FACULTY OF
BIOSCIENCE ENGINEERING

Study of microalgae suspensions as potential biostimulants for sustainable applications in agriculture

Thesis

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List of abbreviations

Abbreviation	Description
ABA	Abscisic acid
BR	Brassinosteroid
CEC	Cationic exchange capacity
CLPs	Cyclic lipopeptides
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
EBIC	European Biostimulant Industry Council
EC	Electrical conductivity
EH	Enzymatic hydrolysis
EH + HPH1C	Enzymatic hydrolysis + 1 cycle of high pressure homogenization
EH + HPH3C	Enzymatic hydrolysis + 3 cycles of high pressure homogenization
GA	Gibberellic acid
HPH1C	1 cycle of high pressure homogenization
HPH3C	3 cycles of high pressure homogenization
HPHmin	Minimal parameters of high pressure homogenization
HS	Humic substances
MBCA’s	Microbiological control agents
NPK fertilizer	Nitrogen, Phosphorus and Potassium fertilizer
NT	No disruption treatment
NVZ	Nitrate vulnerable zone
PBR	Photobioreactor
PGPM	Plant growth promoting microbes
PH	Protein hydrolyzates
PMMA	Polymethyl methacrylate
SICP	Silicium containing products
SPAD	Soil Plant Analysis Development
SWE	Seaweed extracts
TKN	Total Kjeldahl Nitrogen
WWTP	Wastewater treatment plant

Abstract

Natural biostimulants, such as microalgae, have been gaining interest in modern agriculture since they have the potential to enhance crop performance, nutrient use efficiency and resilience to environmental stress. Microalgal biostimulants can play a crucial role in addressing sustainability challeges since they can reduce the dependency on non-renewable fertilizers and pesticides, which is in line with the EU farm to fork strategy (EU Green Deal).

The aim of this work is to investigate the potential of microalgae *Chlorella vulgaris* and *Tetradesmus obliquus* as sources of biostimulants through *in vivo* evaluation. Therefore, the effect of both microalgae on lettuce seedlings was studied by evaluating morphological parameters, such as length of the leaves, number of leaves, fresh and dry weight of the leaves, leaf surface area, and leaf chlorophyll content. The experimental trials were performed by growing lettuce seedlings on black peat substrate (enriched with an NPK fertilizer). The microalgal cells were submitted to different treatments for cell lysis by using different high pressure homogenization (HPH) strengths (HPH3C, HPH1C or HPHmin), enzymatic hydrolysis (EH) or EH combined with HPH. Three mircoalgal concentrations, being 2.0, 0.5 and 0.1 g L^{-1} were prepared with water and the treatments were applied weekly on the substrate. Twelve replicates of lettuce were used per treatment. The results showed that the application of *T. obliquus* suspensions did not affect the lettuce growth substantially, compared to the negative control. However, *C. vulgaris* disrupted with EH + HPH1C 2.0 g L^{-1} (enzymatic hydrolysis combined with 1 cycle of HPH) positively influenced the lettuce growth, by increasing particularly the length of the leaves, dry weight of the leaves and leaf surface area.

Introduction

Recently the total human population reached 8 billion and in 2050 there will be more than 9 billion people (Grafton et al., 2015). Because of this, food production will be even more challenging. This begs the question: how to produce as many crops as possible in an efficient and sustainable way? Agriculture has extensively used N, P, K fertilizers and pesticides for a long time. Furthermore, an overuse of manure has often led to nitrogen-rich-run-off into water courses, polluting rivers, lakes and coastlines. Many areas in Europe are now restricted by the Nitrate Directive and the Nitrate Vulnerable Zone (NVZ) legislation (EEA, 2020). Since Greenpeace detected illegal pesticides and maximum residue levels that exceeded thresholds in Spanish vegetables in Almeria in 2007, the use of pesticides in this province has been reduced by half in the next four years following this scandal (Acebedo et al., 2022). As pesticides have been proven to have harmful effects on the environment and human health, reducing pesticide use has become a goal shared by many countries and a significant concern in public policies (Lee et al., 2019). Moreover, quality control of crops and awareness of farmers about soil health also played a role in the reduction of pesticide use, since farmers want to achieve better and higher productivities.
As part of the Green Deal, Europe wants a decrease in the use of classical fertilizers by 20% and pesticides by 50% by 2030 (Fetting, 2020). To reach this reduction, companies are developing biostimulants to stimulate crop growth and increase the plant resilience (Rouphael et al., 2020). La Bella et al. (2021) demonstrated in a foliar spray experiment a positive effect of *C. vulgaris* on lettuce at the shoot level, but no significant differences at the root level were found.

1. Literature review: What are biostimulants?

A plant biostimulant is a product whose function is to stimulate plant nutrition processes independently of the product's nutrient content. The product should improve one or more of the following characteristics of the plant or the plant's rhizosphere (du Jardin, 2015):

(A) nutrient use efficiency
(B) tolerance to abiotic stress
(C) quality traits
(D) availability of confined nutrients in the soil or rhizosphere

According to the European Biostimulant Industry Council (EBIC), the following characteristics set biostimulants apart from conventional crop inputs: (i) they operate through different mechanisms than those of fertilizers, independently of the presence of nutrients in the products; (ii) they act only on the plant's vigor and not directly against pests or diseases; and (iii) they stimulate crop production in addition to nutrition and protection (De Saeger et al., 2020).

Biostimulants have thus no direct action against pests and are therefore not considered to be pesticides (Calvo et al., 2014). A biostimulant is a product of biological origin that improves plant productivity as a consequence of properties of the complex of constituents. The rationale for biostimulants is related to either the « Stress Hypothesis » or the « Microbiome Hyposthesis » (Rouphael et al., 2020). The **Stress Hypothesis** states that the crops' yield rarely reaches its full potential because of one or other form of abiotic stress: lack of nutrients, drought, temperature, frost, salinity, UV (He et al., 2018; Lucini et al., 2015). An important consideration is: how can we predict the occurrence of stress? Good environmental monitoring and plant monitoring systems combined with appropriate statistics is necessary. It is important to know the crop and its production weaknesses. Biostimulants contribute to the system's resiliency by helping the plant to access and utilize nutrients and water efficiently, enabling plants to tolerate or mitigate these stresses more effectively (Bhupenchandra et al., 2022).

The **Microbiome Hypothesis** states that the microbes in the environment have beneficial effects on crop growth primarily by modulating plant responses to stress. The partnership between plant and microbes is formed providing a mutual benefit. Plants and microbes have co-evolved for approximately a billion years. Microbes rely on plants for energy and often manipulate plant roots in order to obtain organic nutrients such as sugar and lipids. On the other hand, organisms like mycorrhiza-forming fungi provide chelated nutrients (P, Ca, Mg, Zn, N) and water to the plants. Mycorrhizae are usually divided in either endo- and ectotypes. Endotypes live inside and outside the roots and cannot live without the plants. Ectotypes are located outside the root and can survive without the plants. The mycorrhizal fungi increase with their hyphae the vascular surface area within the soil to access, absorb and conduct water and nutrients to the plant root (Rouphael et al., 2020).

1.1 Classes of biostimulants

The main classes of biostimulants are:

=>Humic substances
=>Protein hydrolysates and amino acids
=>Minerals (silicon and phosphite, a reduced form of phosphate)
=>Plant Growth Promoting Microbes (PGPM): mycorrhizae, bacteria & fungi
=>Plant extracts
=>Seaweed
=>Microalgae biomass or suspensions

1.1.1 Humic substances (HS)

Humic substances (humic acid, fulvic acid and humins), are long carbon chains, which are end points of marine, freshwater, and soil organic degradation. They can directly solubilize soil minerals, acting like chelates or micelles.
Humic substances are split up by extraction in humic acid, fulvic acid and humin, with their salts being called humates and fulvates. Soft brown coal with a high oxidation degree is a source of humic substances. When pulverized brown coal is treated with an alkaline

solution, the alkali-insoluble fraction is called humin. However, humic and fulvic acids are soluble in an alkaline solution. Upon acidification, humic acid precipitates but fulvic acids remain soluble (Canellas et al., 2015). Several studies have shown that HS promote plant growth, by increasing nutrient uptake and crop resistance to stress (Chen & Aviad, 1990; Rose et al., 2014).

Table 1. Different humic substances (HS) with their characteristics (Canellas & Olivares, 2014).

Fulvic acid/ Fulvates	Most soluble, lowest molecular weight fraction, used in foliar sprays and soil applications
Humic acid/Humates	Higher molecular weight fraction, typically used in soil applications
Humins	Highest molecular weight fraction, used in soil applications only

Humic substances can act like chelates or micelles. Their presence in the soil increases the cationic exchange capacity (CEC) and the nutrient holding capacity of the soil for molecules such as:

- Cations: K, Ca, Mg, NH_4 (Sonon et al., 2020)
- Micronutrients: Fe, Cu, Mn, Mo, Zn (Dhaliwal et al., 2019)
- Phosphorus complexes with metal ions: Fe, Al, Mg (Ibrahim et al., 2022)

1.1.2 Protein hydrolyzates (PH)

Protein hydrolyzates contain organic acids and amino acids (e.g., glutamic acid and glycine) that can bind minerals (chelates) (Jacob et al., 2022). Chelating Ca results in a stronger vascular system and more pectine in the cell wall. HS and PH can increase lateral root formation. Through the control of plant molecular and physiological processes, which promote growth, enhance yield, and minimize the effects of abiotic stress on crops, it has been shown that PHs frequently play important roles as biostimulants (Colla et al., 2017; Malécange et al., 2023). According to Lachhab et al. (2014), soybean and casein hydrolysates applied on grapevine induced immune responses and resistance against *Plasmopara viticola*, the etiological agent of downy mildew.

1.1.3 Silicium containing products (SICP)

Silicium containing products like AgSil16H from Certis form physical barriers resisting powdery mildew and inhibiting oviposition of thrips and leafminers. They also have sunshine reflective properties reducing temperature, drought stress and sun burn (Savvas & Ntatsi, 2015).

1.1.4 Microbiological control agents (MBCAs)

Microbiological control agents are used as agents against phytopathogens through competition for resources, through production and excretion of metabolites (antibiotics, cell wall degrading enzymes, siderophores), mycoparasitism, induction of defense responses (systemic acquired resistance, induced systemic resistance, and hypersensitive response). Among the potential biocontrol agents, *Bacillus* and *Pseudomonas* are considered to be excellent genera for their wide applicability, diverse natural habitats and various modes of action (Ballio et al., 1996; Fazle Rabbee & Baek, 2020; Köhl et al., 2019). *Bacillus* (*Bacillus velezensis amyloliquefaciens*) and *Pseudomonas* can produce many bioactive molecules including several cyclic lipopeptides. Cyclic peptides are polypeptide chains where the amino acid residues are covalently linked to generate the ring. Cyclic peptides are classified according to the types of bonds within the ring (Bender Carol et al., 1999). The C-terminal carboxylic acid is cycled with a side chain of serine or threonine to create a lactone ring. (Lee & Kim, 2015). Cyclic lipopeptides (CLPs) are composed of a fatty acid tail linked to a cyclized oligopeptide. CLPs such as surfactins, fengycins/plipastatins and iturin possess antifungal, antibacterial, cytotoxic or surfactant properties, that can destroy microbial membranes. One of the ways the CLPs work is by integrating into the membrane forming pores, which results in leakage and an imbalance in the ionic potential across the membrane, which is followed by cell lysis. This is typically seen in the syringomycins secreted by *Pseudomonas syringae* (Lee & Kim, 2015; Raaijmakers et al., 2006). Some CLPs with this mode of action are presented in Figure 1.

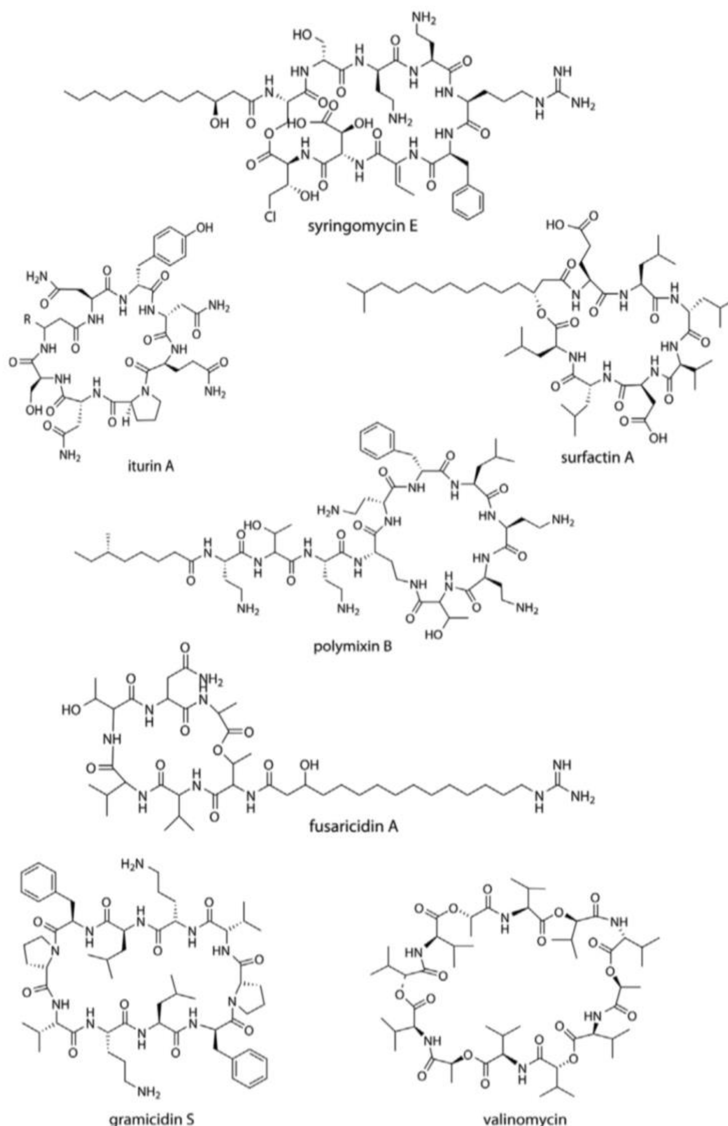
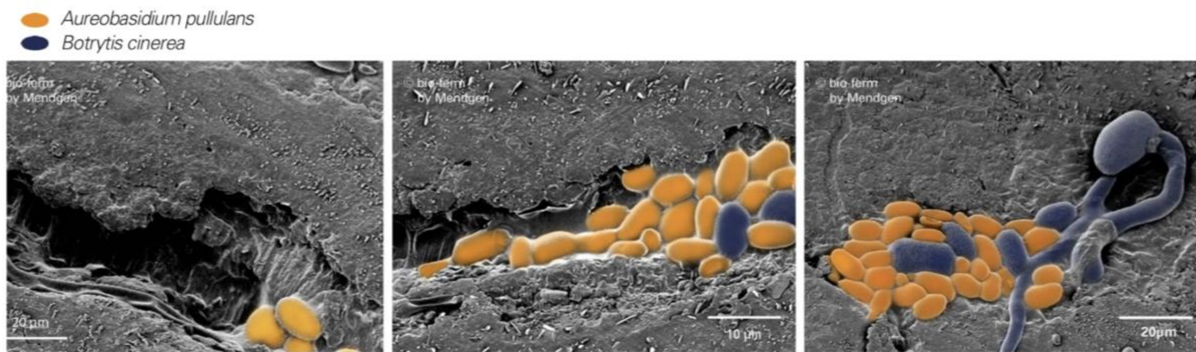


Figure 1. Molecular structures of cyclic lipopeptides that lyse the microbial cell membrane (Lee & Kim, 2015)

All *B. velezensis* strains produce three families of cyclic lipopeptides: surfactins, fengycins and iturins and a variety of other metabolites (Moreno-Velandia et al., 2021). Botector® is a biotechnological fungicide for the control of gray mold (*Botrytis cinerea*) in grapes, berries and tomatoes. Botector® is based on two strains of the species *Aureobasidium pullulans*, DSM 14940 and 14941. The efficacy of *A. pullulans* against different pathogens (e.g. *Erwinia amylovora*, *Botrytis cinerea*, storage pathogens) is based on its antagonistic activity by competing successfully with pathogens for nutrients and space (Weiss et al., 2014).This competition is shown in Figure 2.



