Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Planning,
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Cultivation of *Chlorella vulgaris* in nutrient solution from greenhouse tomato production

- A possibility to reduce nutrient levels and produce commercially interesting metabolites

Odling av *Chlorella vulgaris* i näringslösning från tomatproduktion i växthus

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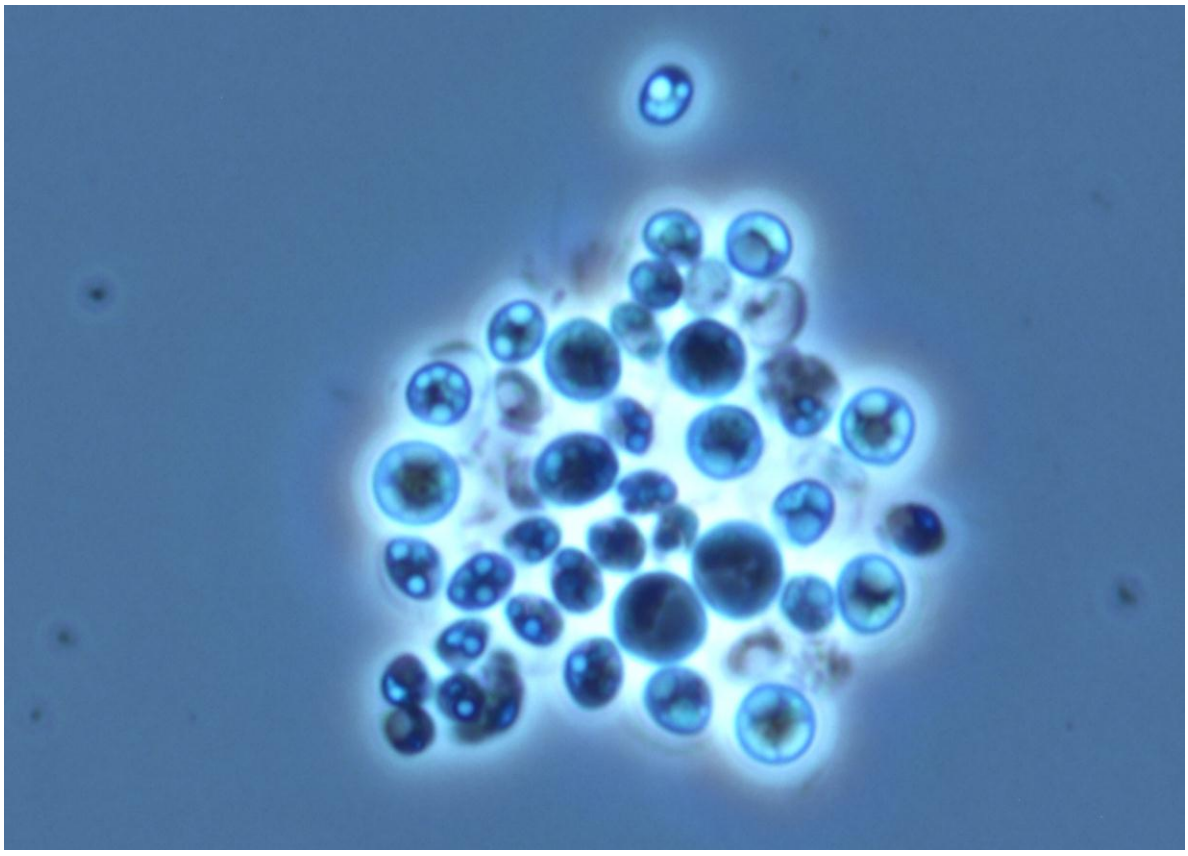
Stina Månsson



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ABSTRACT

The idea of using algae in different areas and exploring its possibilities is nothing new. Already in the early 1950s microalgae was explored as a possible food resource because of its high protein content. Lately there has been an increased interest in its potential for industrial use. Wastewater treatment is a possible use of algae since the algae grow well on the nutrients present in the water. This offers the possibility to grow algae for nutrient reduction and use the biomass for energy or animal feed since many alga species has a good nutritional value.

Approximately 800-1000 m^3 water is applied per 1000 m^2 of greenhouse area during one season in a tomato production. Out of this is about 20-25% drained off and become waste, if there is no recirculation system in the greenhouse. The nutrient composition of the drainage water varies over the season, but is generally high in nitrogen. Considering a sustainable development of the Swedish greenhouse industry, it is therefore important to reduce the nutrient levels in the water before it is drained away.

Chlorella vulgaris is a robust and fast growing microalgae species commonly cultivated and interesting regarding the production of secondary metabolites with health beneficial properties such as omega-3 fatty acids and carotenoids.

A successful alga production requires a high growth rate and a high biomass with a desirable composition e.g. distribution between fatty acids, proteins and pigments, which is influenced by several abiotic and biotic factors. For a grower all those parameters might be hard to control. However the primary purpose of the algae is to reduce the nutrients and less complicated deposition is therefore desirable. This might be as raw material for biogas or animal feed or possibly as biofertilizers.

Two experiments on *Chlorella vulgaris* capability, to grow and reduce nutrient levels in excessive water from greenhouse production, were performed. In experiment 1, Z8 (a standard medium for alga) was compared with an artificial nutrient solution, high in nitrogen. In experiment 2, Z8 was compared with a nutrient solution from a greenhouse tomato production. The growth of *C. vulgaris* was measured by optical density (OD) at 405 nm during two weeks. At the start and at the end of the experiments, nutrient analyses were performed to calculate the reduction. Also a fatty acid analyse was performed with gas chromatography (GC) in order to look for possible interesting fatty acids in *C. vulgaris*.

The results gave a significant reduction of nutrients, which indicate that *Chlorella vulgaris* can grow and reduce nutrient levels in a nutrient solution with a distribution that can be expected from a tomato production. On the other hand it is uncertain if *C. vulgaris* can compete strong enough in a nutrient solution that contains several other microorganisms.

The iron reduction in both experiments was close to 100% and it might be possible that the iron deficiency limited the growth.

The result from the fatty acid analysis was consistent with earlier reported results of fatty acid composition in *Chlorella vulgaris* and no longer fatty acids then 18:3 were found.

In a larger system outside the lab, the amount of biomass and the purity as well as the obtained nutrient reduction will all give a variable result from time to time and between growers due to all culture parameters that are hard to control and environmental conditions which will fluctuate.

SAMMANFATTNING

Idén att använda alger inom olika områden och utforska dess möjligheter är ingenting nytt. Redan på 1950-talet undersöktes möjligheterna att odla mikroalger för matproduktion på grund av dess höga protein innehåll. På senare tid har intresse för olika industriella appliceringar ökat. Behandling av avloppsvatten är ett möjligt användningsområde eftersom algerna växer bra när de konsumerar den näring som finns i vattnet. Detta erbjuder möjligheten att odla algerna i syfte att reducera näringen i vattnet och samtidigt ta vara på biomassan för t.ex. energi produktion eller djurfoder, eftersom många arter av alger har en bra näringsmässig sammansättning.

I en tomatodling i växthus tillsätts ungefär 800-1000 m^3 vatten per 1000 m^2 och säsong. Av detta är det cirka 20-25% som inte tas upp eller avdunstar. Närings förhållandet i vattnet varierar över säsongen men kvävenivåerna är normalt höga. Med hänsyn till en hållbar utvecklig inom den svenska växthusindustrin är det därför viktigt att reducera dessa höga näringsnivåer i vattnen innan det släpps ut.

Chlorella vulgaris är en robust och snabbväxande art av mikroalger som är vanlig i odlingar. Den är också intressant på så sätt att den kan producera sekundära metaboliter med hälsosamma egenskaper så som omega-3 och karotenoider.

En lyckad produktion av alger kräver en hög tillväxthastighet och en hög biomassa med önskvärd fördelning mellan fettsyror, protein och pigment. Detta påverkas av flera abiotiska och biotiska faktorer. Dessa faktorer är kanske inte alltid lätta för en odlare att styra. Dock är algernas huvudsakliga syfte är att reducera näringen i vattnet och någon typ av enklare avsättning för biomassan är därför önskvärt. Exempel på detta är råmaterial till biogas, djurfoder eller som biologiskt gödselmedel.

Två experiment genomfördes för att undersöka om *Chlorella vulgaris* kan växa och reducera näring i vatten från växthus. I experiment 1 jämfördes Z8 (ett standardmedium för alger) med en konstgjord näringslösning med hög kvävekoncentration. I experiment 2 jämfördes Z8 med en näringslösning från en tomatodling i växthus. Den optiska densiteten (OD) vid 405 nm användes för att mäta tillväxten hos *C. vulgaris* under två veckor. I början och slutet av båda experimenten gjordes en näringsanalys för att titta på näringsreduktionen i vattnet. En fettsyreanalys genomfördes också med gaskromatografi (GC) för att undersöka om det finns några intressanta fettsyror hos *C. vulgaris.*

Resultaten visade på en signifikant reducering av näringen. Det indikerar att *Chlorella vulgaris* kan odlas för att reducera näringsnivåer i överblivet vatten från växthus med tomatproduktion. Det är dock oklart om *C. vulgaris* kan konkurrera tillräckligt starkt i näringslösningar som innehåller många andra mikroorganismer.

Reduktionen av järn var nära 100% vid båda experimenten och det är möjligt att järnet begränsade tillväxten hos algerna i näringslösningen.

Resultatet från fettanalysen överensstämde med tidigare rapporterad fettsyresammansättning hos *Chlorella vulgaris* och inga längre fettsyror än 18:3 hittades.

I ett storskaligt system hos en odlare kommer mängden biomassa och dess renhet och lika så uppnådd näringsreduktion att ge ett varierande resultat från gång till gång och mellan olika odlare. Det beror på svårigheten att styra alla odlingsparametrar samt att omgivande förhållanden varierar.

