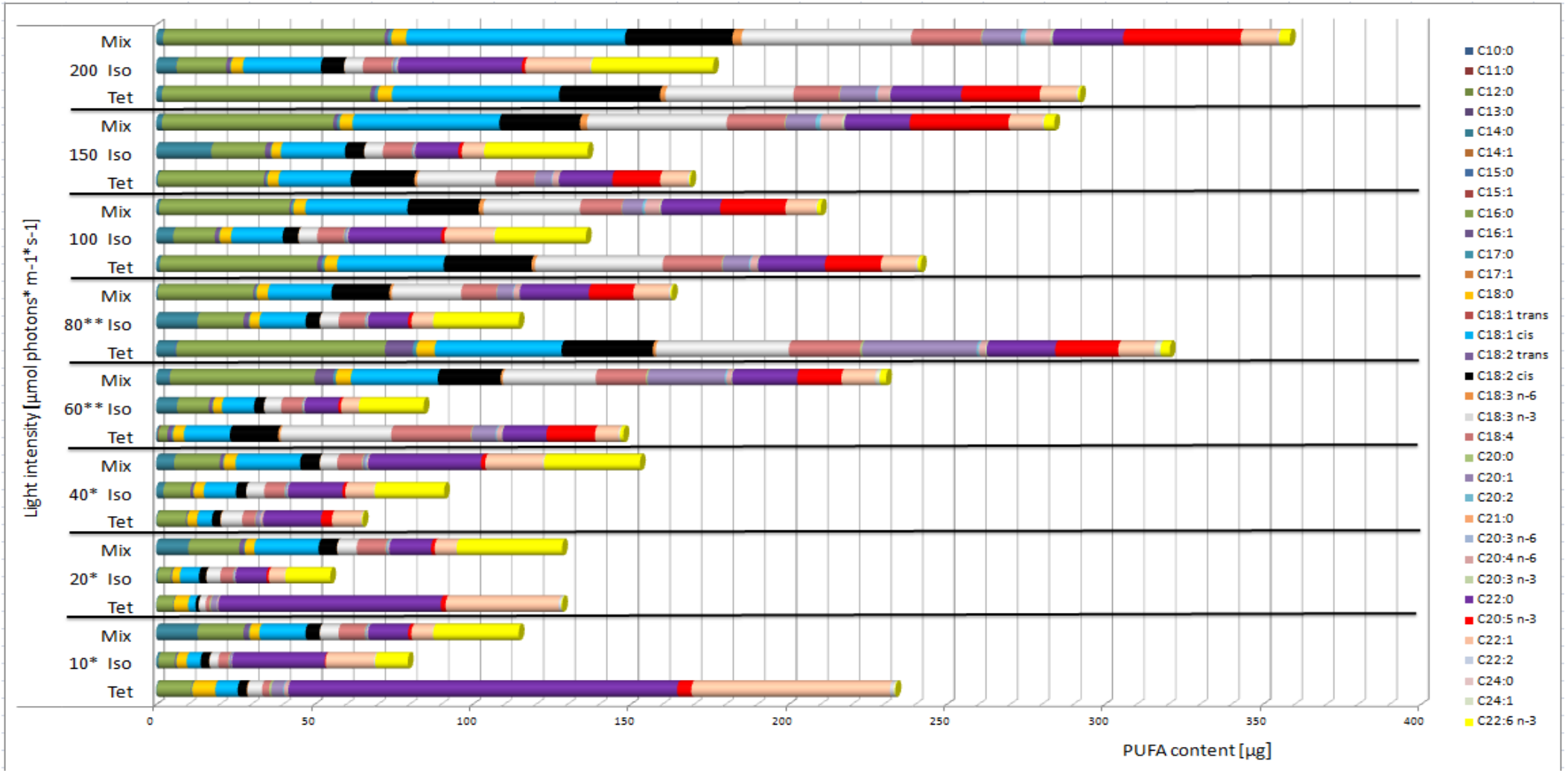

Figure: Bar chart representing quantity of all FA at different light intensities for the 3 cultures.