1. Microscratches on the plant's surface provide a natural entry point for gray mold (*Botrytis cinerea*). These scratches are colonized immediately after applying the highly effective microorganisms (*Aureobasidum pullulans*).

2. The rapid proliferation of *Aureobasidium pullulans* consumes available nutrients and inhibits the development of Botrytis

3. The microscrach is sealed by *Aureobasidium pullulans*, which acts as a natural shield and prevents *Botrytis cinerea* from infecting the plant.

Figure 2. Scanning electron micrograph: Aureobasidium pullulans in competition with Botrutis cinerea, colonizing a microscratch on the plant surface (Bio-Ferm, 2017)

Ampelomyces quisqualis (*Ascomycota pleosporales*) is the most studied biocontrol agent against powdery mildew/oidium (*Erysiphe necator*), a disease caused by ascomycote fungi (order *Erysiphales*) that is easily recognized by the white powdery spots on the leaves. *Ampelomyces* is a parasite of powdery mildew. It infects and forms pycnidia (a spherical asexual fruiting body) in the hyphae and conidia of the disease-causing *Erysiphe necator* (Angeli et al., 2013). The biopesticide AQ10® (Intrachem) is a water-dispersible granule containing 58% (w/w) *Ampelomyces quisqualis* strain M-10 (minimum of 5.0 x 10^9 spores/g). Germinating spores suppress the developing mildews via hyperparasitism: *A. quisqualis* takes the nutrients from its host (Berrie & Xu, 2021; European Food Safety et al., 2017). Several *Trichoderma* (*Ascomycota*, *Hypocreales*)-based fungicides (*T. harzianum and T. virens, T. asperellum* and *T. gamsii*) have been developed by BioWorks (Rootshield and Rootshield Plus) that colonize and protect the root zone (Tyśkiewicz et al., 2022).

1.1.5 Seaweed extracts (SWE)

Seaweed extracts are promising biostimulants. Also termed macroalgae, seaweed are macroscopic, multicellular organisms having a maximum length of 65 m. The species are a diverse array of macroalgae and often divided into red (Rhodophyta), green (Chlorophyta) and brown (Ochrophyta) algae (Battacharyya et al., 2015; Khan et al., 2009). The most widely researched seaweed, used for plant biostimulants is the brown intertidal seaweed *Ascophyllum nodosum*. Intertidal seaweeds are exposed to unfavorable conditions such as extreme variation in temperature, light and salinity, causing them to produce different stress-related compounds. They contain polysaccharides, minerals, oils, fats, antioxidants, acids, vitamins, pigments and hormones (El Boukhari et al., 2020). Different extraction methods have been used to obtain these metabolites. For example, blending and hydrating seaweeds in the presence of water with the solid residues removed through filtration results in an extract rich in phytohormone-like activity (Crouch & Van Staden, 1992). Treating chopped seaweed with acidic solutions (sulphuric, nitric or hydrocloric acids) gives a liquid rich in fucose-containing polysaccharides (Flórez-Fernández et al., 2018). Treating chopped seaweed with alkaline solutions (potassium or sodium hydroxide) breaks down complex polysaccharides into smaller, lower-molecular weight oligomers (Craigie, 2011).

In Northern Europe, mostly brown algae, such as *Ascophyllum nodosum*, are used for the production of biostimulants. Kelp or rockweed (*Ascophyllum nodosum*) harvested along the Northern Atlantic coast are able to yield extracts containing carbohydrates (44.7% of dry weight) mainly in the form of laminarins, fucans and alginates. They also display relatively large amounts of amino acids and protein (5.2% of dry weight), which might be an interesting alternative for beef and soy as a source of protein in animal feed, phenolics (1.4% of dry weight), lipids (3% of dry weight) and other compounds (13% of dry weight) (Moreira et al., 2017; Shukla et al., 2019). In Figure 3, an overview of the positive effects of seaweed extracts on the plant and soil system is presented (Ali et al., 2021).

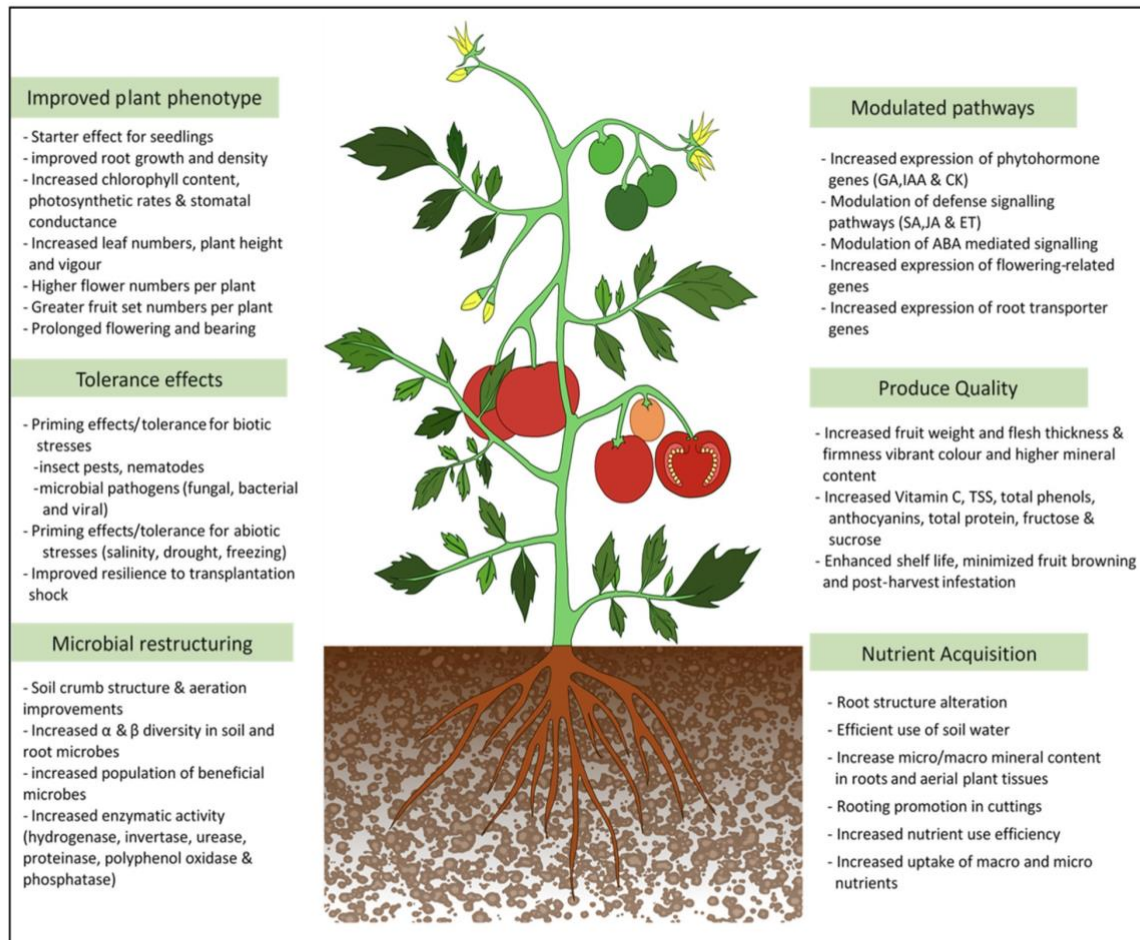


Figure 3. Overview of the positive effects of seaweed extracts on the plant and soil system (Ali et al., 2021).

The application of SWE has shown beneficial effects as, for example: (a) better germination and rooting, (b) greater root and foliage density, (c) increased nutrient uptake, (d) presence of beneficial microbes, and (d) better flowering and fruiting quality. The composition of seaweed depends on parameters such as location, season of harvesting and type of extraction method. The intertidal habitat (variation from low tide exposure with variable temperatures and desiccation to high tide and high salinity conditions) is believed to be responsible for their abiotic stress tolerance. The exact molecular basis of improved growth and stress adaptation of crops induced by *Ascophyllum nodosum* extract treatment proves difficult to unravel (Ali et al., 2021; Sujeeth et al., 2022).

1.1.6 Microalgae

Microalgae are a wide group of mostly photosynthetic phytoplanktonic, benthonic or terrestrial organisms that include cyanobacteria and other eukaryotic organisms (green algae, euglenoids, diatoms and others), which can grow in marine or freshwater systems. Several microalgal genera (*Chlorella*, *Dunaliella*, *Haematoccocus*, *Isochrysis*, *Nannochloropsis*, *Porphyridium* and *Arthrospira*, which is often known as "Spirulina") are industrially exploited as microalgae-based commercial products (Ferreira de Oliveira & Bragotto, 2022; Hachicha et al., 2022).

Application of microalgae as biostimulants in agriculture is done mainly in two ways, by adding its biomass or obtaining its aqueous extracts to be applied. Microalgae as whole or lysed cells, cell extracts, specific metabolites, metabolites alone or in combination with active ingredients show an elevated potential to be used for agricultural purposes as biostimulants and biofertilizers. Studies indicate that microalgae contain plant growth-promoting substances such as auxins, cytokinins, amino acids, vitamins, polyamines (spermine and spermidine), and polysaccharides (β-glucan) from their primary metabolism (Chiaiese et al., 2018). The paper of González-Pérez et al. (2021) refers to the use of different microalgal species as biostimulants for crops like tomato, water cress, bean, cucumber, lettuce, wheat and pepper. Data based on the use of microalgae biostimulants on several plant species show a common response, including better root growth, enhanced nutrient uptake, and improved stress tolerance. However, it is still a challenge to exactly understand their mode of action (Lee & Ryu, 2021; Ronga et al., 2019).

1.2 Focus on microalgae

1.2.1 General

Microalgae (Latin: small seaweed) are single cell, colonial or chain-forming eukaryotic micro-organisms invisible to the naked eye, most of which are photosynthetic. Microalgae are classified mainly considering their pigmentation, life cycle and cell structure. A total of 50,000 species of microalgae have been described out of an estimated 800,000 species. (Suganya et al., 2016). Microalgae are microscopic, unicellular, colonial or even multicellular organisms that can grow both in marine and fresh water, with a small size, from ~1 to ~900 µm (Kurniawati et al., 2014). Some microalgae live on land on rocks in a moist environment and even some are coexisting in the thalli of lichens. For example mircoalga *Trebouxia tramesii* forms a symbiotic relationship with lichen *Ramalina farinacea*. The dominating species of microalgae available commercially are: *Isochrysis spp.*, *Chaetoceros spp.*, *Chlorella spp.*, *Arthrospira spp.* and *Dunaliella spp.* (Rath, 2012). Microalgae are able to produce biomass that might be used in different sectors such as fuel, food, animal feed, pharmaceutical and crop production (Mehariya et al., 2021; Ronga et al., 2019). By means of their photosynthetic activity, they produce metabolites such as carbohydrates, proteins, lipids, pigments, vitamins and minerals (Ahmad et al., 2022). For example, microalgal pigments including carotenoids and chlorophyll are used as food coloring agents (Bhattacharya & Goswami, 2020).

Microalgae are cultivated in open (e.g. raceways) or closed systems, called photobioreactors. Important factors for microalgal growth are CO_2, light, temperature, pH and nutrients. Microalgae can both use CO_2 and/or HCO_3^- via *carbonic anhydrase* (Meier et al., 2022). When looking at the distribution function of carbonic acid in Figure 4, this means that pH should stay lower than 8.5 for optimal growth. For many microalgae species, the working temperature range is between roughly 10 and 40°C, with an optimal range between 28 and 35°C. Increased dark respiration and photorespiration lowers overall algal productivity when temperatures are above optimal (Park, 2010). In many protocols, pH is kept at 6.8 (Cordoba Perez & de Lasa, 2021), while some microalgal species (e.g. *Arthrospira platensis*, better known as “Spirulina”) need a pH of 9 for optimal growth (Belkin & Boussiba, 1991).