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1 INTRODUCTION

The idea of using algae in different areas and exploring its possibilities is nothing new. Already in the early 1950s microalgae was explored as a possible food resource because of its high protein content (Pufelski et al., 2010; Becker, 1994). But lately there has been an increased interest in its potential for industrial use. As an example there is an extensive amount of articles dealing with the possibility to grow algae for production of biofuel (Hu et al., 2008; Putt, 2008). Algae are fast growing and can be cultivated in systems that don't require agricultural land and thereby it doesn't compete with the food supply (Mercer & Armenta, 2011). Wastewater treatment is another possible use of algae since they grow well on the nutrients present in the water (Larsdotter, 2006). This offer the possibility to grow the algae for nutrient reduction and use the biomass for energy or animal feed since many alga species has a good nutritional value. They also produce valuable secondary metabolites such as pigments and polyunsaturated fatty acids, which offers commercial applications within the medical-, cosmetic- and health industry. In some countries like Kina, Japan, India and USA there is a tradition of growing algae (Spolaore et al., 2005), but in Sweden it is still a rather unexplored area.

This study is a part of a larger research project lasting January 2012 to December 2012, at the department of horticulture, at SLU, Alnarp. The aim of the research project is to examine the possibilities to grow algae producing commercially interesting metabolites for nutrient reduction in nursery wastewater. Several greenhouse growers in Sweden are using hydroponic systems, where the greenhouse culture is grown only in a nutrient solution. In a hydroponic system the nutrient is added in a water solution and the excessive nutrient rich water is collected and recirculated or just drained away. It is desirable to have a closed system in the greenhouse where the water is recirculated, but far from all greenhouses in Sweden has this possibility today. Discharging the used nutrient solution, containing high levels of nitrogen and phosphor and sometimes also pesticide residues (Löfkvist et al., 2009), has a negative impact on the environment. According to the Swedish environmental code (Regeringskansliet, 2000) it is forbidden to let wastewater out into the field, water areas or the groundwater. This also includes water from greenhouses with high levels of nutrients (Hansson & Johansson, 2007). If not recirculated, the excessive water from a greenhouse, which in a tomato production of 2000 m^2 can be as much as 500 m^3 during a season (Hansson, 2003), therefore needs

to be cleaned in some way. One way to do this could be with microalgae since they reduce the nutrient level in the water as they grow.

1.1 Objectives

This report looks into the possibilities of growing the microalgae *Chlorella vulgaris* in order to clean excessive nutrient solution from greenhouses. *C vulgaris* is also interesting regarding the production of secondary metabolites with health beneficial properties. The main questions for this study are:

- How well does *Chlorella vulgaris* grow in a nutrient solution that can be expected as waste from a greenhouse with tomato production?
- What effect has the growth of *Chlorella vulgaris* on reduction of nutrients in the water?
- What interesting metabolites can be found in *Chlorella vulgaris* and how can those be utilized?

1.2 Limitations

This study focus only on the microalgae *Chlorella vulgaris,* which is a robust and fast growing species (Pufelski et al., 2010). There are several other algae that could be of interest in a study like this. Different algae have different requirement and the results of the present study are only valid for *C. vulgaris*. Also, there are several metabolites produced by *C. vulgaris* that could be of interest but this study focus on fatty acids and carotenoids. The study do not go into details about how the metabolites can be extracted and purified, since this is still an expensive and energy intensive process (Mercer & Armenta, 2011), neither is the harvest of the cells studied.

2 BACKGROUND

2.1 Greenhouse companies in Sweden

In year 2008 there were 784 greenhouse companies in Sweden with an average greenhouse area of 3 390 m^2 (SJV, 2008). Out of this is approximately 350 000 m^2 tomato production distributed on 198 companies. Between year 1984 and 2008 there has been a decrease in numbers of greenhouse companies and during the same period an increase of the average greenhouse area. Most companies, about 150, have a heated greenhouse in the range of 1000-2000 m^2.

For application and collection of water there are different systems, which can be used. For those growers who grow their plants in a substrate, normally channels are used, either on the floor, on tables or hanging from the sealing (Löfkvist et al., 2009). Some growers use a system with furrows on the ground where excessive water is collected and lead away. There are also growers that have drainage system underneath the greenhouse. According to a study over water collection systems in Swedish greenhouses, performed during the autumn 2006, approximately 2/3 of all greenhouses had some sort of collecting system (Löfkvist et al., 2009). In table 1 and 2 it is specified what collecting systems that were used and how the drainage water was used (only vegetable growers are included).

Another way to cultivate, which becomes more and more popular, is without a substrate. Instead only a nutrient solution is used. This is known as hydroponic systems and requires a recirculation system for the water. According to Löfkvist et al. (2009), the growers put little attention on what is happening to the drainage water. Considering a sustainable development of the Swedish greenhouse industry it is important to avoid leakage of the drainage water to the catchment.

Table 1. Systems used for water collection in greenhouses among vegetable growers in Sweden. The numbers are presented as percentage of companies and total greenhouse area it corresponds to.

Water collecting systems	Vegetables (% of companies)	Area m^2
Channels	24	290 000
Furrows	37	255 000
Other	6	29 000
No collection of water	38	210 000

(Löfkvist et al., 2009)

Table 2. Use of drainage water collected in Swedish greenhouses where vegetables are grown. The numbers are presented as percentage of companies and total greenhouse area it corresponds to.

The use of drainage water	Vegetables (% of companies)	Area m^2
Recirculation	19	268 000
Fertilization of field crops	26	191 000
Drained away	46	304 000
Other	10	36 000

(Löfkvist et al., 2009)

2.1.1 Nutrition composition of the wastewater from greenhouse production

Approximately 800-1000 m^3 water is applied per 1000 m^2 of greenhouse area during one season in a tomato production (Hansson, 2003). Out of this is about 20-25% drained off and become waste, if there is no recirculation system in the greenhouse, which result in an excessive loss of nutrients (table 3). The nutrient composition of the drainage water varies over the season, but from a tomato production a generally distribution is (mg/l): N 375-450, P 35-45, K 375-450, Mg 80-120, S 60-80 and Ca 350-450 with an Ec between 3.5-4.5 (Hansson, 2003). The excessive water needs to be taken care of and potential ways for this is filtration and recirculation in the greenhouse or fertilization of field vegetables. Either way requires tanks with appropriate size for collecting the water.

Table 3. Nitrate, phosphor and potassium added and removed through drainage in a common tomato production.

	N	P	K
Added (kg/1000 m^2/year)	180-220	23-32	240-290
Removal through drainage:			
kg/1000 m^2/year	60-85	6-8	60-85
%	30-40	20-25	23-30

(Hansson, 2003)

2.2 Algae

Algae are a large group of organisms, which has along with the evolution adapted to a wide range of habitats (Harwood & Guschina, 2008). They play an important role as primary producers in the ecosystem and includes around 30 000 species, ranging from unicellular to organisms that are several meters long (Hogg, 2005; Guschina & Harwood,

2006). All algae are eukaryotic and almost all have a cell wall consisting of cellulose. The algae are separated taxonomically mainly based on the pigments they possess, main components in their cell wall and storage compound. Some of the major groups are Euglenophyta, Pyrrophyta, Chrysophyta, Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae).

The group Chlorophyta includes both unicellular and multicellular organisms and are similar to the plants in several ways. They hold chlorophyll a and b and some carotenoids, their main storage compound is starch and their cell wall is made of cellulose (Harwood & Guschina, 2008). Just like plants, algae lives out of carbon dioxide and sun energy.

Microalgae are small organisms, normally unicellular. This heterogeneous group of organisms offers a rich source that could be applied and used for animal feed, food, cosmetics, fuel and pharmaceutical (Olaizola, 2003). On the other hand it is still a less studied group due to its largeness and only a few thousands strains are kept in collections. When it comes to the chemical content, only a few hundred has been evaluated and of those has a handful been cultivated for industrial purposes (Olaizola, 2003).

2.2.1 *Chlorella vulgaris*

The green alga *Chlorella vulgaris* (figure 1) is a commonly cultivated species, according to Spolaore et al. (2005), the annual dry weight production of *Chlorella* is about 2000 t.
It is a small green, micro alga only 2-10 μm in diameter and it has a high content of proteins. According to Spolaore et al. 2005, *C. vulgaris* is estimated to compose of 51-58% protein, 12-17% carbohydrate and 14-22% lipid (calculated as % of dry matter), but it depends on environmental conditions under which *C. vulgaris* has grown and variations can be seen. According to Putt (2008) the lipid content can be up to 30% under certain conditions. Seyfabadi et al. (2010) reported a protein concentration of 33-46% when performing experiments with different light regimes. *C. vulgaris* also produces other metabolites such as pigments and can be grown both autotrophically and heterotrophically (Seyfabadi et al., 2010). Autotrophic means that the cell collects the energy from the light through photosynthesis, while heterotrophic growth only requires a carbon source as energy (Larsdotter, 2006).

According to Putt (2008) *Chlorella vulgaris* has the potential to double in cell number every 8 hour at a temperature between 20-35°C during autotrophic growth, provided that nutrients and light is not limiting the growth.