Light intensity [µmol photons* m-1*s-1]		FA	C10:0	C11:0	C12:0	C13:0	C14:0	C14:1	C15:0	C15:1	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1 tran	C18:1 cis	C18:2 tran	C18:2 cis
		Tet	0,00	0,00	0,02	0,00	0,06	0,00	0,03	0,00	4,63	0,06	0,09	0,00	3,11	0,02	3,01	0,00	1,24
	10*	Iso	0,00	0,00	0,00	0,00	0,86	0,06	0,08	0,00	6,46	0,46	0,21	0,00	4,07	0,00	5,48	0,02	3,40
		Mix	0,00	0,00	0,01	0,01	11,20	0,00	0,18	0,00	12,69	1,40	0,28	0,00	2,74	0,00	12,71	0,01	3,88
		Tet	0,00	0,00	0,02	0,00	0,08	0,00	0,04	0,00	4,06	0,11	0,09	0,00	3,52	0,00	1,80	0,00	0,78
	20	Iso	0,00	0,00	0,00	0,00	0,84	0,01	0,06	0,00	7,57	0,57	0,25	0,00	4,33	0,00	10,95	0,05	4,17
		Mix	0,00	0,01	0,00	0,00	7,79	0,00	0,13	0,00	12,51	1,18	0,24	0,00	2,35	0,00	15,83	0,00	4,50
		Tet	0,00	0,00	0,03	0,02	0,28	0,00	0,11	0,00	13,98	0,46	0,28	0,00	4,68	0,09	7,14	0,00	4,02
	40	Iso	0,00	0,00	0,00	0,00	2,45	0,00	0,10	0,00	9,37	0,89	0,25	0,00	3,53	0,00	11,15	0,04	3,49
		Mix	0,00	0,00	0,01	0,00	3,62	0,00	0,09	0,00	9,43	0,66	0,18	0,00	2,45	0,00	13,34	0,00	4,05
		Tet	0,00	0,00	0,00	0,00	0,29	0,02	0,07	0,15	1,86	0,92	0,33	0,02	2,30	0,00	9,84	0,00	10,34
	60	Iso	0,04	0,00	0,00	0,00	7,64	0,00	0,15	0,00	11,88	1,26	0,30	0,00	3,35	0,00	11,99	0,02	3,70
		Mix	0,01	0,00	0,00	0,00	1,67	0,00	0,17	0,00	19,84	2,57	0,36	0,00	2,04	0,00	11,92	0,00	8,60
		Tet	0,01	0,00	0,00	0,00	1,79	0,00	0,18	0,00	20,58	2,75	0,37	0,00	1,79	0,00	12,51	0,00	9,00
	80	Iso	0,00	0,00	0,01	0,01	11,20	0,00	0,18	0,00	12,69	1,40	0,28	0,00	2,74	0,00	12,71	0,01	3,88
		Mix	0,00	0,00	0,00	0,00	0,29	0,00	0,05	0,00	18,49	0,36	0,32	0,00	2,18	0,00	12,31	0,00	11,23
		Tet	0,00	0,00	0,00	0,00	0,40	0,00	0,06	0,00	20,61	0,59	0,35	0,00	1,65	0,00	13,93	0,00	11,48
	100	Iso	0,00	0,00	0,00	0,00	3,90	0,00	0,10	0,00	9,64	0,95	0,21	0,00	2,64	0,00	12,01	0,00	3,60
		Mix	0,01	0,00	0,02	0,00	0,38	0,00	0,05	0,00	19,60	0,26	0,32	0,00	1,80	0,00	15,35	0,00	10,72
		Tet	0,00	0,00	0,00	0,00	0,29	0,00	0,05	0,00	19,82	0,36	0,33	0,00	2,07	0,00	13,46	0,00	11,94
	150	Iso	0,00	0,00	0,02	0,01	12,57	0,00	0,17	0,00	12,46	1,23	0,22	0,00	2,21	0,00	14,87	0,00	4,43
		Mix	0,00	0,00	0,01	0,00	0,58	0,00	0,06	0,00	19,08	0,41	0,30	0,00	1,44	0,00	16,35	0,00	8,98
		Tet	0,00	0,00	0,00	0,00	0,56	0,00	0,06	0,00	22,55	0,48	0,33	0,00	1,54	0,00	18,09	0,00	10,93
	200	Iso	0,00	0,00	0,00	0,00	3,59	0,01	0,08	0,00	8,89	0,69	0,17	0,00	2,15	0,00	14,01	0,00	4,14
		Mix	0,00	0,00	0,02	0,00	0,54	0,00	0,05	0,00	19,53	0,31	0,30	0,00	1,29	0,00	19,31	0,00	9,47