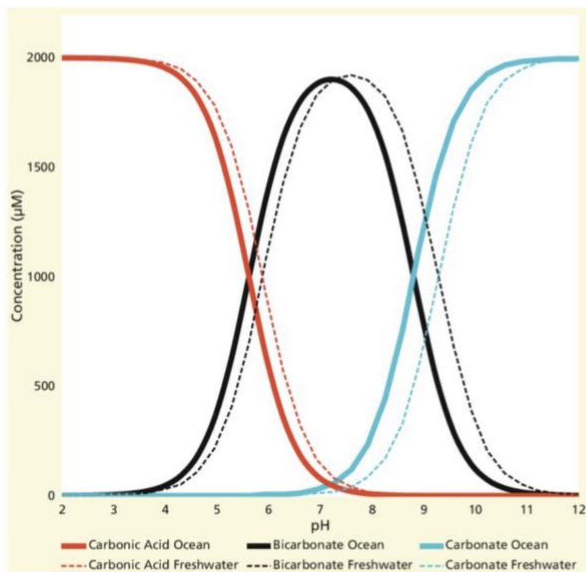


Figure 4. Bjerrum plot showing the distribution of carbonic acid (red), bicarbonate (black) and carbonate (blue) ions as a function of pH in freshwater (dashed line) and seawater (solid lines) (Middelburg, 2019).

Photoautotrophic microalgal growth refers to microalgae that use light and inorganic carbon such as CO_2 as an energy and carbon source, respectively, for photosynthesis to produce organics (Cao et al., 2023). Some algae use a metabolic process other than photosynthesis. There are algae species able to grow heterotrophically, unlike obligate photoautotrophs, which can only grow in the presence of light and are unable to survive by taking up organic carbon from the external environment as an energy source. Additionally, some microalgae being grown under hetero- and mixotrophy are able to ingest particles and even other cells, a process that is known as phagotrophy, or by taking up some dissolved organic compounds, such as glucose, glycerol and others (osmotrophy) (Beamud et al., 2014; Tittel et al., 2009). This last mechanism has been proven to happen in coccolithophore populations (Balch et al., 2023). Another type of microalgae are facultative mixotrophic algae, which can either grow by phototrophy or heterotrophy. The use of either trophy often depends on the amount of light and organic carbon that is available (Patel et al., 2019). For microalgae to grow properly they also need nitrogen and phosphorus sources. The required Redfield ratio depends on the microalgae. For *C. vulgaris* a Redfield ratio of 16:1 (N:P) was considered to be an average value according to Silva et al. (2015).

When high cell concentrations are reached in microalgal cultures, cells undergo self-shading due to increased light attenuation caused by the photosynthetic pigments in the antennas and photosystems. At a certain point, when light becomes limiting,

microalgae switch to respiration. The compensation point is the light intensity at which the rate of photosynthesis equals the rate of respiration (Saccardo et al., 2022).

In recent years, biotechnological and bioengineering techniques have enabled algae to become more efficient 'cell factories' for carbon sequestration and food production (Barati et al., 2022; Caporgno & Mathys, 2018). The rapid growth of the algal bio-economy has been driven by significant advances in algal biotechnology that have turned algae into an efficient 'cell factory' for food production (Kusmayadi et al., 2021). Instead of adding nutrients obtained from mining and chemical processes and using CO_2 exclusively from the atmosphere, it might be interesting to see if wastewaters can be a source of these compounds.

1.2.2 Wastewater nutrients for microalgae

In Europe, water stress affects about 30% of the population on an annual basis. Because of climate change, which is increasing the frequency, severity, and impact of droughts, the situation is anticipated to get worse (European Environment Agency, 2021). Wastewater contains nutrients such as nitrogen, phosphorus, and potassium, which act as fertilizers and are essential for the growth of photosynthetic organisms (Griffiths et al., 2016).

The use of wastewater as feed allows microalgal biomass production. In a perspective of circular bio-economy, microalgae can recover the nutrients from wastewaters, like for instance from pig production facilities. Ferreira et al. (2021) selected three microalgae (*Tetradesmus obliquus*, *C. protothecoides*, *C. vulgaris*) and one cyanobacterium (*Synechocystis sp.*) to treat diluted piggery wastewater (1:20). The nutrient removals were 62-79% for COD (chemical oxygen demand), 84-92% for TKN (total Kjeldahl nitrogen), 79-92% for NH_4^+ and over 96% for PO_4^{3-}. *T. obliquus* and *C. protothecoides* were the most efficient ones. After treating the piggery wastewater, the produced biomass was assessed as biostimulant for seed germination, root/shoot growth, and pigment content for tomato, watercress, cucumber, soybean, wheat and barley seeds. Especially in *T. obliquus* and *C. vulgaris* treatments, longer roots were observed (Ferreira et al., 2021).

Additionally, microalgae have the potential to be used for tertiary wastewater treatment. Morais et al. (2022) conducted a study on a pilot installation in a wastewater

treatment plant (WWTP) in Portugal, where microalgae were able to remove up to 95% of NH_4^+ after secondary treatment. The biomass that was produced contained high amounts of proteins and carbohydrates and had the potential to be used as biofertilizers or to make biofuels like biogas. However, the installation will not be replacing the conventional WWTP because of the long retention times and the consequently high land area occupation. Still, the pilot installation had an environmental impact two to three times lower than a conventional treatment, making it an interesting system to combine with conventional treatment.

1.2.3 *Chlorella vulgaris* and *Tetradesmus obliquus*

1.2.3.1 *Chlorella vulgaris*

C. vulgaris is a green eukaryotic spherical microalga with 2-10 µm diameter, very rich in proteins (42 - 58 % dry weight) and often used as a food source in Japan (Safi et al., 2012; Yamamoto et al., 2004). It is also used in medical treatments having immuno-modulating and anti-cancer properties (Morris et al., 2009). In addition, it has been reported to lower the risk of atherosclerosis and stimulate collagen synthesis for skin (Caicedo et al., 2020). Furthermore, *C. vulgaris* is capable of accumulating important amounts of lipids, especially after nitrogen starvation with a fatty acid profile suitable for biodiesel production (Zheng et al., 2011).

The name *Chlorella* comes from the Greek word "*Χλωρος*", which means green, and the Latin suffix "*-ella*" refers to its microscopic size (Safi et al., 2014).

C. vulgaris is ideal for production because it is remarkably resistant against contaminants and harsh conditions. Under unfavorable growth conditions the lipid and starch contents increase and biomass productivity ceases or decreases. These unfavorable growth conditions are nitrogen and phosphorus limitation, high CO_2 concentration, excessive exposure to light, excess of iron in the medium or increase in temperature (Safi et al., 2014). There is a relation between the growth technique and productivity. When autotrophic growth of microalgae is carried out in open ponds (15-20 cm in depth) environmental control is difficult. There is a risk of pollution, invading bacteria, contaminants and water evaporation. Other limiting factors are the

fluctuations in temperature, excess sunlight exposure and low CO_2 concentration. Since cells are surrounded by other cells across the water column, they have lower mass yields because some cells at the bottom are not exposed to enough sunlight for optimal growth. Closed photobioreactors (20 cm or less in diameter with transparent walls of a few millimeters) can offer a controlled environment (pH, light intensity, temperature, CO_2 concentration) to obtain higher growth and a better quality of the harvested products, but this requires higher manufacturing costs. The CO_2 for microalgae is typically supplied in a dispersible form through a perforated porous sparger at the bottom of the bioreactor or in a separate reservoir (Moraes et al., 2020). In flat panel green walls, which are thin plastic bags held by a metal grid, CO_2 is fed to the biomass by bubbling into the culture (Carone et al., 2022). The same happens in a bubble column. If the tubes of the photobioreactor are not sufficiently exposed to sunlight, artificial lights are used (Blanken et al., 2013).

Heterotrophic growth is possible when the microalgae are fed in an continuous stirred tank reactor (CSTR) or fermenter. The carbon sources used for *C. vulgaris* are glucose, acetate, glycerol and glutamate with maximum specific growth rate being obtained with glucose. Barros et al. (2019) published that growing *C. vulgaris* cells heterotrophically in fermenters led to the highest biomass concentration, 174.5 g L^{-1}, ever reported for this microalga. Apart from the large quantity of proteins *C. vulgaris* also contains lipids (5-40% lipids per dry weight of biomass), carbohydrates (12-55 % per dry weight of biomass), pigments (chlorophyll 1-2 % dry weight and carotenoids), minerals and vitamins (especially vitamin C, E and B2) (Safi et al., 2014). The tough cell wall of *C. vulgaris* is a significant obstacle to the extraction of all internal components and to the ability to be digested. It is usually necessary to cool the system during mechanical cell breaking because the high energy input overheats the fractured microalga and puts the integrity of target metabolites in danger by oxidizing or damaging them (Safi et al., 2014). *C. vulgaris* has shown a great potential to be used as a biostimulant when a concentration of 0.1 g L^{-1} was applied to watercress. It increased the germination index of watercress seeds by 3.5%. When concentrations of 0.5 g L^{-1} and 2.0 g L^{-1} were used, the root formation in soybean seeds were increased by 220% and 493% respectively (Morillas-España et al., 2022). In a study of Park et al. (2022), three types of *C. vulgaris* treatments were carried out on kale (suspension, biomass and filtered supernatant). Total chlorophyll and carotenoid

contents were increased by 1.57 and 1.41 respectively when *C. vulgaris* was used in a suspension. Notably, the filtered *C. vulgaris* supernatant negatively influenced the growth of kale by 37% (Park et al., 2022).

1.2.3.2 *Tetradesmus obliquus*

Cells in a colony of *Tetradesmus* occur in multiples of two with four or eight cells being most common (Afify et al., 2018). *T. obliquus* is a freshwater microalga that has exceptional vitality in different wastewaters and huge possibilities for commercial uses since it grows quickly and can withstand a wide range of temperature and pH (Duan et al., 2020). *T. obliquus* is an interesting microalga for triacylglycerol accumulation under nitrogen starved stress conditions (Breuer et al., 2013). It also seems to be a suitable species for CO_2 biofixation (Tang et al., 2010).
A few studies exist on brewery wastewater treatment using *T. obliquus* (Ferreira et al., 2018; Marchão et al., 2018; Wu et al., 2017). Ferreira et al. (2019) demonstrated an enhanced capacity of germination of wheat and barley seeds by *T. obliquus* culture cultivated in brewery effluent. In a study of Navarro-López et al. (2020), the use of *T. obliquus* biomass at 0.1 g L^{-1} resulted in an increased germination index of watercress seeds by 40%. This is significantly more than the effect of *C. vulgaris* biomass (0.1 g L^{-1}) on the germination index of watercress seeds, which resulted in an increase by only 3.5% (Morillas-España et al., 2022; Navarro-López et al., 2020).

1.3 Screening for properties when used on crops

Microalgae are considered to be a viable source of environmentally friendly antifungal agents that may decrease the need for synthetic fungicides. Schmid, Coelho, et al. (2022) investigated the properties of aqueous extracts from *Nannochloropsis sp.*, *Phaeodactylum tricornutum*, *T. obliquus*, *C. vulgaris* and *Spirulina sp.* regarding their antagonistic activity towards the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea* and *Alternaria alternata*. Their results showed that microalgae with fungicidal activity could replace chemical agents as an environmentally friendly alternative (Schmid, Navalho, et al., 2022).

Many factors influence the biostimulant action: source (plant or animal origin), extraction/hydrolysis technique, composition and solubility, application type (soil application, foliar spray), timing (phenological stages of the crop), concentration applied, type of cultivar/crop, and leaf permeability (Kapoore et al., 2021).
Whatever the reason for a beneficial effect, the effect itself can be evaluated by monitoring:

=> **morphological changes in roots and shoots** (length, leave number, fresh weights of leaves and roots, dry weights, ash content, relative growth rate)

=> **pigment content** (chlorophyll content and carotenoids)

=> **total protein content** (Bradford, 1976)

=> **enzymatic activities** (e.g., glutamate synthase and glutamine synthetase, enzymes involved in nitrogen metabolism)

1.3.1 Chlorophyll content determination in treated crops

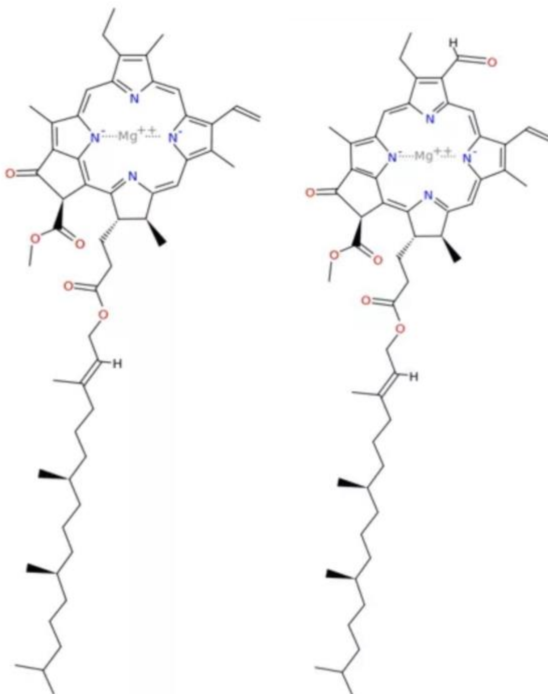


Figure 5: Structures of chlorophyll a (left) and b (right) .

Chlorophyll, the pigment that gives plants their distinctive green color, is crucial to the process of photosynthesis, which provides energy for the metabolism, growth, and reproduction of the plant (Li et al., 2018). The light-harvesting chlorophyll molecules have a chlorin ring (cyclic tetrapyrroles) system with a central Mg^{2+} ion and different

side chains on the chlorin ring (chlorophyll *a*, *b*, *c*, *d* and *f*). The structures of chlorophyll *a* and *b* are presented in Figure 5. Chlorophyll *b* is slightly more polar due to the presence of a polar carbonyl group on one of the pyrrole rings and is more yellow-green colored. In Figure 6, it is shown that chlorophyll *a* is best at absorbing photons at 400-450 nm and 650-700 nm, whereas chlorophyll *b* is best at 450-500 nm and 600-650 nm (Guidi et al., 2017; Porra et al., 1989).

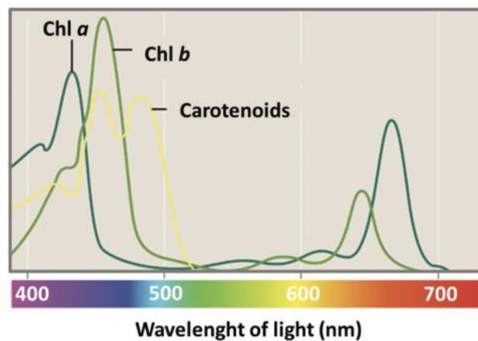


Figure 6: Absorbance spectra of chlorophyll a and b and carotenoids (Guidi et al., 2017)

Chlorophyll concentrations in leaves serve as an indicator of chloroplast development, photosynthetic capacity, leaf nitrogen concentration, and general plant health (Pavlovic et al., 2014).