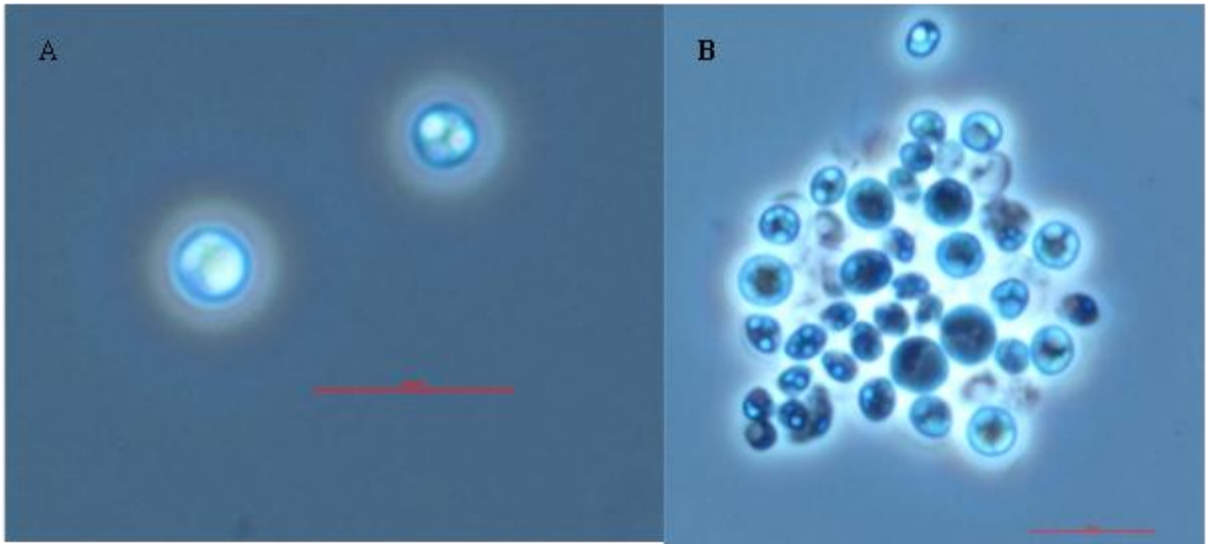


Figure 1. *Chlorella vulgaris* seen in microscope magnified 100 times. The red line is equal to 10 µm. A: Two separate cells. B: A group of cells, different in size and generation phase. (Photo S. Månsson)

2.2.2 Fatty acids in alga

Fatty acids are divided in saturated, unsaturated and polyunsaturated. The saturated ones has no double bindings like 16:0 (palmitic acid), unsaturated fatty acids has one double bound, e.g. 18:1 (oleic acid) and the polyunsaturated fatty acid (PUFA) has more than one double binding. Omega-3, which is a type of polyunsaturated fatty acids, has repeatedly been proved to be essential for the human body and important for preventing cardiovascular diseases (Rymer et al., 2009; Cannon, 2009; Harris, 2004).The omega-3 fatty acid alpha-linolenic acid (ALA) act as precursor for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which has an anti-inflammatory effect in the body (Cannon, 2009). Also omega-6 fatty acids are important for a balanced diet. Linoleic acid is an important omega-6 fatty acid that acts as a precursor for arachidonic acid (AA). Both omega-3 and omega-6 fatty acids have an essential role in the human body as components of membranes and receptor sites.

Neither omega-3 nor omega-6 can be synthesised by the human body and need to be assimilated through our diet. With the western diet of today the risk of omega-6 deficiency is minimal since the ratio of intake between omega-6 and omega-3 is suggested to be about 10:1, while the recommended ratio are 3:1 (Cannon, 2009).

Omega-3 is normally assimilated from fish through our diet. Fish oil is an excellent source of omega-3 since it contains already synthesised DHA and EPA, which is the active form of omega-3 in the body. The human body can convert ALA to DHA and EPA but very inefficient. Also, omega-6 and omega-3 go through the same delta-6 desaturase enzymatic pathway and thereby compete with each other (Cannon, 2009).

Today there is a huge market of nutritional supplements, which offering the possibility to consume fish oil. But increasing demand for omega-3, due to health aspects and increasing population, is raising the question about the sustainability of today's production of omega-3 (Cannon, 2009). Many fish species are threatened by extinction due to overfishing, which has a great impact on the ecosystem and on the living conditions for humans resident in certain areas. A necessary development is to change the human behaviour concerning fishing but also to find alternative sources for production of omega-3.

The fish does not synthesise DHA and EPA themselves but receive these fatty acids by eating microalgae (Harris, 2004), i.e. certain microalgae can be a possible source for essential omega-3 fatty acids. According to Cannon (2009) there is collected evidence that oil from microalgae can meet the human need for essential omega-3 fatty acids. Over the past years PUFAs in microalgae (both marine and freshwater) has been subjected to an increased research and commercial interest. Already some species are used to a large extent in order to produce PUFAs for aquaculture (Guschina & Harwood, 2005).

2.2.3 Pigments in *Chlorella vulgaris*

Except from fatty acids, other interesting secondary metabolites produced in *Chlorella vulgaris* are pigments. The main pigments are chlorophyll a and chlorophyll b but also carotenoids such as betacaroten, zeaxanthin and astaxanthin have been reported in *C. vulgaris* (Gouveia et al., 1995). The characteristic orange colour of carotenoids is due to its absorbance between 400 and 500 nm (Taiz & Zeiger, 2006). The synthesis of carotenoids is restricted to the plant world and found mainly in fungi, algae and higher plants. Animals need to assimilate those through the food (Gouveia et al., 1995).

In plants carotenoids acts as accessory pigments in photosynthesis and can protect against oxidation and light stress (Taiz & Zeiger, 2006). The biosynthetic pathway of

carotenoid formation is well understood but still its duration will be affected by abiotic stress factors such as salinity, light and nutrient deficiency (Gouveia et al., 1995).

Interestingly it has been reported that it is possible to stimulated production of carotenoids in *Chlorella vulgaris* when they are cultivated under certain stress conditions such as high light intensity and low nutrient levels (Gouveia et al., 1995).

2.3 Perspectives on algal cultivation in the horticultural sector

2.3.1 Maintains and requirement for the alga production

A successful alga production requires a high growth rate and a high biomass with a desirable composition e.g. distribution between fatty acids, proteins and pigments, which is crucial for the nutritional value of the alga biomass (Seyfabadi et al., 2010). This is influenced by several abiotic factors:

Light: The light is important since it drives the photosynthesis and both duration and intensity has an impact (Seyfabadi et al., 2010). The day length affects the growth rate indirect by affecting the circadian rhythm of photosynthesis, respiration and cell division. In natural conditions alga are exposed to changes in day length over the year. Studies have shown that response in growth rate and photosynthesis to day length varies with species, but also depends on other abiotic factors such as temperature and phosphor availability (Litchman et al., 2003). According to results from experiments performed by Litchman et al. (2003), the maximum rate of photosynthesis, in green algae, is very sensitive to phosphor limitations. Light can often be a limiting factor in algal mass cultivation system since the cells shadowing each other (Seyfabadi et al., 2010).

pH: A natural pH is desirable, or slightly acid, in order to prevent precipitation of nutrients (Becker, 1994). The pH will rise as the algae grow and consume the carbon dissolved in the water. If CO_2 is not added to the nutrient solution and the buffering capacity is low, pH will reach over 9 which will inhibit the algae growth.

Temperature: A suitable temperature range for most algae is 15-25^{o}C (Larsdotter, 2006). To high temperature will inhibit the growth, but this is normally not a problem in Sweden. Another problem, which are of concern in Sweden, might be photoinhibition that occur when light intensity is high but the temperature is low (Larsdotter, 2006).

Nutrients: Nitrogen is a very important nutrient since more than 10% of the alga biomass can consist of it and since it is a main problem during eutrophication. Algae can normally assimilate nitrogen both from nitrate, ammonia and nitrite, but the latter one is toxic at too high levels. The chemical composition of *Chlorella vulgaris* varies depending on N-source and concentration. According to Becker (1994) the lipid content of *Chlorella vulgaris* decreased with a higher concentration of nitrogen. At the same time there was an increase in protein content. The amount of lipids and proteins also varied depending on if the nitrogen was supplied as NH_4Cl or KNO_3.

Phosphor is another key nutrient important for energy transfer and synthesis of nucleic acids in the cell. Sulphur is important since it has an essential role in several amino acids. Also magnesium is important due to its key position in the chlorophyll molecule. Trace elements are important even though the required amounts are very small.

Carbon source: A desirable ratio, suitable for most algae, between N:P:C in the substrate are 8:1:50 or even 16:1:106 (Pufelski et al., 2010). About 50% of the alga biomass is made up of carbon and is therefore needed to a large extent for good growth (Becker, 1994). The algae need the carbon for the photosynthesis, just like plants. A carbon source could be added as CO_2 into the growing solution, or as an organic source such as sugar. The natural CO_2 present in the air is not sufficient to maintain optimal growth.

Biotic factors that will influent the rate of success are the presence of pathogens such as bacteria and viruses, predation by zooplankton and competition (de la Noue et al., 1992).

Today there are some systems worked out for large-scale production of microalgae. The two main systems are open ponds and closed photobioreactors that are divided into covered raceways and tubular reactors (Larsdotter, 2006). Open ponds are harder to control since they are more affected by the surrounding conditions and can easy be contaminated. On the other hand they are more easy to construct and operate and can be preferable from an economic aspects. A third and less tested way to grow the algae is by immobilise them. They are trapped in a solid medium allowing water and nutrient diffuse to the cell. The greatest advantage with this method is that the harvest is

taken care of, which normally can be rather complicated, especially for *Chlorella vulgaris* due to its small size (Pufelski et al., 2010).

2.3.2 Possible use of the alga biomass

As can be seen in figure 2 there are several potential ways to use the alga biomass. Some of them already established like food and food additives, and others still explored such as fuels and biogas. Becker (1994) suggests several specified applications of commercially produced algae e.g. food (protein supplements), feed (protein and vitamin supplement for poultry, cattle, pigs and fish), pigments (β-carotene as food colour and food supplement as provitamin A), fine chemicals (fatty acids, lipids, amino acids, vitamins), fuel (oil, biogas), hormones (auxins, gibberllins, cytokines) and others (bio fertilizer, waste treatment).