Light intensity [µmol photons* m-1*s-1]		FA	C18:3 n-6	C18:3 n-3	C18:4	C20:0	C20:1	C20:2	C21:0	C20:3 n-6	C20:4 n-6	C20:3 n-3	C22:0	C20:5 n-3	C22:1	C22:2	C24:0	C24:1	C22:6 n-3
		Tet	0,12	1,94	0,96	0,29	1,62	0,12	0,02	0,03	0,43	0,07	52,59	1,93	26,87	0,32	0,09	0,24	0,07
10*		Iso	0,15	3,46	3,98	0,29	0,76	0,11	0,03	0,12	0,09	0,07	36,80	0,37	19,23	0,16	0,11	0,20	12,96
		Mix	0,07	5,13	7,19	0,15	0,23	0,17	0,02	0,17	0,13	0,08	11,04	0,93	5,79	0,10	0,08	0,00	23,60
		Tet	0,07	1,66	0,97	0,31	1,42	0,05	0,02	0,03	0,23	0,07	54,79	1,21	27,84	0,35	0,09	0,26	0,13
20		Iso	0,16	7,90	7,14	0,27	0,45	0,23	0,05	0,21	0,26	0,12	17,78	1,27	9,12	0,15	0,15	0,08	25,86
		Mix	0,09	4,79	7,11	0,13	0,18	0,20	0,02	0,19	0,16	0,05	10,27	0,90	5,27	0,06	0,06	0,00	25,98
		Tet	0,31	10,28	6,21	0,29	1,99	0,24	0,03	0,08	0,90	0,28	28,13	5,26	14,10	0,15	0,17	0,14	0,37
40		Iso	0,07	6,19	6,96	0,22	0,41	0,20	0,03	0,18	0,15	0,09	18,92	1,00	9,78	0,16	0,10	0,10	24,14
		Mix	0,07	3,55	5,09	0,18	0,51	0,20	0,02	0,17	0,21	0,06	23,29	0,89	11,90	0,12	0,07	0,07	19,80
		Tet	0,48	23,69	17,05	0,17	5,07	0,22	0,02	0,09	0,93	0,21	9,45	10,38	4,96	0,06	0,14	0,21	0,73
60		Iso	0,08	6,32	7,76	0,19	0,20	0,19	0,03	0,17	0,14	0,07	12,60	1,00	6,47	0,11	0,09	0,00	24,24
		Mix	0,30	12,72	6,89	0,20	10,58	0,18	0,02	0,09	0,58	0,14	8,91	6,06	4,58	0,07	0,09	0,51	0,90
		Tet	0,31	13,11	7,03	0,19	11,30	0,18	0,01	0,09	0,58	0,13	6,76	6,22	3,50	0,05	0,08	0,53	0,92
80		Iso	0,07	5,13	7,19	0,15	0,23	0,17	0,02	0,17	0,13	0,08	11,04	0,93	5,79	0,10	0,08	0,00	23,60
		Mix	0,46	13,45	6,79	0,10	3,13	0,09	0,01	0,09	1,02	0,10	13,42	8,62	6,87	0,15	0,12	0,09	0,26
		Tet	0,46	16,69	7,72	0,12	3,43	0,11	0,01	0,11	0,86	0,10	8,76	7,32	4,54	0,07	0,09	0,10	0,42
100		Iso	0,08	4,35	6,13	0,17	0,39	0,18	0,02	0,16	0,13	0,05	21,58	0,80	11,38	0,13	0,07	0,08	21,24
		Mix	0,70	14,53	6,26	0,08	3,04	0,33	0,01	0,13	2,13	0,21	8,89	9,82	4,55	0,04	0,11	0,04	0,60
		Tet	0,51	14,64	7,26	0,09	3,14	0,09	0,01	0,10	1,09	0,11	10,00	8,95	5,11	0,12	0,12	0,07	0,25
150		Iso	0,07	4,28	6,67	0,12	0,16	0,15	0,02	0,17	0,14	0,05	10,00	0,79	5,06	0,07	0,06	0,00	23,98
		Mix	0,78	15,55	6,48	0,09	3,32	0,46	0,01	0,10	2,39	0,27	7,25	11,01	3,74	0,03	0,11	0,04	1,17
		Tet	0,58	13,85	4,96	0,09	3,87	0,11	0,00	0,13	1,25	0,09	7,67	8,50	3,93	0,04	0,05	0,10	0,23
200		Iso	0,06	3,31	5,16	0,16	0,38	0,21	0,02	0,17	0,15	0,05	22,24	0,60	11,61	0,12	0,06	0,08	21,87
		Mix	0,80	14,96	6,16	0,08	3,43	0,34	0,01	0,11	2,14	0,22	6,22	10,37	3,19	0,05	0,11	0,03	0,96