An organic solvent, such as acetone or dimethyl formamide, is commonly used in the laboratory to extract the pigments. One way to measure the effect of a biostimulant application is to check if there is an increase in chlorophyll content in the crop. The chemical method to determine the concentration of chlorophyll is a destructive method: leaf material is collected, chopped and ground and treated with organic solvents such as acetone, aqueous acetone or dimethylformamide upon which the content is determined photometrically (Porra et al., 1989). The leaf tissue used for these measurements are often obtained using a cork borer, e.g. a cork borer with diameter of 0.5 cm gives a surface area of 0.19635 cm^2. The leaf discs are also weighed after the excision, allowing the chlorophyll data to be expressed in relation to both fresh weight and leaf area. Inskeep and Bloom (1985) also determined extinction coefficients for chlorophyll a and b in DMF (dimethylformamide) and 80% acetone.

Table 2: Equations for quantifying Chl in plant tissue from measured absorbance values (A), using 1.00 cm cuvettes (Inskeep & Bloom, 1985)

80% Acetone	DMF
Chl $b^{a} = 20.47A_{647}^{b} - 4.73A_{664.5}$	Chl $b = 20.70A_{647} - 4.62A_{664.5}$
Chl $a = 12.63A_{664.5} - 2.52A_{647}$	Chl $a = 12.70A_{664.5} - 2.79A_{647}$
Total Chl $= 17.95A_{647} + 7.90A_{664.5}$	Total Chl $= 17.90A_{647} + 8.08A_{664.5}$

[a] All Chl components in $mg \cdot L^{-1}$. [b] A_{647} = absorbance at 647 nm (maximum for Chl *b*); $A_{664.5}$= absorbance at 664.5 nm (maximum for Chl *a*)

Wellburn (1994) determined some formulas to quantify chlorophyll *a* and *b* with different extraction solvents taking into account the resolution range of the spectrophotometer:

Table 3: Equations to determine concentrations of chl a *(C_a) and* b *(C_b) as well as total carotenoids (C_{x+c}) in µg/ml (Wellburn, 1994).*

Solvent	Spectrophotometer resolution range	
	0.1–0.5 nm	1–4 nm
80% Acetone	$C_a = 12.25A_{663.2} - 2.79A_{646.8}$ $C_b = 21.5A_{646.8} - 5.1A_{663.2}$ $C_{x+c} = (1000A_{470} - 1.82C_a - 85.02C_b)/198$	$C_a = 12.21A_{663} - 2.81A_{646}$ $C_b = 20.13A_{646} - 5.03A_{663}$ $C_{x+c} = (1000A_{470} - 3.27C_a - 104C_b)/198$
Chloroform	$C_a = 11.47A_{665.6} - 2A_{647.6}$ $C_b = 21.85A_{647.6} - 4.53A_{665.6}$ $C_{x+c} = (1000A_{480} - 1.33C_a - 23.93C_b)/202$	$C_a = 10.91A_{666} - 1.2A_{648}$ $C_b = 16.38A_{648} - 4.57A_{666}$ $C_{x+c} = (1000A_{480} - 1.42C_a - 46.09C_b)/202$
Diethyl-ether	$C_a = 10.05A_{660.6} - 0.97A_{642.2}$ $C_b = 16.36A_{642.2} - 2.43A_{660.6}$ $C_{x+c} = (1000A_{470} - 1.43C_a - 35.87C_b)/205$	$C_a = 10.05A_{662} - 0.77A_{644}$ $C_b = 16.37A_{644} - 3.14A_{662}$ $C_{x+c} = (1000A_{470} - 1.28C_a - 56.7C_b)/205$
Dimethyl-formamide	$C_a = 12A_{663.8} - 3.11A_{646.8}$ $C_b = 20.78A_{646.8} - 4.88A_{663.8}$ $C_{x+c} = (1000A_{480} - 1.12C_a - 34.07C_b)/245$	$C_a = 11.65A_{664} - 2.69A_{647}$ $C_b = 20.81A_{647} - 4.53A_{664}$ $C_{x+c} = (1000A_{480} - 0.89C_a - 52.02C_b)/245$
Dimethyl-sulphoxide	$C_a = 12.47A_{665.1} - 3.62A_{649.1}$ $C_b = 25.06A_{649.1} - 6.5A_{665.1}$ $C_{x+c} = (1000A_{480} - 1.29C_a - 53.78C_b)/220$	$C_a = 12.19A_{665} - 3.45A_{649}$ $C_b = 21.99A_{649} - 5.32A_{665}$ $C_{x+c} = (1000A_{480} - 2.14C_a - 70.16C_b)/220$
Methanol	$C_a = 16.72A_{665.2} - 9.16A_{652.4}$ $C_b = 34.09A_{652.4} - 15.28A_{665.2}$ $C_{x+c} = (1000A_{470} - 1.63C_a - 104.96C_b)/221$	$C_a = 15.65A_{666} - 7.34A_{653}$ $C_b = 27.05A_{653} - 11.21A_{666}$ $C_{x+c} = (1000A_{470} - 2.86C_a - 129.2C_b)/221$

Mzibra et al. (2018) used the following procedure to determine chlorophyll in their study of Moroccan seaweed extracts as tomato plant growth promotors: 0.1 g of uniformly sized and colored tomato leaves from the middle of each plant were homogenized with 80% acetone and 0.1% (w/v) CaCO3. Centrifugation was used, for ten minutes at 3000xg, to separate the homogenized mixture. The absorbance (A) of chlorophylls *a* and *b* was measured, being 663 and 646 nm respectively, and the amounts of both pigments was determined, using the same formulas as in the table above:

Chlorophyll *a* (μg mL^{-1}) = 12.5 A_{663} – 2.79 A_{646}

Chlorophyll *b* (μg mL^{-1}) = 21.5 A_{646} – 5.10 A_{663}

The extracts used, contained 17 algae species with different polysaccharide levels. After 30 days of application, plants were healthier and taller, as there was a significant increase in leaf chlorophyll *a* and *b* (Mzibra et al., 2018).

1.3.2 The use of a SPAD-502 meter

The Soil Plant Analysis Development (SPAD) is a diagnostic tool to measure the crop's nitrogen status (Zheng et al., 2015). With this hand-held SPAD-502 Konica Minolta device (the sensor of the SPAD-502 is only 2x3mm, suitable for even small or narrow leaves), one measures the leaf transmittance in the red (650 nm where chlorophyll absorbs) and infra-red (940 nm for the correction of leaf thickness) wavelength ranges of the electromagnetic spectrum in a non-destructive way. These transmittance values are used to define "a SPAD value" that is proportional to the amount of chlorophyll and hence nitrogen in the sample (Ling et al., 2011).

1.4 Some relevant classes of molecules present in microalgae extracts

The beneficial biostimulating effect can be related to different classes of molecules present in the extracts. The following classes of molecules are found in microalgae extracts. However, only the classes that are more relevant for biostimulation are mentioned.

1.4.1 Phytohormones

Microalgae are capable of accumulating phytohormones in the cells. These phytohormones are signaling molecules that are naturally produced in low concentrations and function as chemical messengers to control and stimulate growth in terrestrial plants (Pan et al., 2019). Lu and Xu (2015) indicated that phytohormones have similar regulatory roles in microalgae as those in higher plants. The following major phytohormones are of commercial interest and found in algae: auxins (e.g., indole-3-acetic acid), cytokinins, gibberellic acids (GAs), ethylene, abscisic acid, polyamines (phytohormone-like compounds), brassinosteroids (BRs), lunularic acid,

jasmonic acid, betaines, rhodomorphin and salicylic acid (Kapoore et al., 2021). Some important phytohormones are presented in Figure 7.

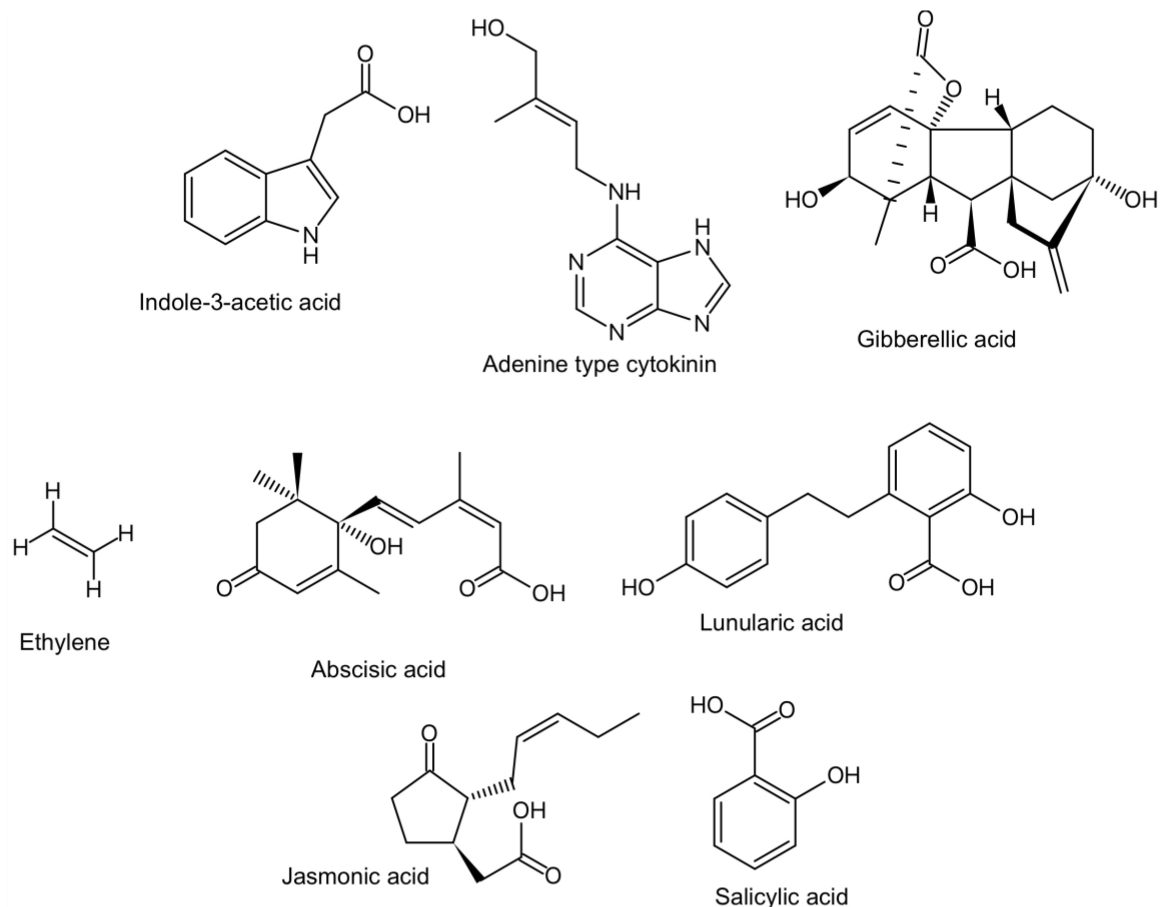


Figure 7: Some important phytohormones (drawn in Chirys Draw)

Auxins (from Greek auxein = to grow) are found in brown algae (*Undaria, Laminaria*), green algae (*T. obliquus, C. vulgaris, C. pyrenoidosa, S. quadricauda*) and red algae (*Neopyropia yezoensis, Polysiphonia urceolata*) and are involved in cell growth, cell division and resistance to biotic and abiotic stresses (Liu et al., 2016; Singh, 2014; Uji & Mizuta, 2022). Additionally, auxins are known to be used in agriculture because of their herbicide and pesticide properties (Munira et al., 2018).

Cytokinins are a class of plant hormones active in promoting cell division or cytokinesis, shoot and root morphogenesis and stress tolerance (Aremu et al., 2020). The ratio of cytokynins to auxins is important. Whereas auxins promote shoot growth and restrict lateral branching by inhibiting axillary buds (apical dominance), cytokinins move from the roots into the shoots signaling lateral bud growth (Müller & Leyser, 2011).

Gibberellins (GAs) are crucial plant endogenous growth regulators that affect a variety of fundamental physiological and developmental processes, including seed germination, stem elongation, flowering, leaf expansion, and fruit formation (Castro-Camba et al., 2022). According to Chen et al. (2021), foliar application of gibberellins on lettuce restored part of root morphology and vigor of lettuce under Cd stress by increasing the total root length and surface area. As a result, the lettuce also showed an increased biomass.

Foliar application of brassinosteroids (BRs) to tomato and snap bean plants can reduce the negative impacts of heat stress and increase overall plant growth by enhancing antioxidants in leaves (El-Bassiony et al., 2012; Ogweno et al., 2008).

An ethylene precursor (1-aminocyclopropane-1-carboxylic acid) is used for several agricultural purposes, such as enhancement of seed germination, auxin transport, seedling and root hair growth (Lu & Xu, 2015; Pan et al., 2019). Furthermore, ethylene has a role in regulating fruit ripening, opening of flowers and leaf abscission (Lin et al., 2009). *Arthrospira sp.* was reported to contain the highest concentration of ethylene precursor in case of microalgae with 546 ng g^{-1} (Plaza et al., 2018).

When a plant drops a leaf, a fruit or a flower, it is called abscission (from Latin ab-anscindere: cut off). Abscisic acid (ABA) is to a certain extent believed to be involved in abscission. In order to survive the winter, terminal buds release ABA, which slows plant growth and helps in protecting the dormant bud. ABA is also produced in the roots in case of low water availability, upon which it translocates to the leaves and helps to close the stomata, reducing in this way transpiration (Marusig & Tombesi, 2020). Sprays of ABA are suggested to cause partial closure of stomata for few days, to reduce transpirational loss of water. Seed germination is inhibited by ABA in antagonism with gibberellin.

Although ABA is shown to maintain seed dormancy to regulate leaf senescence and to inhibit cell elongation, it can be used in cases of severe environmental stress (drought, extreme temperatures, salinity and pathogens) since it triggers stomatal closing and reduces growth to allocate resources for stress tolerance (Chen et al., 2020).