Since there is a lack of PUFAs in today's western diet, Rymer et al. (2009) suggest that one way to increase the consumption of PUFAs in the human diet is by the enrichment of staple foods e.g poultry meat. In fact, incorporation of alga into animal feed is a rather common application. About 30% of today's total algae production is used for this purpose. The main use is in aquacultures but the biomass can also be used in feed for pets or farm animals (Spolaore et al., 2005). Aspect important to consider for this kind of application is toxics, distribution between DHA, EPA and AA and vitamins present in the algae. Pigment content is also important and opens up for applications as natural food colorant, e.g. in orange juice and additives in animal feed mainly for poultry and fish. According to Gouveia et al. (1995) the biomass of *Chlorella vulgaris* can be used as natural colorant in eggs and trout flesh. For the purpose of colouring egg yolk it is important to have a maximum level of lutein/zeaxanthin in the biomass at harvest.

The greenhouse sector provides already existing recourses that could be used to grow algae e.g. competence among growers, water rich in nutrients as a waste product, greenhouses, artificial lightning etc. There is a growing market for alga in Sweden and companies specialised in production of algae start to emerge. One of the few Swedish companies for commercial production of alga started in year 2010 in Simrishamn (Simrisalg, n.d). The company offers algae for energy and environmental purposes, such as refining of carbon dioxide from fumes of recycling of nutrients from wastewater and greenhouse production. They also offer consulting service with in the area.

AstaReal AB is another company with Swedish originators. Now the company is hold by foreign owners but still with some production in Sweden. The company is

specialized in producing astaxanthin from the micro alga *Heamatococcus pluviailis,* used as supplement in food and animal feed (BioReal, n.d).

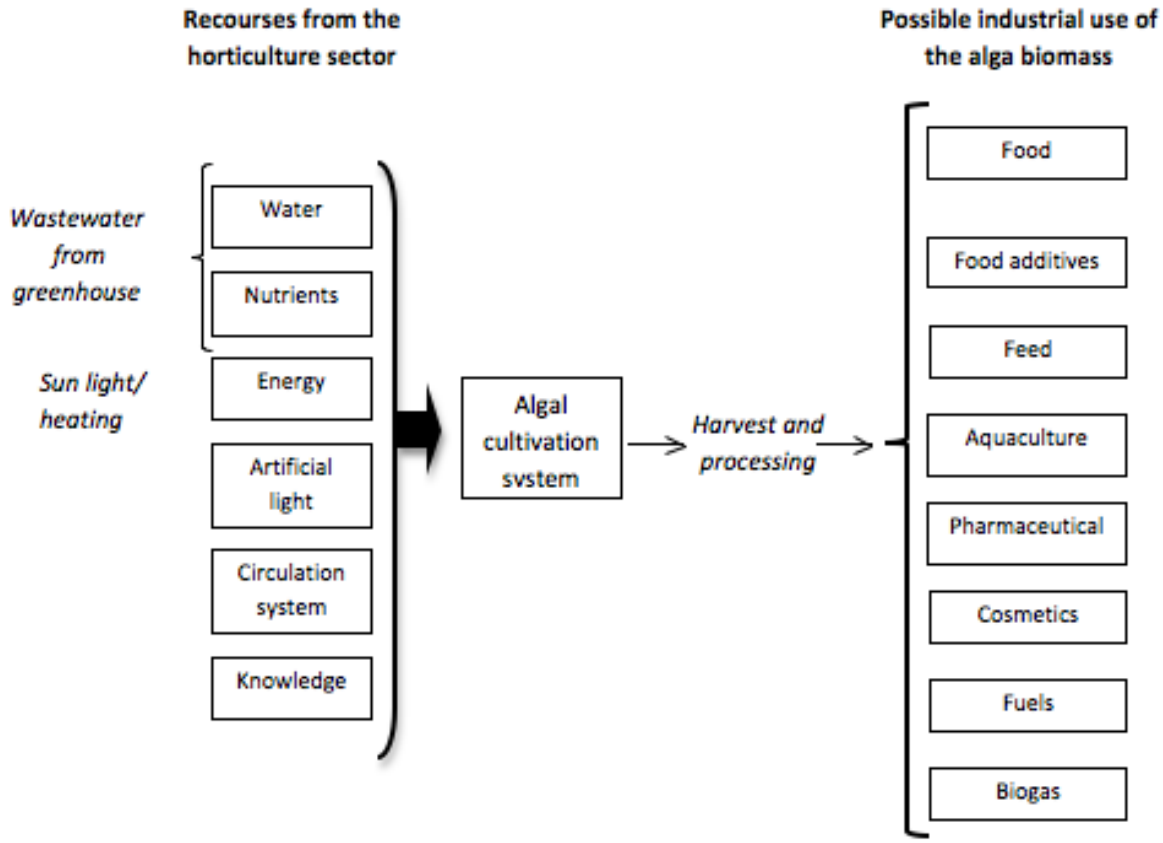


Figure 2. Resources available from the horticultural sector that could be used for algal cultivation and possible use of the recovered algal biomass.

3 MATERIAL AND METHODS

3.1 Microalgae

The microalga used in the experiments was *Chlorella vulgaris,* strain 211/11b, which is a green alga belonging to the division *Chlorophyt*a and class *Trebouxiophyceae* (CCAP, n.d). The strain has its origin from freshwater in Holland and was ordered from SAMS – CCAP (the Scottish association for marine science - culture collection of algae and protozoa). The strain was routinely cultured in Z8 (Niva, 1976) for long time storage. Z8 is a standard growth medium for green algae with the following composition of (mg/l): N 87 (provided as nitrate), P 5.6, K 14, Mg 2.4, S 3.2, Ca 14, Na 135.7, Cl 10.9 and Fe 5.7, trace element (µg/l): $Na_2WO_4 \cdot H_2O$ 3.3, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 8.8, KBr 12, KJ 8.3, $ZnSO_4 \cdot 7H_2O$ 28.7, $Cd(NO_3)_2 \cdot 4H_2O$ 15.5, $Co(NO_3)_2 \cdot 6H_2O$ 14.6, $CuSO_4 \cdot 5H_2O$ 12.5, $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ 19.8, $Cr(NO_3)_3 \cdot 9H_2O$ 4.1, V_2O_5 0.89, $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$ 47.4, H_3BO_3 310, $MnSO_4 \cdot 4H_2O$ 223.

3.2 Cultivation of *Chlorella vulgaris*

The experiments were performed in a greenhouse with a 16h/8h day/night regime with an added light, from 400 W high pressure sodium (HPS) lamps, of 100 µmol m^{-2} s^{-1}, measured as photosynthetic active irradiation. The irradiation on the greenhouse was 553 ± 83 Wh/m^2/d during experiment 1 and 1640 ± 670 Wh/m^2/d during experiment 2. It is estimated that out of this irradiation 60% reaches the greenhouse chamber. The temperature in the greenhouse was set to 20°C. Both experiments were performed as batch cultures in 0.5 l flasks for each sample. Four replicates were used in each treatment and the samples were continuously aerated to prevent settling of the algae.

3.2.1 Experiment 1

The growth in the standard medium Z8 (Niva, 1976) was compared with growth in an artificial nursery drainage solution, described in Christensen & Hansson (2010), with the following composition (mg/l): N 520 (provided as nitrate), P 63.6, K 442, Mg 44.7, S 88.2, Ca 499, Na 99, Cl 9.23, Fe 4.1, Mn 1.1, B 0.1, Zn 0.8, Cu 0.1, Mo 0.1 and pH 6.4. The start density was 2.0 x 10^5 cells/ml. The microalga growth was monitored every 24 hours by counting the cells in a haemocytometer and by reading the optical density at 405 nm with a spectrophotometer. The experiment was terminated after 20 days.

3.2.2 Experiment 2

The growth in the standard medium Z8 (Niva, 1976) was compared with growth in nutrient solution from a tomato cultivation with the following composition (mg/l): N 383 (provided as nitrate), P 33.9, K 387, Mg 67, S 36, Ca 335, Na 22.4, Cl 13.9, Fe 2.15, Mn 0.358, B 0.330, Zn 0.500, Cu 0.169, Mo 0.083 and pH 6.3. The start density was 1 x 10^4 cells/ml. The microalgae growth was monitored by reading the optical density at 405 nm at day 0, 2, 5, 7, 9, 12 and 14. At the same time was also pH and Ec measured. The evaporation was compensated with distilled water and the experiment was terminated after 14 days.

3.2.3 Analysis of nutrient reduction

For nutrient analysis the samples were filtered in GF/C filters followed by sterile filtration. The nutrient concentration were analysed by the company LMI AB, Helsingborg (table 4). LMI AB is an accredited laboratory that analysis the nutrition concentration with ICP-OES (inductively coupled plasma - optical emission spectrometer) according to the ISO 11885:2009 standard.

3.3 Production of metabolites

3.3.1 Fatty acids

For analysis of fatty acids alga biomass from experiment 1 was used. When stationary phase was reached *C. vulgaris* was centrifuged down at 3250 g for 20 min. The analyses were performed on both fresh and lyophilized biomass with gas chromatography (GC), by Anders Carlsson, SLU, Alnarp. The method is described in Banas et al. (2006) and gives a picture of the fatty acid distribution in a sample.

3.3.2 Induction of carotenoid production

Chlorella vulgaris was cultivated under the conditions described above for five days in Z8. The culture was then diluted with an equal volume of water (aq. dest.) and allowed to grow under LED light of six different colours, in order to examine the possibility to stimulate pigment production. The light colours used were blue (460 nm), green (525 nm), red (620 nm), yellow (585 nm), purple (eight parts of 660 nm and one part of 460 nm) and white (430-730 nm). Seven microtitreplates with 12 wells was used, one per each light treatment (green, blue, red, purple, yellow, white and one reference in greenhouse light). Each well were filled with 5 ml, 2.5 ml distilled water and 2.5 ml cell

solution, which gave a start density of 1.7 x 10^7 cells/ml. The six LED luminaries (Trädgårdsteknik AB, Ängelholm, Sweden) were switched on 24 h/day and placed at different distance, over a cart (0,5 x 0,5 m), in order to give the same irradiance of 60 µmol $\cdot m^{-2} \cdot s^{-1}$. Each luminar had a rated power of 90 W but measurements showed some differences between the actual powers. In order to avoid contamination from other wavelength, each cart was wrapped in plastic, black on the outside and white on the inside for maximal reflection.