Figure b: Table 1 and 2 show the content of all FA [µg/ml] that were analysed at different light irradiances for the 3 cultures.

Test for equal means			C18:2 cis	LA	
	Sum of sq	df	Mean squ	F	p (same)
Rows:	1220,54	7	174,363	4,2	0,01079
Columns:	3545,34	2	1772,67	42,7	1,10E-06
Error:	581,239	14	41,5171		
Total:	5347,11	23			

Test for equal means			C20:5 n-3	(EPA)	
	Sum of sq	df	Mean squ	F	p (same)
Rows:	434,552	7	62,0789	2,672	0,05572
Columns:	4886,94	2	2443,47	105,2	3,68E-09
Error:	325,235	14	23,2311		
Total:	5646,73	23			

Test for equal means			C18:3 n-3	ALA	
	Sum of sq	df	Mean squ	F	p (same)
Rows:	1731,9	7	247,414	2,909	0,04229
Columns:	8215,1	2	4107,55	48,29	5,22E-07
Error:	1190,91	14	85,0652		
Total:	11137,9	23			

Test for equal means			C22:6 n-3	(DHA)	
	Sum of sq	df	Mean squ	F	p (same)
Rows:	200,934	7	28,7048	0,9441	0,5047
Columns:	2710,16	2	1355,08	44,57	8,49E-07
Error:	425,651	14	30,4036		
Total:	3336,75	23			

Test for equal means			PUFA essential mean		
	Sum of sq	df	Mean squ	F	p (same)
Rows:	10549,1	7	1507,01	5,087	0,00476
Columns:	34740,1	2	17370	58,64	1,57E-07
Error:	4147,14	14	296,224		
Total:	49436,3	23			

Test for equal means					
	Sum of sq	df	Mean squ	F	p (same)
Rows:	68253,5	7	9750,5	4,029	0,01275
Columns:	243042	2	121521	50,22	4,10E-07
Error:	33877,2	14	2419,8		
Total:	345172	23			

Figure c: Two-way ANOVA without replication – test

RGR					
	Sum of sq	df	Mean square	F	p (same)
Light:	1,69123	7	0,241604	1,56	0,1703
Sp:	2,13281	2	1,0664	6,887	0,002347
Interactio	1,85445	14	0,132461	0,8555	0,6087
Within:	7,43247	48	0,154843		
Total:	13,111	71			

Figure d: Two – way ANOVA – test