Salicylic acid controls defense responses against biotrophic pathogens (i.e., requiring living plant tissue), while jasmonic acid controls defense responses against herbivorous insects and necrotrophic pathogens (i.e. feeding on dead matter) (Caarls et al., 2015). In addition, jasmonic acid has been reported to alleviate salt stress in sweet potato plants (Zhang et al., 2017). The highest concentrations of jasmonic acid and salicylic acid were reported in *Tetradesmus sp*., 75.13 ng g^{-1} and 156.714 ng g^{-1}, respectively (Plaza et al., 2018). Polyamines like putrescine, spermine and spermidine accumulate in stressed plants. When using polyamines as biostimulants, plants become more tolerant to stress conditions (Papenfus et al., 2013). Betaines such as glycine betaine, γ-aminobutyric acid betaine, δ-aminovaleric acid betaine and laminine are believed to regulate defense mechanisms against abiotic stresses such as salinity, frost and drought (Lamaoui et al., 2018; Quan et al., 2004). In Table 4, an overview is given of phytohormones present in several microalgae.

Table 4: Phytohormones present in some microalgae with their physiological activities (Kapoore et al., 2021).

Phytohormone	Physiological activity	Examples of microalgae sources
Auxins	Cell elongation, differentiation of phloem, apical dominance, stress tolerance (heat, salinity and drought), tropisms and initiation of root formation	*Chlorella, Acutodesmus, Scenedesmus, Coenochloris*
Cytokinins	Control of cell division, chloroplast and vascular tissue development, bud, development, promoting protein and chlorophyll synthesis, fruit and flower development, senescence, stress tolerance and apical dominance	*Chlorella, Desmococcus, Euglena, Myconastes, Scenedesmus, Nannochloropsis, Protococcus*
Gibberellins	Stem elongation, floral organ development and initiation of flowering, initiation of seed germination and influence protein biosynthesis	*Scenedesmus, Chlorella, Nautococcus, Nannochloropsis, Stigeoclonium*
Brassinosteroids	Regulation of elongation, division and differentiation of vascular system, promotion of stress tolerance and ethylene production	*Klebsormidium*
Ethylene	Regulation of fruit ripening, opening of flowers, cell division, cell elongation, senescence and stress tolerance	*Chlorella, Scenedesmus*
Abscisic acid	Generates stomatal closure, protein storage in seeds with seed dormancy and inhibits shoots growth	*Scenedesmus, Chlorella, Dunaliella, Nannochloropsis*
Jasmonic acid	Regulates plant defense responses, tuber formation, senescence and synthesis of proteinase inhibitors	*Chlorella, Scenedesmus, Dunaliella, Euglena, Gelidium*
Salicylic acid	Regulates plant defenses when a pathogen develops	*Scenedesmus*
Polyamines	Regulates stress responses, cell proliferation, differentiation and growth	*Chlorella, Euglena, Cyanidium*

1.4.2 Protein hydrolysates and amino acids

The hydrolysis of proteins (chemical, thermal, enzymatic) from animal waste or plant biomass results in a mixture of smaller peptides and amino acids, containing small proportions of other elements. The small peptides exert a similar activity in plants like auxins and gibberellins (Colla et al., 2017) and amino acids, which are good chelators for metals contributing to nutrient mobility or mitigate stress via chelating effects of heavy metals (Huang et al., 2022). Application of algal extracts containing individual amino acids is known to increase the synthesis of key phytohormones (Bulgari et al., 2019; Ronga et al., 2019). According to a number of studies, amino acids like glutamate, phenylalanine, cysteine, histidine, proline, glycine, and glycine betaine are crucial for metabolic signaling and for reducing the effects of environmental stresses like heavy metals, nutrients, oxidative stress, heat, cold, drought, and salinity (Paul et al., 2019; Teixeira et al., 2017).Hempel et al. (2012) demonstrated that strains like *Chlorella sp.* can contain more than 40% dry weight of amino acids. Foliar application of protein hydrolysate (obtained via enzymatic hydrolysis) of *Arthrospira* and *Tetradesmus* biomass (at 10 g L^{-1}) to *Petunia x hybrida* plant resulted in increased number of flowers, the flower fresh and dry matter per plant and the root dry weight (Plaza et al., 2018).

1.4.3 Humic substances

The natural breakdown of plants, animals, and microbial residues as well as the metabolic activity of soil microorganisms generate HSs, which make up around 60% of the organic matter in soil. Results from the use of HSs on plants indicate variable but generally positive effects on plant growth, with the majority of the impacts being connected to the improvement of root nutrition (du Jardin, 2015).

1.4.4 Polysaccharides

In macroalgae, the most common polysaccharides are ulvans, galactans, fucoidans, laminarans, alginates and oligoalginates. The key polysaccharides in microalgae are heteropolymers of galactose, xylose, mannose, arabinose and glucose, which are linked in different proportions by glycosidic bonds. The only exception is a

homopolymer of galactose (in *Gyrodinium impudicum*) and *β*-(1,3)-glucan in *C. vulgaris* (De Jesus Raposo et al., 2015; Farid et al., 2019). According to Chanda et al. (2019), the proportional differences in neutral sugars among the constituents of microalgal polysaccharides, as well as other elements such as the level of sulfation, the presence of uronic acid, and molecular weight, significantly influence the biostimulant activity of these compounds. Elarroussia et al. (2016) observed an overall increase in plant growth when a foliar spray of a polysaccharide rich extract of *A. platensis* was applied to tomatoes and paprika.

1.4.5 Antioxidants

Several substances that can be categorized as antioxidants are found in algae, including vitamins C and E, carotenoids, chlorophylls, and phenolics. (Shebis et al., 2013). One of the most significant classes of natural antioxidants is phenolic compounds. It is known that a number of algae species produce phenolic compounds in a reaction to stress and/or as a form of defense (Kapoore et al., 2021).

1.5 Harvesting and drying methods

Harvesting of algae begins at the end of the growth cycle when the algal cells reach their maximum concentration (Tan et al., 2020). Harvesting of algae is often done by filtration and centrifugation followed by drying (Kumar, 2021). The choice of drying method depends on the production scale and the properties and application of the product that needs to be dried (Ryckebosch et al., 2011).

Freeze-drying (lyophilization) and spray-drying are the most commonly used techniques for high value products. Freeze-drying is the most gentle drying method. The liquid is frozen and a vacuum is applied for sublimation of the ice. Because of the expensive equipment needed and its high energy consumption, it is however only used for applications that require the conservation of the biochemical composition. Because of the high cost, this technique is only used to make products with a high added value (Ratti, 2001). Spray-drying has its application in fast and uninterrupted drying of solutions and emulsions. In spray drying, a suspension of very small droplets is produced (tens to hundreds of micrometers in diameter) with a large surface area available for heat and mass transfer. An atomizer disperses the liquid stream as a mist of fine droplets into a heated chamber where the small droplets dry into granules.

Just like with freeze-drying, the applications are limited to products with a high added value because of the high cost. When the spray-dried product is vacuum packed, it can be stored for several months. However, spray-drying can lead to damage of different thermolabile components. (Cal & Sollohub, 2010; Grima et al., 2004).

Although direct biomass exposure to sunlight typically results in the loss of functional characteristics, solar drying is thought to be a more environmentally friendly and economical option. In order to combine the environmentally friendly benefits of using the sun indirectly (to heat air) while preserving high biomass quality, indirect solar-drying techniques were created. The authors obtained further process stability by applying an indirect and hybrid solar dryer that uses sun irradiance as the primary heat source, coupled with a fan heater, a dehumidifier, and a ventilation system. The hybrid drying system consumes energy to achieve optimum values if the environmental conditions are unfavorable to attain the specified set points (Schmid, Navalho, et al., 2022).

1.6 Cell disruption methods

Because of the fact that some microalgae have a very rigid cell wall, the improvement of cell disruption processes has been the subject of research in recent decades. One of these promising disruption techniques is high pressure homogenization (HPH) (Ferreira et al., 2022). Cell disruption is needed to extract valuable compounds inside of the microalgal cells, such as lipids, proteins and polysaccharides. HPH is an efficient and sophisticated technology that pushes liquids through a narrow gap at a high pressure that can range from 100 to 2000 bar. Particles then accelerate in a very short distance and collide in the impact ring, undergoing disruption (Figure 8). Cell disruption is dependent on the microalgae species due to differences, especially in cell wall composition and size. Therefore, the pressure and number of passages has to be optimized for each strain.

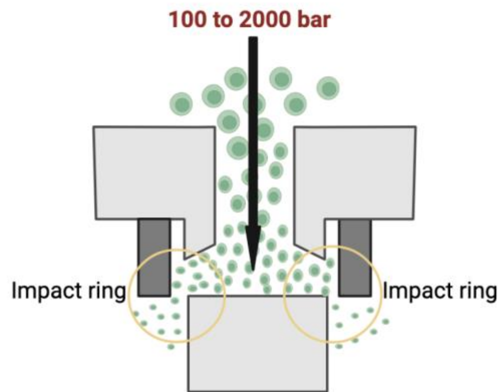


Figure 8: Illustration of the process going on in a high pressure homogenizer (made in BioRender.com).

The following cell disruption methods were carried out with *C. vulgaris* (Table 5) at GreenCoLab:

For method HPH3C, high pressure homogenization (HPH) was used with 3 cycles and a pressure of 100 bar. The high-pressure homogenizer PandaPlus 2000 (GEA, Germany) was used and afterwards the disrupted microalgal biomasses were analyzed by flow cytometry using the CytoFLEX S (Beckman Coulter Life Sciences, USA), equipped with a blue argon laser (488 nm). Cell disruption method HPH1C consisted of 1 cycle of HPH and a pressure of 1150 bar was used. This was done to study if the high pressure (resulting in a higher temperature) could also compromise the bioactive compounds of the algal cells. For method HPHmin, the minimal parameters of the high pressure homogenizer were used, being 100 bar and 1 cycle. For method NT, no cell disruption treatment was carried out. However, the microalgal biomass arrived at GreenCoLab as a frozen paste. It had to be thawed and this process has also partially compromised the cell walls due to ice crystals.

For method EH, enzymatic hydrolysis with commercial Alcalase was used for cell disruption of the cell walls of the microalga. This was performed at 50°C for 24h with a pH of 8. For method EH + HPH1C, first HPH was carried out at 1150 bar with 1 cycle and next enzymatic hydrolysis was used as an double cell disruption step, with successively Viscozyme, Alcalase and Flavourzyme (at 50°C for 3.5h). Alcalase is a serine endopeptidase, an enzyme that cleaves proteins in the middle of the amino acid chain (Tacias-Pascacio et al., 2020). Viscozyme is a commercial mixture of carbohydrases that breaks down carbohydrates and facilitates extraction of proteins

from the microalgal cells. Flavourzyme is a blend of exo- and endopeptidases that breaks peptide chains at the N-terminal (Figueiredo et al., 2018).

Table 5: The six cell disruption methods of C. vulgaris *carried out at GreenCoLab.*

C. vulgaris	Cell disruption abbrevation	Cell disruption method	Alga cell concentration (g/L)	Pressure (bar)	Number of cycles
	HPH3C	HPH 3 cycles	120	100	3
	HPH1C	HPH 1 cycle	120	1150	1
	HPHmin	Minimal parameters HPH	120	100	1
	NT	No disruption treatment	/	/	/
	EH	Enzymatic hydrolysis with Alcalase	/	/	/
	EH + HPH1C	HPH 1 cycle + enzymatic hydrolysis with Viscozyme, Alcalase and Flavourzyme	120	1150	1

The following cell disruption methods were carried out with *T. obliquus* (Table 6) at GreenCoLab:

For method HPH3C, HPH was used with 3 cycles and a pressure of 258 bar. Cell disruption method HPH1C consisted of HPH with 1 cycle and a pressure of 1200 bar was used. For methods HPHmin and NT, the same processes were used as for the treatments HPHmin (100 bar, 1 cycle) and NT of *C. vulgaris*. For method EH, enzymatic hydrolysis with cellulase, pectinase and xylanase was used for cell disruption. This was performed at 45°C for 24h with a pH of 4.4. Cellulase has the ability to degrade cellulose and making it easier to release proteins, whereas pectinase can hydrolyze pectic substances (Maffei et al., 2018; Zhang et al., 2022). The hydrolytic enzyme xylanase can cleave the complex cell wall polysaccharide xylan (Bhardwaj et al., 2019). For method EH + HPH3C, first HPH was carried out at 600 bar

with 3 cycles and next enzymatic hydrolysis was used with successively visoczyme, alcalase and flavourzyme (at 50°C for 3.5h).

Table 6: The six cell disruption methods of T. obliquus carried out at GreenCoLab.

T. obliquus	Cell disruption abbrevation	Cell disruption method	Alga cell concentration (g/L)	Pressure (bar)	Number of cycles
	HPH3C	HPH 3 cycles	120	258	3
	HPH1C	HPH 1 cycle	120	1200	1
	HPHmin	Minimal parameters HPH	120	100	1
	NT	No disruption treatment	/	/	/
	EH	Enzymatic hydrolysis with cellulase, pectinase and xylanase	/	/	/
	EH + HPH3C	HPH 3 cycles + enzymatic hydrolysis with Viscozyme, Alcalase and Flavourzyme	120	600	3

1.7 Effects of microalgae on lettuce and other crops

Lettuce (*Lactuca sativa* L.) is a crop that is relatively sensitive to salt in comparison with other vegetable crops and therefore often requires the use of biostimulants or chemical fertilizers to reach a high productivity (Ekinci et al., 2012). In a study performed by La Bella et al. (2021), a foliar spray of *C. vulgaris* extract was applied to examine the effect on the growth of lettuce seedlings. The effect of *C. vulgaris* was evaluated by monitoring morphological features, chlorophylls, carotenoids, total protein contents and various enzymatic activities.

Their experiment was composed of five replicates per treatment and control, where each replicate contained 10 seedlings. The lettuce seedlings were randomly placed in a container, containing pumice as inert substrate that was wetted with 1 L Hoagland solution. Next, they were placed in a growth chamber for 6 days at 25°C ± 2°C with a 16h photoperiod. The containers were irrigated daily with 100 mL distilled water and three treatments (one week apart) of the seedlings were performed by spraying them with a Hoagland solution (500 mL) containing *C. vulgaris* extract (1 mg of organic carbon per liter). The control plants were sprayed with a 500 mL Hoagland solution without the *C. vulgaris* extract. At the end of the 21 days (after the first treatment) in a growth chamber, five plants per treatment were used to analyze the morphobiometric parameters.