Measurements of the spectra shows were each LED luminar has its wavelength pike (figure 3). The measurements were performed with a spectrometer (Li-Cor Li-1800, Lincoln, USA) by Göran Nilsson, Biotronen, SLU Alnarp. The white light included some yellow-green light and had a pike in the blue wavelength area.

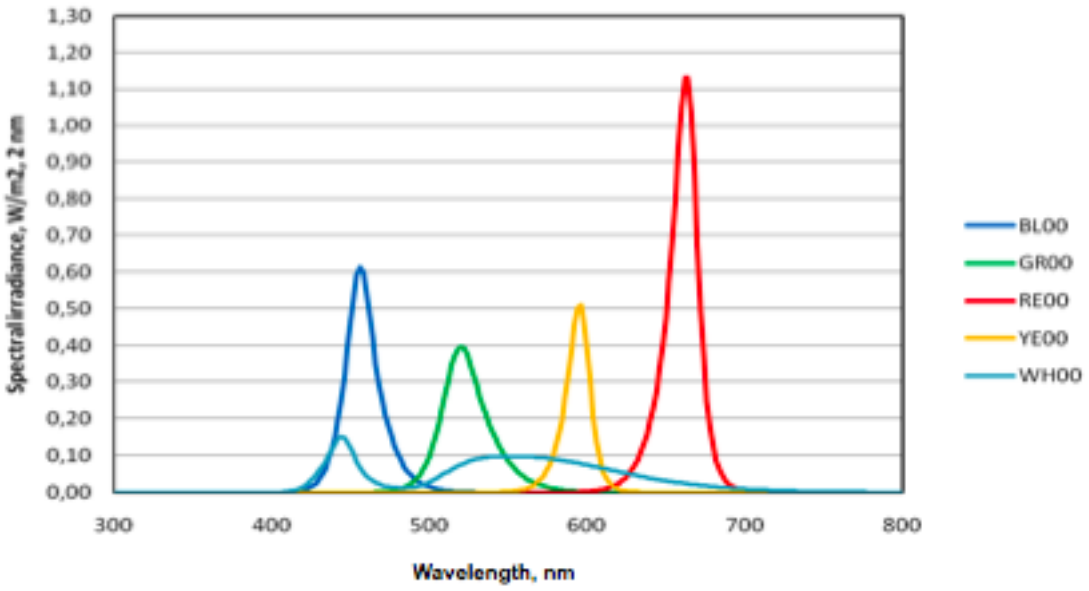


Figure 3. Distribution of the wavelength for five of the LED light used in the experiment. BL00 = blue, GR00=green, RE00=red, YE00=yellow and WH00=white.

The alga cells were counted in a haemocytometer at day 0, 6, 12, 13 and 23. After 23 days the concentration of chlorophyll a, b and carotenoids was determined as described by Seyfabadi et al. 2010. In short, 2.5 ml of cell solutions from each well were filtered with GF/C filter. Each filter with algae was put in a test tube of glass and 2 ml acetone (80%) was added. The test tubes were left in the cooler, on ice, over night. At day 2 the test tubes were placed in an ultrasonic bath for 90 s and 1.5 ml were moved from the test tube to an eppendorf tube. Each sample were centrifuged for 5 min at 10 000 rpm.

The absorbance was measured at 3 different wavelengths 470 nm, 647 nm and 663 nm. The following calculations presented by Lichtenthaler & Wellburn (1983) were used to determine the amount of pigment in the samples:

$$Chlorophyll\ a = 12.21*A_{663}-2.81*A_{647}$$

$$Chlorophyll\ b = 20.13*A_{647}-5.03*A_{663}$$

$$Carotenoid = (1000*A_{470}-3.27*C_a-104*C_b)/229$$

3.4 Statistic analyses

The greenhouse experiments on growth and nutrient reduction was performed with four replicates and repeated once. The reduction in nutrient concentration was compared with paired t-test (Minitab 16). The experiment with different light quality was performed with three replicates. The number of cells and amounts of pigment were analysed with a one-way ANOVA followed by Tukey's multiple comparison test (Minitab 16). Differences were considered significant at $P<0.05$.

4 RESULTS

4.1 Cultivation of *Chlorella vulgaris* in greenhouse

At the end of experiment 1 the obtained biomass, measured as dry weight, was 0.45± 0.13 mg/ml of nutrient solution. At the end of experiment 2 the obtained biomass, measured as dry weight, was 0.42± 0.04 mg/ml of nutrient solution. The growth rate in experiment 1 and 2 is shown in figure 4. In experiment 2 the exponential phase is more evident in Z8 compared to experiment 1. The growth in the nutrition solution was slow in both experiments. At the end of experiment 2, OD had not reached more then 0.17 compared to Z8 were OD was about five times higher.

The pH was monitored during experiment 2 (figure 4) and reached over 10 in Z8 before it started to drop again. In the nutrient solution the pH was much lower and a minor increase could be observed as the number of algae cells increased.

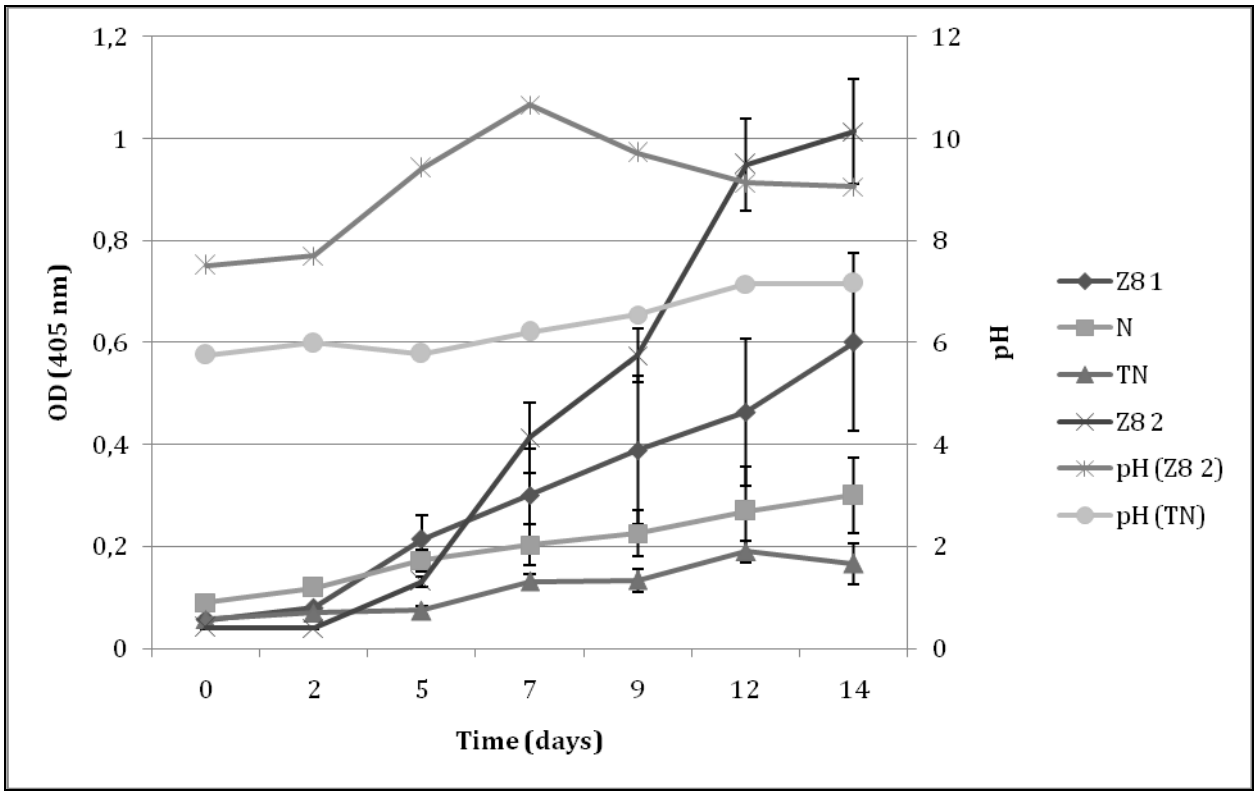


Figure 4. Average value of OD ± sd. measured over time in the two experiments. Z8 1=Z8 nutrition solution used in experiment 1. N=artificial nutrition solution used in experiment 1. TN=nutrition solution from tomato cultivation used in experiment 2. Z8 2=Z8 nutrition solution used in experiment 2. pH (Z8 2)=pH in nutrition solution used in experiment 2. pH (TN)=pH in nutrition solution from tomato cultivation used in experiment 2.

There were visual differences in colour between Z8 and the nutrient solution in experiment 1 (figure 5C).

Visual observations in microscope during experiment 2 confirmed the difference in viability and growth phase between Z8 and the nutrition solution (TN) (figure 5A and 5B). There were a higher cell density in Z8 at day 7, some of the cells were already dead and parts of broken cells can be observed in figure 5A. At day 14 there was a decline in OD in the nutrition solution (TN) and microorganisms larger then *Chlorella vulgaris,* and with two flagella, was observed (figure 5D).

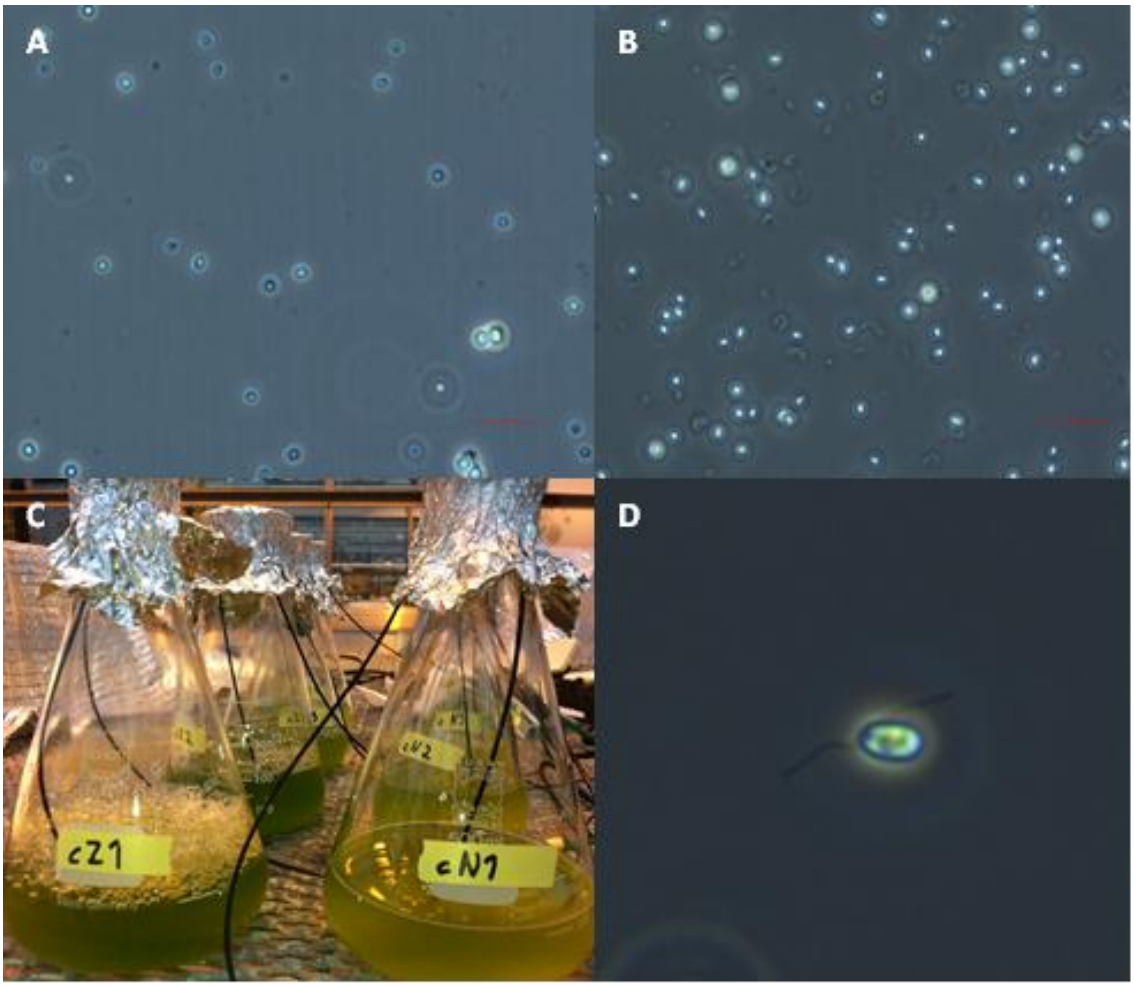


Figure 5. A: *Chlorella vulgaris* in nutrient solution, experiment 2. The red line equals 10 µm. B: *Chlorella vulgaris* in Z8, experiment 2. The red line equals 10 µm. C: colour difference between Z8 (left side) and nutrient solution (right side) during experiment 1. (Photo: S. Månsson) D: unidentified microorganism observed in the nutrient solution during experiment ,2 (photo: A. Persson).

4.2 Nutrient reduction

In experiment 1, a very low reduction of nitrogen was observed (table 4), however there was a large and significant reduction of phosphor. There was also a significant reduction in calcium, iron, manganese and zinc. A slightly increase in pH from 6.4 to 6.7 was observed, but the Ec (electric conductivity) was unchanged. In experiment 2, Ec was

monitored over time but no large changes were observed. At the end Ec had decreased by 0.66 and significant reduction could be observed for several of the nutrients. Nitrate, potassium, manganese and sulphur had been reduced by 15-20%. Phosphor had been reduced by nearly 50% and several of the micronutrients had a great reduction. However, there was a slight increase in boron and chloride. In both experiments a significant and large reduction of iron was observed.

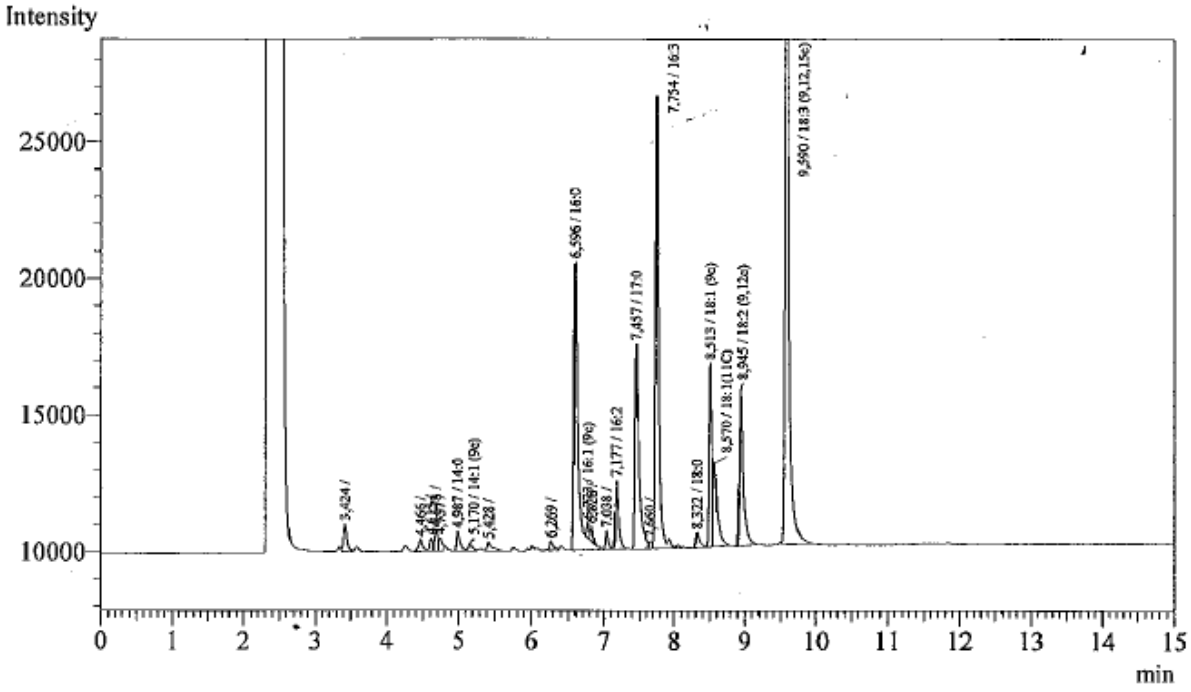
Table 4. Nutrient concentration in solution at the start and at the end of the experiments, including pH and Ec (electric conductivity) values. The obtained nutrient reduction is also presented as percentage. Reduction in nutrient concentration was analysed with paired t-test (Minitab 16) and significant decrease compared to the control is indicated with a letter.

	N^1	N^2	Reduction	TN^1	TN^2	Reduction
pH	6,4	6,7		6,3	7,3	
Ec (mS/cm)	4,70	4,57		3,66	3,00	
	mg/l	**mg/l**	**%**	**mg/l**	**mg/l**	**%**
NO3-N	520 a	504 b	3,1	383 a	321 b	16,2
NH4-N	<0,100	<0,100		3,6	0,5	86,1
P	63,9 a	15,3 b	76,1	33,9 a	18 b	46,9
K	442 a	455 a	-2,9	387 a	323 b	16,5
Mg	44,7 a	46,4 b	-3,8	67 a	56 b	16,4
S	88,2 a	95 b	-7,7	36 a	29 b	19,4
Ca	499 a	453 b	9,2	335 a	283 b	15,5
Na	99 a	108 b	-9,1	22,4 a	25,2 b	-12,5
Fe	4,06 a	0,004 b	99,9	2,15 a	0,002 b	99,9
Mn	1,1 a	0,27 b	75,5	0,36 a	0,09 b	74,9
B	0,12 a	0,15 b	-19,7	0,33 a	0,37 b	-12,1
Zn	0,75 a	0,46 b	39,3	0,50 a	0,23 b	60,0
Cu	0,12 a	0,12 a	0,0	0,17 a	0,13 b	24,9
Mo	0,101 a	0,107 b	-5,9	0,083 a	0,070 b	15,7
Cl	9,2 a	11,4 b	-23,8	13,9 a	15,7 b	-12,9

N^1=Nutrient solution used in experiment 1, analysed 2012-02-13. N^2=Nutrient solution used in experiment 1, analysed 2012-03-09. TN^1=Nutrient solution used in experiment 2, analysed 2012-03-29. TN^2=Nutrient solution used in experiment 2, analysed 2012-04-27.

4.3 Fatty acid composition

No difference was observed between the fatty acid profiles of fresh or lyophilized material. The fatty acid analyse of fresh biomass from *C. vulgaris* grown in Z8 is shown in figure 6. The dominating fatty acid was α-linolenic acid (18:3), which was 34% of total amount of fatty acids. Hexadecatrienoic acid (16:3) was 16% of the total amount of fatty acids and palmitic acid (16:0) was 13%. Other fatty acids found in the sample were oleic acid (18:1) and linoleic acid (18:2).