The foliar spray treatment with *C. vulgaris* had a strong effect on the growth of lettuce seedlings mostly at shoot level (height, number of leaves, fresh and dry weight). However, at the root level there were no significant differences found in length and fresh weight, although the dry weight of the treated seedlings was higher than the ones of the control plants. The following results were obtained (La Bella et al., 2021):

Table 7: The morfological traits of lettuce seedlings after a treatment with C. vulgaris *by foliar application, after 21 days from the first treatment. Ctr: control. CV: treatment with* C. vulgaris*. FW: fresh weight. DW: dry weight (La Bella et al., 2021)*

	Shoot Height (cm)	Leaves (N°)	Shoot FW (g)	Shoot DW (g)	Root Length (cm)	Root FW (g)	Root DW (g)
Ctr	19.37 ± 0.89 b	14.67 ± 1.15 b	13.66 ± 1.05 b	0.43 ± 0.04 b	11.72 ± 0.56 a	1.95 ± 0.20 a	0.099 ± 0.02 b
CV	23.53 ± 0.75 a	18 ± 1.15 a	16.85 ± 0.95 a	0.60 ± 0.05 a	11.33 ± 0.89 a	1.89 ± 0.16 a	0.154 ± 0.03 a

Other researchers used living cells of *C. vulgaris* and *C. pyrenoidosa* to study the effect on lettuce seedlings, supplied in the irrigation water of the culture. This resulted in strongly improved dry weights and chlorophyll content of the lettuce. A similar effect

was proven for other crops such as cucumber, rice and eggplant (Yerrapragada & Elhafiz, 2015).

More recently, Puglisi, Barone, et al. (2020) have shown that living cells of microalgae *C. vulgaris* and *S. quadricauda* have a biostimulant effect on tomato plants by increasing several growth parameters such as length, mean root diameter, root volume, surface area (Puglisi, Barone, et al., 2020). In another study of Puglisi, La Bella, et al. (2020), the lettuce seedlings were treated with *S. quadricauda* extract by irrigating the substrate (such as pumice) with a Hoagland solution (500 mL) containing the *S. quadricauda* extract with a concentration of 1 mg Corg L^{-1}. After one week, the treatment was repeated and the seedlings were grown for 14 days in total in a growth chamber at 25°C ± 2°C, with a 16h photoperiod and daily irrigation (100 mL of distilled water). Their results showed that *S. quadricauda* extract had a positive effect on the growth of the lettuce seedlings, mainly working at the shoot level. Furthermore, pigments such as chlorophyll *a*, chlorophyll *b* and carotenoids were measured. Almost all pigments, at every sampling time, showed higher values than the control values (Puglisi, La Bella, et al., 2020).

2. Objectives

The goal of this study is to determine the biostimulant effect of two microalgae, namely *C. vulgaris* and *T. obliquus* on the growth of lettuce crops. This is done by adding different concentrations of the microalgal extracts to the substrate. Morphological parameters such as length and number of leaves, fresh and dry weight and leaf surface area are evaluated. Furthermore, the chlorophyll content is measured using a SPAD apparatus. To get a better understanding of the biostimulant activity, the total amino acid content, reducing sugar content and total Kjeldal nitrogen of the microalgal biomass is determined. Additionally, the pH, electrical conductivity (EC), moisture content and mineral matter of the substrate was analyzed. This will enable the determination of which generated algae-based extract has the most promising biostimulant potential.

Hence, the main research questions are:

1) Do the microalgae, processed at GreenCoLab, have a biostimulating effect?

2) Which of the two microalgae has the most pronounced effect on lettuce growth?

3) What is the optimal concentration of the added suspension?

4) Is there an optimal microalgal cell disruption method?

3. Material & Methods

All experiments were carried out in the greenhouses, soil lab and GreenCoLab at Campus of Gambelas, University of Algarve, Portugal (37°02’35.45”N, 7°58’20.64”W). The biostimulant potential of the microalgae *C. vulgaris* and *T. obliquus* was evaluated by performing *in vivo* tests.

3.1 Microalgal biomass cultivation and harvesting

Cultures of *C. vulgaris* and *T. obliquus* were grown at the company Allmicroalgae S.A. (Pataias, Portugal) using 15 m^3 tubular photobioreactors (PBR). Polymethyl methacrylate (PMMA) tubes with an internal diameter of 56 mm made up the photosynthetic zone of each PBR. The PBR’s were cooled by a sprinkling water system and the pH was kept below eight by injecting CO_2. Centrifugation was used to harvest the microalgae at a late exponential phase and the biomass was stored as fresh paste at -18°C.

3.2 Experimental design and preparation of microalgal extracts

The experimental design consisted of eight treatments, six of them being treatments with microalgal extracts (Figure 9). Each treatment with microalgal extracts was applied in three concentrations (0.1 g L^{-1}, 0.5 g L^{-1} and 2.0 g L^{-1}) and two controls were used (one positive and one negative). The positive control was Algaman B (Hubel, Portugal) and the negative control was without biostimulant (Table 8). Algaman B is a biostimulant formulated with *Ecklonia maxima* seaweed extract and boron that includes a range of natural hormone-like substances. To prepare the positive control, 2.0 g L^{-1} of Algaman B was used, following the manufacturer’s recommendations. Per treatment, four replicates (pots) were used, each containing one lettuce seedling, with four true leaves. Three repetitions were carried out per treatment, making it twelve replicates per treatment. In Figure 10, the setup of the experiment is shown, which indicates the random design of the treatments.

To prepare microalgal extracts with different concentrations, 0.624 g of each microalga was weighed and mixed with 312 mL of water. This was the stock solution.

The volume of microalgal suspension needed to pour 20 mL on each replicate (pot) is the following:

$$12\ pots_{per\ treatment} = 20\ mL * 12 = 240\ mL$$

The volume V_1 that you need from the stock solution for 0.5 g L^{-1} and 0.1 g L^{-1} suspensions:

$$C_1 * V_1 = C_2 * V_2$$
$$2\ gL^{-1} * V_1 = \mathbf{0.5\ gL^{-1}} * 240\ mL$$
$$V_1 = 60\ mL$$

$$C_1 * V_1 = C_2 * V_2$$
$$2\ gL^{-1} * V_1 = \mathbf{0.1\ gL^{-1}} * 240\ mL$$
$$V_1 = 12\ mL$$

The total volume of the stock solution:

$$V_{stock\ solution} = 240\ mL_{for\ 2\ gL^{-1}} + 60\ mL_{for\ 0.5\ gL^{-1}} + 12\ mL_{for\ 0.1\ gL^{-1}}$$
$$= 312\ mL\ of\ the\ 2\ gL^{-1}\ suspension$$

Weight of each type of microalgal biomass:

$$\frac{2\ g}{1000\ mL} * 312\ mL = 0.624\ g$$

Dilution of 0.5 g L^{-1} and 0.1 g L^{-1} suspensions:

$$C_{0.5} = 60\ mL\ (of\ stock\ solution)\ +\ 180\ \text{mL of}\ H_2O$$
$$C_{0.1} = 12\ mL\ (of\ stock\ solution)\ +\ 228\ \text{mL of}\ H_2O$$

Weekly applications of microalgal extracts were carried out during the entire experiment. Each application consisted of 20 mL on the substrate of each replicate (pot). Lettuce seedlings, at four true leaves, were seeded in 250 mL pots, with black peat as substrate that was enriched with a NPK fertilizer. The packaging of the substrate showed the following characteristics: a pH range between 5.0 - 6.5, 180 - 450 mg L^{-1} nitrogen, 200 - 500 mg L^{-1} P_2O_5 and 200 - 600 mg L^{-1} K_2O. The lettuce plants were watered twice a day, with a twelve hour interval, by sprinklers set with a timer.

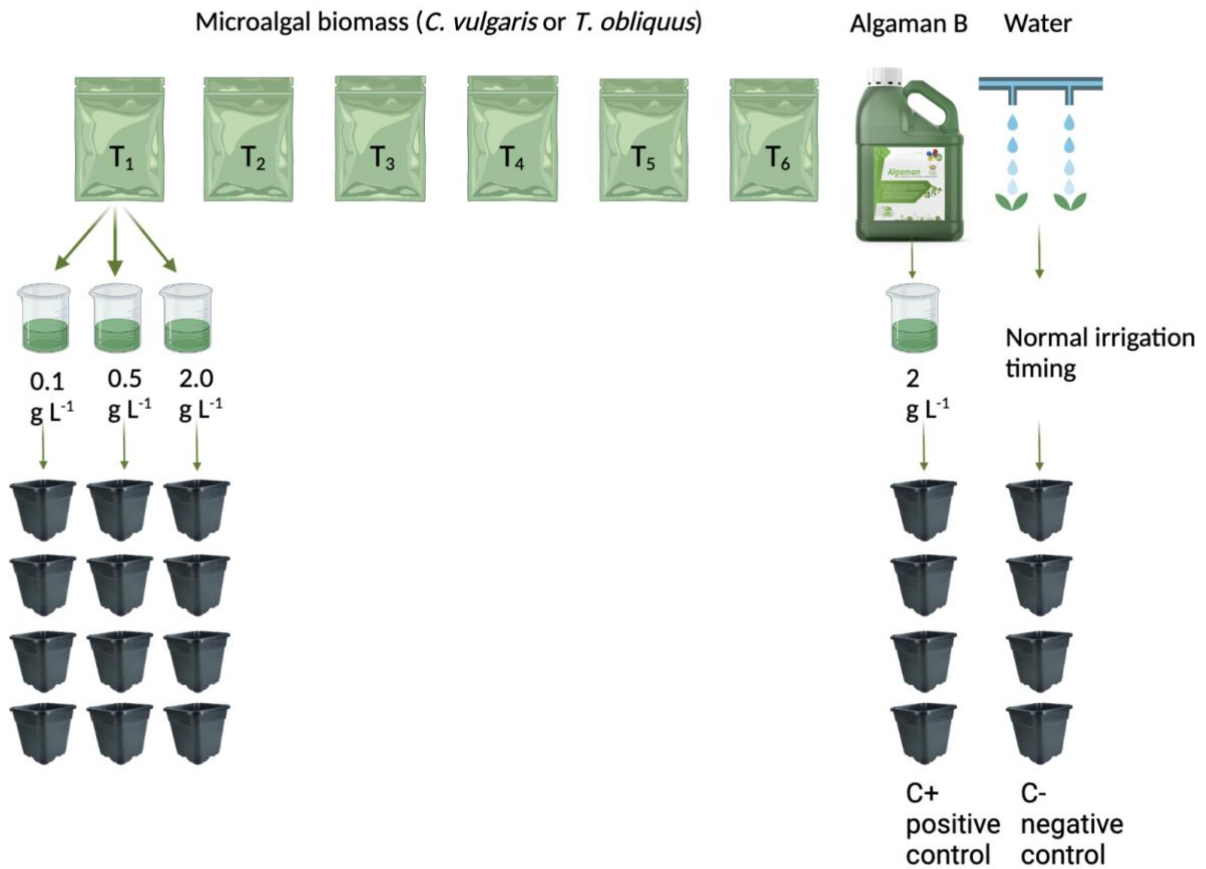


Figure 9: Illustration of the six treatments with microalgal extracts and two controls with their concentrations for each microalga and for Algaman B. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. This set-up was repeated three times to assure the statistical power. See Table 9 for more information about the cell disruption methods (made in BioRender.com).

Table 8: The different treatments with microalgal extracts and controls used with their concentrations.

Plant	Treatment	Concentrations (g L^{-1})
Lettuce	*C. vulgaris*	0.1, 0.5, 2.0
	T. obliquus	
	Algaman B (positive control)	2.0
	Water, normal irrigation (negative control)	-

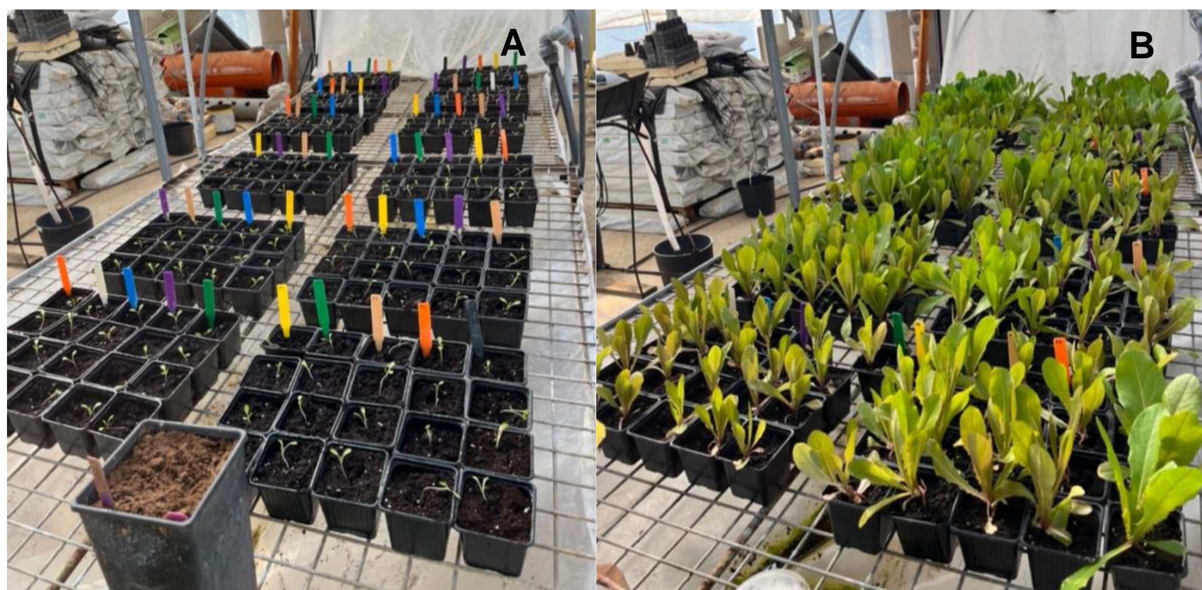


Figure 10: The setup of the experiment with lettuce and C. vulgaris *used as potential biostimulant in the beginning (A) and end (B) of the trial. On image A, the eight colors refer to the six different treatments with microalgal extracts and to the postive (black label) and negative control (white label).*

3.3 Cell disruption methods

The frozen pastes of *C. vulgaris* and *T. obliquus* from the company Allmicroalgae S.A. (Pataias, Portugal) were pretreated in the biorefinery of GreenCoLab (Faro, Portugal). Six different cell disruption methods were carried out, as mentioned in part 1.6 and displayed in Table 9. After cell disruption, the biomass was dried and stored in a desiccator at room temperature (22°C) to protect them from water vapour in the air. All storage containers were also covered with aluminium foil to avoid exposure to sunlight.