Peak#	Cmpd Name	Ret.Time	Area	Area%	Conc.	Unit
1		3,424	3668	1,2273	0,000	
2		4,466	1826	0,6109	0,000	
3		4,615	1259	0,4213	0,000	
4		4,678	2363	0,7907	0,000	
5		4,737	1547	0,5176	0,000	
6	14:0	4,987	2751	0,9203	4,999	nmol
7	14:1 (9c)	5,170	1018	0,3405	1,849	nmol
8		5,428	1593	0,5329	0,000	
9		6,269	1221	0,4084	0,000	
10	16:0	6,596	39941	13,3631	72,591	nmol
11	16:1 (9c)	6,773	1153	0,3857	2,095	nmol
12		6,828	1234	0,4127	0,000	
13		7,038	1823	0,6100	0,000	
14	16:2	7,177	7365	2,4639	13,385	nmol
15	17:0	7,457	27511	9,2044	0,000	nmol
16		7,660	1012	0,3385	0,000	
17	16:3	7,754	47239	15,8046	85,853	nmol
18	18:0	8,322	1968	0,6585	3,577	nmol
19	18:1 (9c)	8,513	18749	6,2728	34,075	nmol
20	18:1(11C)	8,570	11465	3,8357	20,837	nmol
21	18:2 (9,12c)	8,945	20293	6,7892	36,880	nmol
22	18:3 (9,12,15c)	9,590	101895	34,0910	185,189	nmol
Total			298894	100,0000		

Figure 6. Fatty acid composition analyse of *Chlorella vulgaris*. The upper part of the figure is an image illustrating the peaks for each fatty acid. The lower part specifies in a table the retention time, peak area and percentage for each fatty acid.

4.4 Growth and pigment formation under LED light

The growth in the different light conditions is presented in figure 7. The red light gave the fastest growth rate. At the end of the experiment the white light treatment had the highest concentrations of cells and the fewest cells were observed in the greenhouse treatment. This treatment is, however, not comparable due to different environmental conditions, such as day length and temperature. The cell number in the red LED

treatment differed significantly from the yellow and green treatment at day 6 and from the white, purple and green treatment at day 12. From day 13, no significant difference in cell number could be observed between any of the LED treatment or the greenhouse.

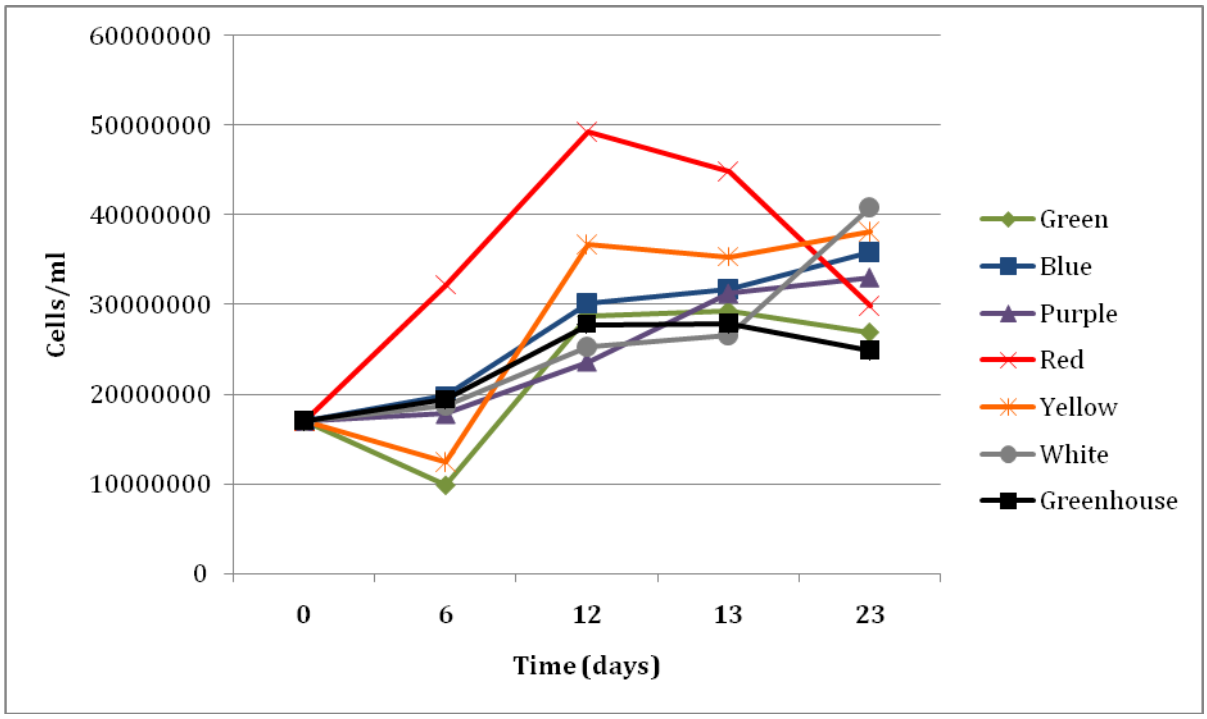


Figure 7. Number of cells in the LED light treatments over time.

There were visible colour differences between the light treatments (figure 8). The algal solution in the red LED light treatment had a lighter and less green colour. In table 5 the pigment concentration, calculated as described in material and method, are shown. The highest concentration of carotenoids was observed in the reference sample kept in greenhouse. The cells in the yellow and white LED light seemed to contain the highest concentration of carotenoids when the LED treatments were compared. The concentration of carotenoids was, however, generally low in all treatments. The highest levels of chlorophyll were found in the red and the green LED treatments, however, no significant differences could be observed between the treatments considering chlorophyll concentration.

Figure 8. Colour differences could be observed visually at the end of the LED experiment. From left: white LED treatment, yellow LED treatment and red LED treatment. (Photo: S. Månsson)

Table 5. Calculated values of chlorophyll a, chlorophyll b and carotenoids for each light treatment.

	Chlorophyll a		Chlorophyll b		Carotenoids	
	µg/ml	***µg/cell***	***µg/ml***	***µg/cell***	***µg/ml***	***µg/cell***
Green	1,223 a	4,6E-08	1,739 a	6,5E-08	0,021 ab	7,7E-10
Blue	0,677 a	1,9E-08	0,929 a	2,6E-08	-0,025 ab	
Purple	0,900 a	2,7E-08	1,317 a	4,0E-08	0,016 ab	4,9E-10
Red	1,365 a	4,6E-08	2,195 a	7,3E-08	-0,087 b	
Yellow	0,407 a	1,1E-08	0,483 a	1,3E-08	0,062 ab	1,6E-09
White	0,438 a	1,1E-08	0,944 a	2,3E-08	0,060 ab	1,5E-09
Greenhouse*	0,853 a	3,4E-08	0,476 a	1,9E-08	0,144 a	5,8E-09

* The greenhouse can only be seen as a reference and not be compared with, since those cells had different environmental conditions e.g. the day length, irradiance and temperature were different.

5 DISCUSSION

The faster growth rate in Z8 compared to the growth rate in the nutrient solution can be explained by an adaption period. Since the nutrient solution contained higher level of nutrients it might have been necessary for the cells to adapt to these levels before they could start their growth. All cells had been maintained in Z8 before the experiment started and were well adapted to these conditions. It might have be possible to receive a faster growth from start in the nutrient solution if the cells had been grown in this solution for acclimatized before the experiment, this approach will be tested in later experiments.

In experiment 1 an artificial nutrient solution, which had not been in contact with plant roots, was used. This experiment showed that growth was possible, however, there was a concern that a used nutrition solution would contain several other microorganisms and thereby provide too much competition for *Chlorella vulgaris.* To sterilize the nutrient solution would be too costly and the nutrient solution, used in experiment 2, was therefore filtered in order to remove larger grazing microorganisms. Using a non-sterile solution also means that the organisms have to compete for nutrients and light. In order to obtain a monoculture of a certain alga species, it has to be a very competitive and strong species. For nutrient reduction a monoculture is not required since other microorganisms also consume the nutrients during growth, but if the cells should be harvested and used for a certain purpose a monoculture is required.

In experiment 2, unidentified microorganisms were observed in the microscope and the decreasing value of OD after 14 days might suggest predation. According to Larsdotter (2006) protozoa and rotifers are the biggest threat to the alga in wastewater treatment. Bacteria can also be a problem but since the majority of algae are autotrophic and not assimilate organic carbon, unlike most bacteria, they do not compete for the same carbon source (Pufelski et al., 2010). However, they still compete for the nutrients such as nitrogen and phosphor. In this present study this competition is not believed to be of any problem since there were very high concentrations of nutrients available in the nutrient solution.

In order to establish *Chlorella vulgaris* as a monoculture in a nutrient solution from a greenhouse production, other microorganisms would preferable need to be killed. This could be done with e.g hydrogen peroxide since it has the advantage of being broken down fast. On the other hand, this will introduce an additional step for the grower and

dealing with chemicals is always negative from a work environmental point of view. Methods discussed in the literature (Larsdotter, 2006) are e.g decrease the pH to about 2 in the nutrient solution for a short time, which kills rotifers and protozoa but not algae, or increase the ammonia concentration, which can lower the contamination by zooplankton. However those methods are hard to implement in large scale cultivations (Larsdotter, 2006).

In the present work growth was monitored by measuring change in the optical density. This method does not tell anything about what is in the sample or the state of the cells but works well within a certain range. When cells start to die, OD will continue to increase to some extent, but it does not tell anything about the number of cells present any longer. It is also impossible to tell with this method whether the sample contain many small cells or fewer larger cells. This can be seen in a parallel experiment performed by Ardal (2012) where the density for cells of *Chlorella vulgaris* was measured at 405 nm with a spectrophotometer and also counted in a haemocytometer. It was clear that the density and number of cells over time follow each other in the beginning, but when stationary phase was reach the optical density continued to rise. Already before the stationary phase was reached OD was higher in Z8 then in the nutrition solution. As discussed by Ardal (2012) this had to do with the fact that the cells in Z8 were larger, possibly indicating a lack of a certain nutrients in the nutrient solution. In fact, also the unsatisfactory growth in the nutrient solution might suggest a deficiency. Although the solution contains high concentrations of nutrients, the balance between the nutrients is not optimized for algal growth and therefore there is a risk that some nutrient becomes limiting.