Table 9: Cell disruption methods used for C. vulgaris *and* T. obliquus*.*

Cell disruption abbrevation	**Cell disruption method *C. vulgaris***	**Cell disruption method *T. obliquus***
HPH3C	HPH (high pressure homogenization) 3 cycles	HPH 3 cycles
HPH1C	HPH 1 cycle	HPH 1 cycle
HPHmin	HPH with minimal parameters	HPH with minimal parameters
NT	No disruption treatment	No disruption treatment
EH	Enzymatic hydrolysis with Alcalase	Enzymatic hydrolysis with cellulase, pectinase and xylanase
EH + HPH1C/HPH3C	HPH 1 cycle + enzymatic hydrolysis with Viscozyme, Alcalase and Flavourzyme	HPH 3 cycles + enzymatic hydrolysis with Viscozyme, Alcalase and Flavourzyme

3.4 Biometric measurements

The SPAD index was recorded weekly during the trial using the SPAD-502 (Minolta Camera Co., Osaka, Japan), from the moment that the lettuce leaves were sufficiently large enough to do the measurements, which was starting from week three in the experiment with *C. vulgaris* and from week two in the experiment with *T. obliquus*. It differs since the first experiment with *C. vulgaris* started in February and the temperature in the greenhouse was not as high as in April, when the second experiment with *T. obliquus* started.

The trials were completed based on the number of leaves per plant and then the height, fresh weight and number of leaves were determined after cutting the plants just above the root system. The experiment with *C. vulgaris* was completed five weeks after planting the seedlings, while the experiment with *T. obliquus* was completed after four weeks. After the fresh weight measurement, the leaf area was recorded for two out of four replicates, for every repetition. These replicates were stored in plastic bags in the fridge for one night to make sure that the moisture stayed in the leaves. The computer program WinDias 2.0 (Delta-T Devices, UK), which was connected to a video camera, was used to measure the leaf surface area (Figure 11). The dry weights were obtained by placing the samples in a VENTICELL (mmm Medcenter, Germany) drying oven at 60 °C for four days until a constant weight was reached.

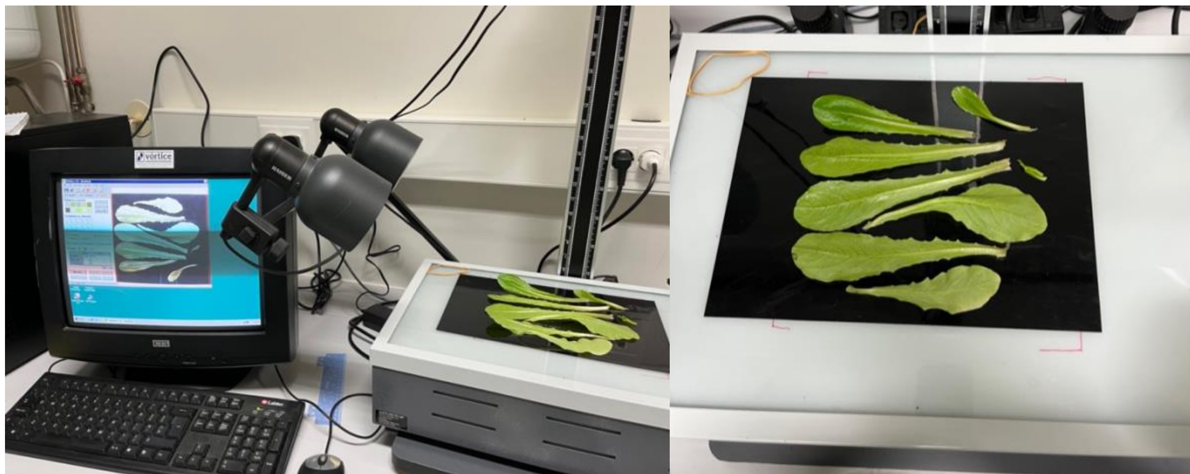


Figure 11: Illustration of the measurement of the leave surface area using the computer program WinDias 2.0.

3.5 Analysis of the substrate

The substrates of three out of four replicates of each treatment were collected in plastic bags and mixed to form one homogenized sample. The samples were stored at 4°C in the plastic bags, and analyzed within one week. The analysis of the substrate was carried out before and after the experimental period.

3.5.1 pH determination

First, 50 g of substrate were weighed for each treatment and placed in 500 mL Erlenmeyer's with 250 mL of distilled water. Next, the suspensions were agitated using a multi-flask shaker (Edmund Bühler GmbH, Germany) at 125 rpm for 20 minutes. Before measuring the pH with a potentiometer (Crison Micro pH2001, Spain), the suspensions were left to rest for 1 hour at room temperature. In between measurements the electrode was carefully cleaned to reduce the risk of errors.

3.5.2 Determination of electrical conductivity

The above mentioned suspensions, prepared for the pH determination, were used to measure the electrical conductivity (EC) of the substrate. The suspensions were filtered using 125 mm filters (filtraTECH, France) to obtain clear suspensions. A conductimeter (Crison Conductimeter 522, Spain) was used to obtain the EC at room temperature (22°C).

3.5.3 Moisture content of substrate

The moisture content of the substrates was determined by the oven drying method (O'Kelly, 2004). Between 40 and 50 g of each substrate was weighed in a paper box and placed in the VENTICELL (mmm Medcenter, Germany) oven at 60°C for four days. Afterwards, the dry weight was measured and the moisture content could be determined using the following formula:

$$Moisture\ \% = \frac{fresh\ weight\ (g) - dry\ weight\ (g)}{fresh\ weight\ (g)} * 100$$

3.5.4 Total Kjeldahl nitrogen determination

The method, used to determine the total Kjeldahl nitrogen of the substrate, consists of three steps: digestion, distillation and titration. Before starting with this method, 40 g of the substrate was dried in the VENTICELL oven at 105°C. Four days later, exactly 1 g of substrate was weighted using a Sartorius A-120S analytical balance (Sartorius, Germany) with a resolution up to 0.0001 g and placed in a Kjeldahl digestion tube. This was repeated three times, but it was only carried out for the substrate at the start of the experiment. The digestion of the organic material is achieved by adding 25 mL of H_2SO_4 in each tube and using heat (350°C) and a Se catalyst to speed up the reaction. The digestion ended from the moment that the solution in the Kjeldahl tube became green/yellow and translucid. The digested solutions are shown in Figure 12. This reaction converts any nitrogen in the sample to ammonium sulfate. After the digestion, the samples are neutralized using a 35% NaOH solution and placed in the BÜCHI B-315 Kjeldahl distiller (BÜCHI, Switzerland). This distiller converts ammonium sulfate to ammonia which is distilled off and collected in a receiving flask of excess (10 mL) boric acid (4%) forming ammonium borate. Then, the titration follows in which the borate anions formed are titrated with HCl (0.0968 M), using a methyl-red indicator (Sáez-Plaza et al., 2013). The formula to calculate the total Kjeldahl nitrogen is the following:

$$Total\ Kjeldahl\ N\ \%\ (w/w) = \frac{V * M * 14}{W} * 100$$

With V, the volume (L) of HCl that was needed to get to the endpoint in the titration step. M in the formula stands for the molarity of HCl, being 0.0968 mol L^{-1}. The molar mass of nitrogen is 14 g mol^{-1}. Also the weight (g) of the substrate is used, indicated with W.

Figure 12: Illustration of the digestion of normal substrate for which the reaction ended since the solution is green and translucid.

3.5.5 Analysis of total mineral matter content

The total mineral matter content of the substrate was determined by performing the following steps. Around 10 g of the substrates from all treatments were weighted and placed in the VENTICELL oven (MMM Medcenter, Germany) at 105°C for 4 days. Afterwards, approximately 1 g of each substrate was weighted in a crucible. The crucibles were placed in a muffle furnace at 560°C until a constant weight was reached. The following formula was used to calculate the total mineral matter content of the substrate (Haryono et al., 2021):

$$\%\,Mineral\ matter = \frac{Initial\ weight\ (g) - Final\ weight\ (g)}{Initial\ weight\ (g)} * 100$$

3.6 Chemical characterization of microalgal biomass

3.6.1 Total Kjeldahl nitrogen determination

The total Kjeldahl nitrogen was determined for both microalgae biomasses of *C. vulgaris* and *T. obliquus*. Three repetitions of each disrupted microalga biomass were carried out in the same way as was done for the substrate, explained in part 3.7.4.

3.6.2 Amino acids determination

The amount of free amino acids present in the microalgal biomass, disrupted with different cell disruption methods, was determined following the OPA (o-phthaldialdehyde) method. First, 0.045 g of each microalgal biomass was weighted to get concentrations of 30 g L^{-1} in a 1.5 mL Eppendorfs. Before diluting the samples for the absorbance readings, the samples were rapidly mixed using the Intllab vortex mixer VM-370 (Intllab, Malaysia). Next, the samples were placed in the Eppendorf centrifuge 5430 R (Eppendorf, Germany) during five minutes at 10621 x g and at room temperature (23°C).

After centrifugation, the samples were diluted in a 1:50 ratio with distilled water. Thus, 20 µL of the microalgal sample was mixed with 980 µL of distilled water. L-serine, with a concentration of 1 mg/L, was used as a standard and a calibration curve was established for the different concentrations of the standard (0, 10, 25, 50, 75, 100, 250 and 500 µg/L) and their absorbance at 340 nm. To measure the absorbance of the microalgal samples at 340 nm, 1.5 mL of the OPA reagent was mixed with 20 µL of each the diluted sample. As absorbance changes somewhat with time, the absorbance of each sample was measured at exactly two minutes after mixing with the OPA reagent. For the absorbance measurements the double beam UH5300 spectrophotometer (Hitachi, Japan) was used.

3.6.3 Reducing sugar determination

To analyse the reducing sugar concentration in the microalgal biomass, the DNS (3,5-Dinitrosalicylic acid) method was used. This method detects the presence of free carbonyl groups (C=O) of reducing sugars. It involves an oxidation reaction in which DNS is reduced to 3-amino-5-nitrosalicylic acid (ANS). When ANS is exposed to alkaline conditions, it transforms to a complex with a reddish brown color which has an absorbance maximum of 540 nm (Figure 13). To prepare the microplate, 25 µL of the diluted samples (1:50), previously used for the OPA method, was added together with 25 µL of the DNS standard. The microplate was incubated at 105°C for ten minutes. Next, it was cooled down by putting it in an ice bucket for five minutes. After that time, 250 µL of distilled water was added to each cell. As a blank distilled water was used (Gonçalves et al., 2010). The absorbance of the microplate was read at 540 nm by the BioTek Synergy H1 microplate reader (Agilent, US).

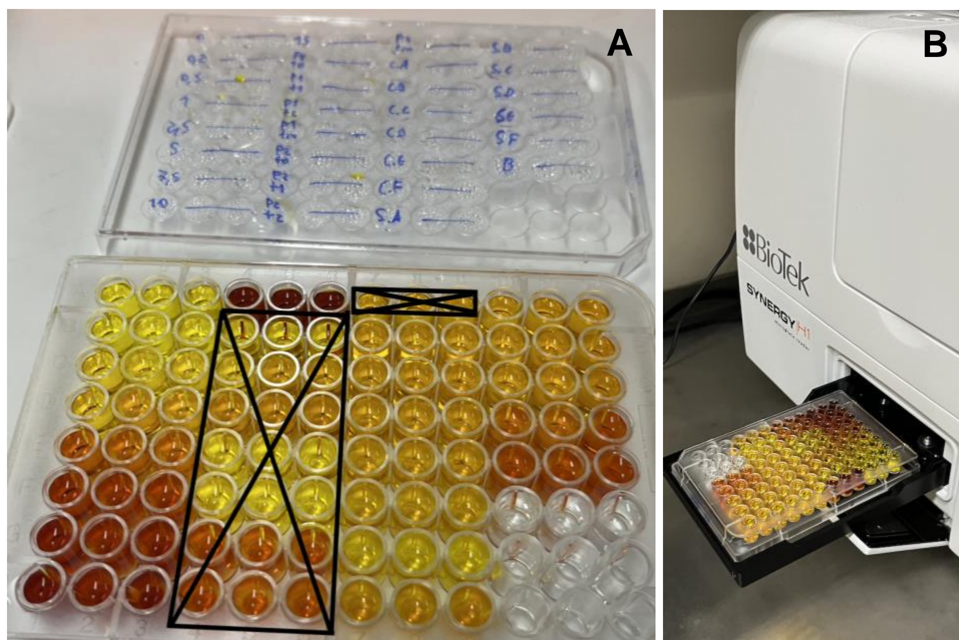


Figure 13: A. The microplate with at the left the standard DNS concentrations. The darker the color, the higher the reducing sugar concentration. Each sample was repeated over 3 cells for a larger statistical power. The crosses are samples of a colleague. At the right of the crosses, the different disrupted microalgal samples can be seen, starting with C. vulgaris*, followed by* T. obliquus*. Cells F 10-12 were filled with a blank (distilled water). B. Introduction of the microplate in the BioTek microplate reader.*

3.7 Statistical analysis

After the above mentioned measurements, the results were subjected to statistical analysis. Data were analyzed using the statistical software IBM® SPSS® Statistics, version 28. The data are presented as a mean ± standard error. Standard errors are represented with error bars. The effects of each microalga were evaluated by analysis of variance (ANOVA; F test) and when ANOVA yielded a significant F value, the individual means were compared using a Duncan's Multiple Range Test (DMRT) at $P < 0.05$. All true outliers were taken out due to natural variations in the population and for more normally distributed data.

4. Results & Discussion

4.1 Growth and development of lettuce plants

In this part, the results of the measured growth parameters are represented for each treatment in Figure 14 - Figure 26. The biostimulant effect of six treatments with microalgae *C. vulgaris* and *T. obliquus*, displayed on the x-axis of the graphs by HPH3C (high pressure homogenization with 3 cycles), HPH1C (high pressure homogenization with 1 cycle), HPHmin (high pressure homogenization with minimal parameters), NT (no disruption treatment), EH (enzymatic hydrolysis), EH + HPH1C/HPH3C (enzymatic hydrolysis combined with HPH), was evaluated and compared with the positive (Algaman B) and negative control (normal irrigation). Each treatment with microalgal extracts was tested in three concentrations, 0.1, 0.5 and 2.0 g L^{-1}.