Iron has an important role in the photosynthetic electron transport over the membranes (Behrenfeld et al., 2006). According to Liu et al. (2007) increased attention has been put on the function of iron on microalgae growth over the last years. However the article also point out that, as far as they know, it is still unknown if iron deficiency is one of the main factors limiting alga growth productivity in batch cultures. In both experiments in this study the iron reduction was close to 100%. Therefore it might be possible that the iron deficiency limited the growth. It might also be that the observed colour difference between Z8 and the nutrient solution in experiment 1 had something to do with a nutrient deficiency.

The experiments in the present study were performed in a simple cultivation system, in small scale and the results in an up scaled system might have looked different. De la Noue et al. (1992) pointed out the fact that it is a great difference between small-scale trails in the laboratory compared to outdoor large-scale systems where it is hard to control both the abiotic and biotic factors influencing the growth of microalga due to large variations in effluent composition and environmental conditions. On the other hand those simple conditions used in the present study are more or less equivalent with the reality in a greenhouse company. There is natural sunlight, quite often artificial light to some extent and nutrient solution is not sterile. Still this is only in small scale and in order for it function in a greenhouse and on a larger scale several issues has to be solved. However, as stated by Olaizola 2003, "scale up is an engineering problem, not a biological one". The application has to be fast and easy for the grower and give a result worth the effort. In some way circulation, airing and harvest has to be worked out.

In this small-scale experiment, centrifugation can be used to separate the cells from the nutrient solution. This method is both too complicated and economically ineffective in a large-scale cultivation (Larsdotter, 2006). Suggested methods for harvest from the literature are e.g. sedimentation, filtration or immobilised systems.

If the weight from the obtained biomass in this study were used to calculate the potential biomass that could be reached in an average greenhouse company with tomatoes, it would approximately be 180 kg of alga biomass measured as dry weight. This calculation is done with a greenhouse of 2000 m^2 tomato production where 1000 m^3 water/1000 m^2/year are applied with a loss of 20%. This will give $4*10^8$ ml nutrition enriched wastewater per season. Assume that the obtained biomass as dry weight is the same as in experiment 1, 0.45 mg/ml, this will give 180 kg of alga biomass over one season. If 20% of this dry weight is lipids and the result from the fatty acid analyse in this study is used, this will give about 12 kg of α-linolenic acid (18:3).

Since optimal algae growth require a high ration of carbon (Becker, 1994), the airing in this experiment is not enough since air contain a relatively low concentration of CO_2. It would therefore been possible to obtain a higher biomass in the experiment if CO_2 were added. The reason why it was not added is the effort to keep the cultivation system as simple as possible.

The large reduction of phosphor in experiment 1 is probably due to a precipitation of phosphor with calcium and maybe also with iron. In this experiment several of the nutrients looked like they have increased but this has to do with the fact that there was no compensation for the evaporation, which led to a concentration of the nutrient solution during this experiment. In experiment 2 the significant reduction shows that it is possible to remove nutrients from greenhouse water by using algae. Still it is a very small reduction and inefficient considering the total concentration. Possibly a system with a repeated growth and harvest would be potential development.

Considering phosphor in experiment 2, a slightly cloudy colour was observed in the nutrient solution after start. This might be a precipitation of phosphor due to the airing. If there is a precipitation also in this experiment this will be misleading since the result will reflect a greater reduction in phosphor then the algae actually has accomplished. However, the potential to precipitated phosphor by an increase in pH due to algal growth can possibly be of interest for reduction and recovery of phosphor.

There is a discussion about which start density of algae that is suitable for an effective nutritional removal. According to Lau et al. (1994), who observed the nutrient removal in waste water with three different inoculum densities of *Chlorella vulgaris*, the larger inoculum density of $1x10^7$ cells/ml gave the best result. This result was explained by the fact that the efficiency of the nutrient removal is correlated to the physiological state of the cells, the higher alga density, the faster growth and thereby a faster removal. In this current study a lower start density was used based on the believe that it is more sustainable to produce the biomass from the nutrients present in the actual system. The obtained nutrition removal (table 4) might appear unsatisfying in this present study but too much attention should not be put on the percentage number. Lau et al. (1994) reported a nitrogen reduction close to 90%, but the actual amount of nitrogen present in the water at the beginning of the experiment were 10 times lower the in this study.

The fact that excessive water from greenhouses contains high levels of nutrients and the fact that fare from all growers have a purification or recirculation system, makes it even more urgent to find possible and sustainable solutions.

If the grower should use alga to reduce the nutrition level in the excessive water, deposition for the alga biomass is needed. Otherwise it just becomes a waste product.

One way is to sell the biomass to a specialized company, but this requires that there is a company like that. It requires a fairly large amount of biomass in order to make it economically feasible. It also requires high quality of the biomass i.e. purity and right chemical composition. As taken up in the background of this study, environmental conditions and culture parameters affect the chemical composition of the algae at harvest. For a grower all those parameters might be hard to control. However the primary purpose of the algae is to reduce the nutrients and less complicated deposition is therefore desirable. This might be as raw material for biogas or animal feed or possibly as biofertilizers.

The result from the fatty acid analysis is consistent with earlier reported results of fatty acid composition in *Chlorella vulgaris* (Petkov & Garcia, 2005). Due to lack of time, the fatty acids composition from experiment 2 has not been analysed yet. But probably it would have been some difference between the results in experiment 1 and 2 due to the fact that the nutrient solution used in experiment 2 had been used for tomato cultivation and thereby had been in contact with the root area, which has an intense microbial life. The fatty acid analyse would therefore have included some other microorganisms like bacteria, which has another fatty acid composition. Fatty acids with odd numbers e.g. 15:0 is a clear sign that the sample is contaminated by bacteria (Petkov & Garcia, 2005).

The problem with contamination and thereby reports about wrong fatty acid composition of *Chlorella vulgaris* are discussed by Petkov & Garcia (2005). There are no long fatty acids (>20 carbon) to be found in *C. vulgaris*, instead those fatty acids mainly appear in marine algae (Petkov & Garcia, 2005).

The fact that no longer fatty acids then 18:3 were found makes the algae biomass less valuable as feed or food. According to Harris (2004), α-linolenic acid (18:3) (ALA) is mainly used as energy in the human body. It is a precursor of DHA but only about 1-9% is converted. Consume ALA in comparison to already synthesized DHA is therefore rather ineffective.

Since light intensity has been shown to affect pigment composition in *Chlorella* (Seyfabadi et al. 2010) we determined to examine if it was possible to induce pigment formation by different light quality. The reason why the cell solution was diluted and kept in an abnormally long photoperiod was with the intention to stress the cells.

According to Gouveia et al. (1995) the biosynthesis of carotenoids in algae can be affected by stress e.g. salinity, light and nitrogen availability.

For the cells in the LED experiment there were a visible difference between the treatments (figure 8), and a pigment extraction was therefore performed. The result from this extraction did not correspond to the visual appearance of the sample, which indicated a higher carotenoid production in the red treatment. Since the lights had different wavelength a difference in growth and development could be expected due to the fact that certain wavelengths are easily absorbed during the photosynthesis. No literature was found on the subject, which indicates that it is still a new and unexplored subject. Other articles (Seyfabadi et al., 2010; Litchman et al., 2003) only pointing out the fact that light intensity and durability has an important impact on the growth, but obviously this also should include the quality of the light.

The cells kept in greenhouse cannot be compared with those under the LED-light since those differences might have to do with variance in day length conditions, irradiance and temperature. When focusing on the cells under the LED light both visual differences and variation in pigment composition were observed. The obtained amount chlorophyll a was very low in all treatments (table 5) compared to results reported by Seyfabadi et al. (2010). At an irradiance level of approximately 60 μmol $\cdot m^{-2} \cdot s^{-1}$, the same as in this experiment, reported levels of chlorophyll a were about ten times higher than the obtained results in this study. Also a few of the results from the pigment measurement had a negative value. This is likely an effect of the method. The approach and calculations might not have been suitable for the small amount cell solution used and therefore the obtained results might not reflect the actual levels of pigment in the cells. An improvement could be to use a larger amount cell solution and to be more careful with exposing the cells to light since carotenoids are easily broken down by light when outside of the cell. The question still remains why there was a clear visible difference observed in the red light, though the method possibly can be blamed as discussed above, further experiments are needed.

6 CONCLUSIONS

6.1 Conclusions

- *Chlorella vulgaris* can grow and reduce the nutrient levels in a nutrient solution with a distribution that can be expected from a tomato production. A nutrient solution from a real tomato production includes several other microorganisms, despite of filtration, and that is a problem. It is uncertain if *C. vulgaris* can compete strong enough under those conditions.

- It is possible to obtain a significant reduction in nutrient levels by cultivation of *Chlorella vulgaris* under simple conditions in a nutrient solution from greenhouse tomato production. The initial nutrient levels though, which should be reduced are high and will influence the result.

- The amount of biomass and the purity as well as the obtained nutrient reduction will all give a variable result from time to time and between growers due to all culture parameters that are hard to control and environmental conditions which will fluctuate.

- There are interesting metabolites in *Chlorella vulgaris* such as carotenoids that can be used for different industrial purposes. The fatty acids on the other hand, to be found in *C. vulgaris* is not long enough to be truly interesting.

6.2 Further studies

The experiments from this study can be developed in several ways, some examples are:

- maintain the alga in a nutrient solution that are sterile filtrated, instead of Z8, in order for the algae to adapt
- add CO_2
- examine the effect of adding iron

This study is performed with only one strain of *Chlorella vulgaris* and others can be examined. It might also be possible to use genetic modification (GM) in order to improve the quality and concentration of desirable metabolites produced by *C. vulgaris.*

The use of LED light is still a rather unexplored area regarding algae and might be used as a tool in order to control the production of specific metabolites. That requires detailed knowledge about how microalgae respond to certain wavelengths. One way to begin is to cultivate larger volumes of algae under LED light of different wavelengths for pigment and fatty acid analyses.

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