4.1.1 Length of leaves

Treatment HPH3C 2.0 g L^{-1} of *C. vulgaris* (3 cycles of HPH) was the one with the highest average length of leaves, with a size of 17.83 ± 1.61 cm, being 38.11% larger in size compared to that of the negative control (Figure 14). The second highest length was observerd for treatment HPHmin 0.5 g L^{-1} (minimal HPH parameters) with a size of 17.71 ± 1.36 cm. Additionally, treatments HPHmin 2.0 g L^{-1}, NT 0.1 g L^{-1}, EH 2.0 g L^{-1} and EH + HPH1C 2.0 g L^{-1} were also higher than the negative control (12.91 ± 1.23 cm). Treatment EH 0.1 g L^{-1} (enzymatic hydrolysis with alcalase) showed the lowest average length, with a size of 10.50 ± 0.35 cm.

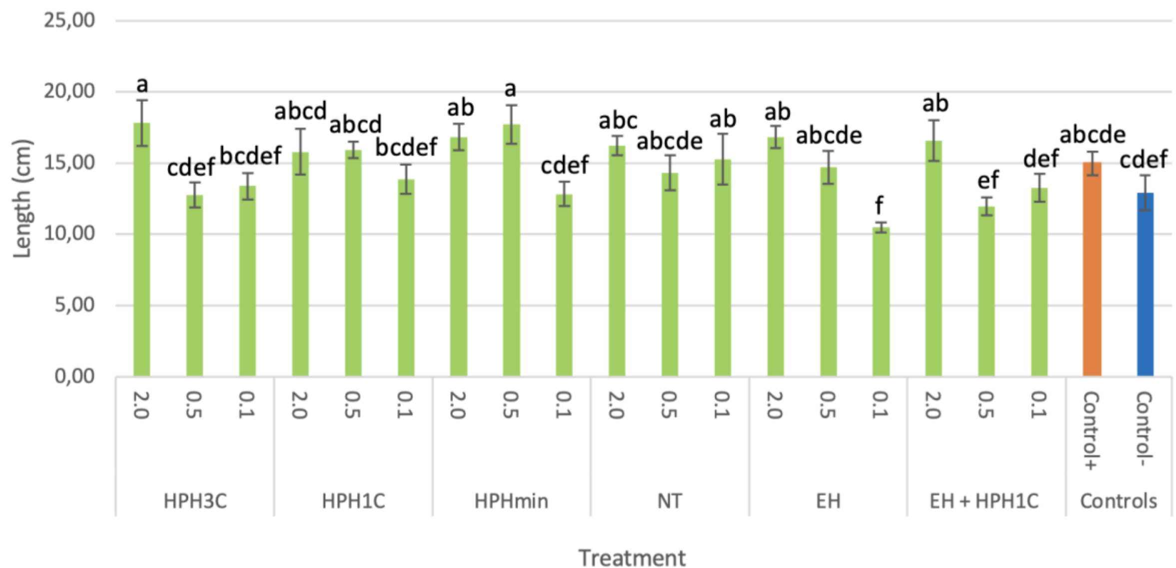


Figure 14: Length (cm) of leaves obtained, when applying six treatments with the microalga C. vulgaris *on lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

The experiment with *C. vulgaris* started in February and therefore the temperatures were not yet ideal for the lettuce plants, ranging between 4°C during the night to 18°C during the day. However, in this experiment several treatments obtained better results than the negative control. The large difference in temperature between day and night, might have caused some abiotic stress for the plants. Therefore, this experiment lasted five weeks compared to four weeks that were needed for the experiment with *T. obliquus*. It can be said that the treatments with higher results, compared to the negative control, were capable of overcoming this abiotic stress. For the experiment with *T. obliquus*, which started in April, the temperatures were higher than in February, ranging from 11 to 30 °C. A week after planting the lettuce seedlings, eight seedlings had died, all in pots of different treatments, because of the disease *Botrytis cinerea* (Figure 15).

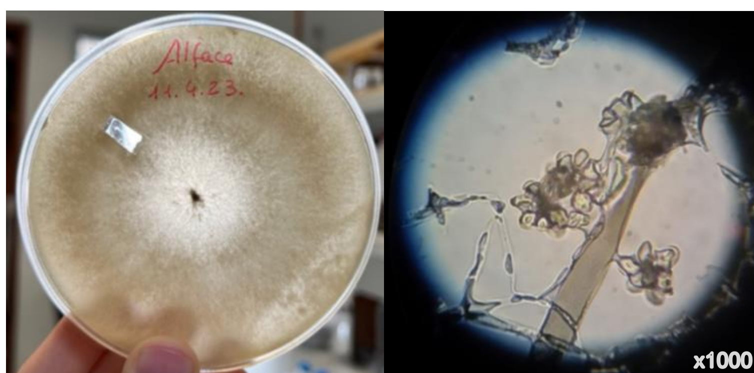


Figure 15: Botrytis cinerea *found in the root of a lettuce seedling. On the right figure, spores can be observed with a Leitz Labovert FS microscope (Leitz,Germany) (x1000).*

As can be seen in Figure 16, Figure 20, Figure 22, Figure 24 and Figure 26 for the experiment with *T. obliquus*, there were no higher results than the negative control for the measured length of the leaves, fresh and dry weight of the leaves, leaf surface area and SPAD value. Thus, *T. obliquus* did not show a biostimulant effect on lettuce growth. What can be noticed is that for all measured parameters the average values of treatments with *T. obliquus* were higher than the values of treatments with *C. vulgaris.* This can be explained by the fact that the higher temperatures increased the growth rate of the lettuce plants during the experiment (Fallovo et al., 2009). Additionally, the following hypothesis could explain why the plants from the negative control were as large as the plants from the treatments with *T. obliquus*. The root systems of the lettuce plants that received the microalgal extracts were possibly growing so fast that at a certain moment there was no space in the little pot for the root system to grow more. While the plants from the negative control still had space for the root system to grow and the more time passed, the more they had a chance to keep up with the plants from the treatments with microalgal extracts. Therefore, it was important to select the right moment to complete the trial. This was done by looking at the number of leaves. However, the roots were not analyzed due to a lack of time but it would be recommended to investigate them in a subsequent study.

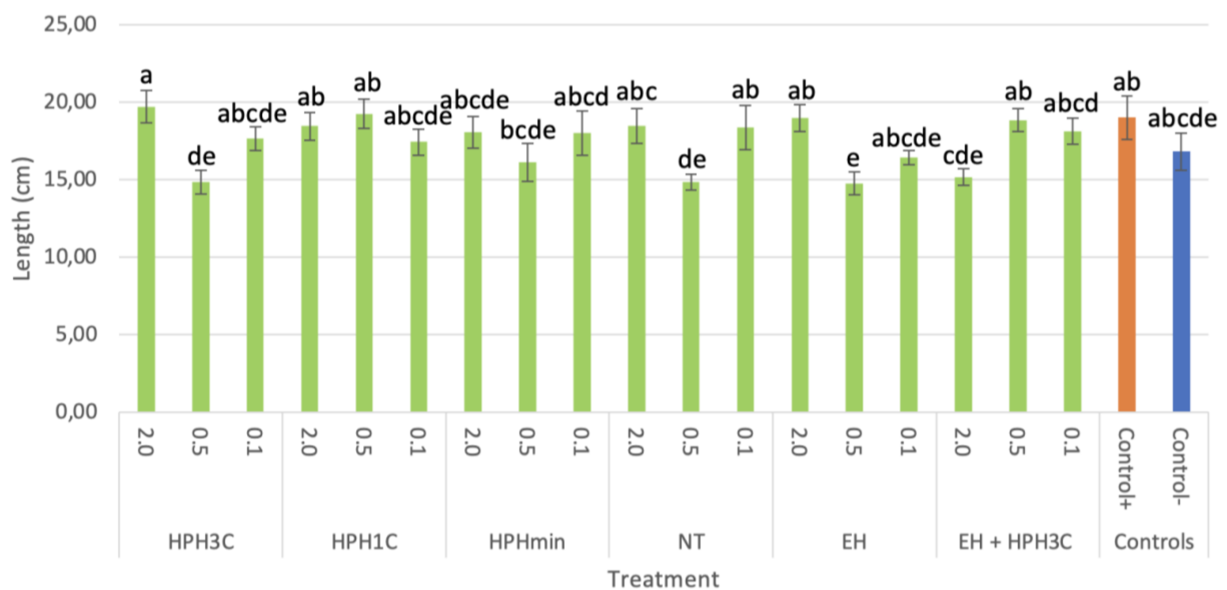


Figure 16: Length (cm) of leaves obtained, when applying six treatments with the microalga T. obliquus *on lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH3C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.1.2 Number of leaves per plant

When comparing the number of leaves between treatments, treatment NT 2.0 g L^{-1} (no disruption treatment) of *C. vulgaris* was the only one that was higher than the negative control, with approximately 9 leaves (Figure 17). The remaining treatments were approximately 8 leaves per plant.

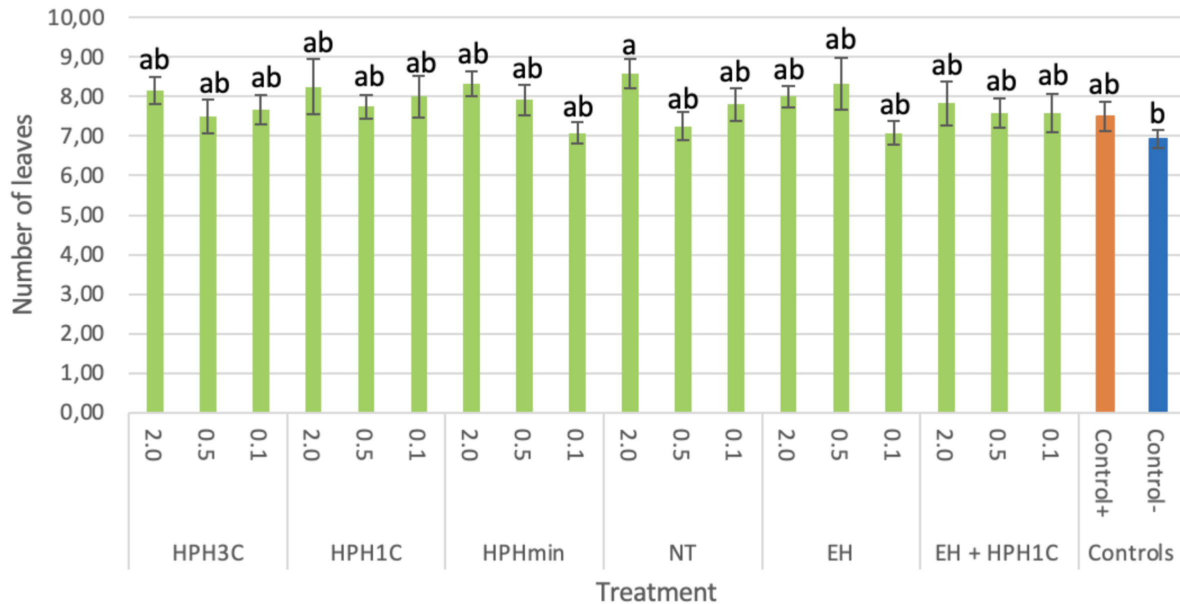


Figure 17: Number of leaves obtained per plant, when applying six treatments with the microalga C. vulgaris *on lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

Only treatments HPH3C 2.0 g L^{-1} and NT 2.0 g L^{-1} of *T. obliquus* had a higher result than the negative control, with approximately 10 leaves. The others treatments resulted in approximately 7 to 8 leaves per plant (Figure 18).

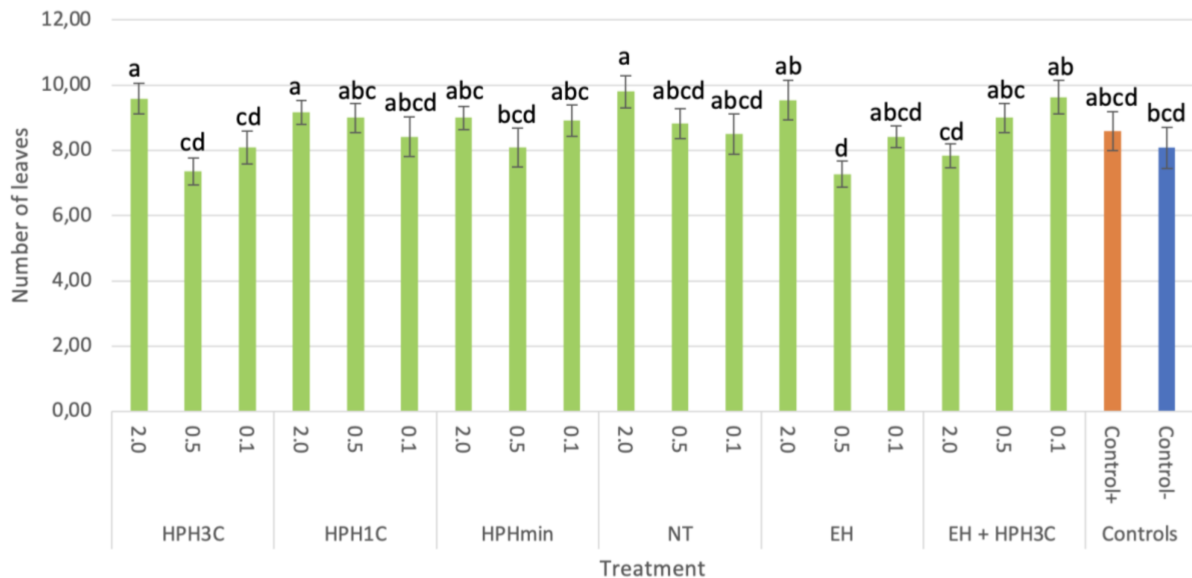


Figure 18: Number of leaves obtained per plant, when applying six treatments with the microalga T. obliquus *on lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH3C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.1.3 Fresh weight of leaves

There were no higher results for the fresh leaf weight of treatments with *C. vulgaris* compared to the negative and positive control. As shown in Figure 19, in all six treatments with microalgal extracts, the concentration of 2.0 g L^{-1} resulted in a higher fresh weight of the leaves than concentrations 0.1 and 0.5 g L^{-1}, although only for treatments HPH3C, EH and EH + HPH1C this correlation was significant.

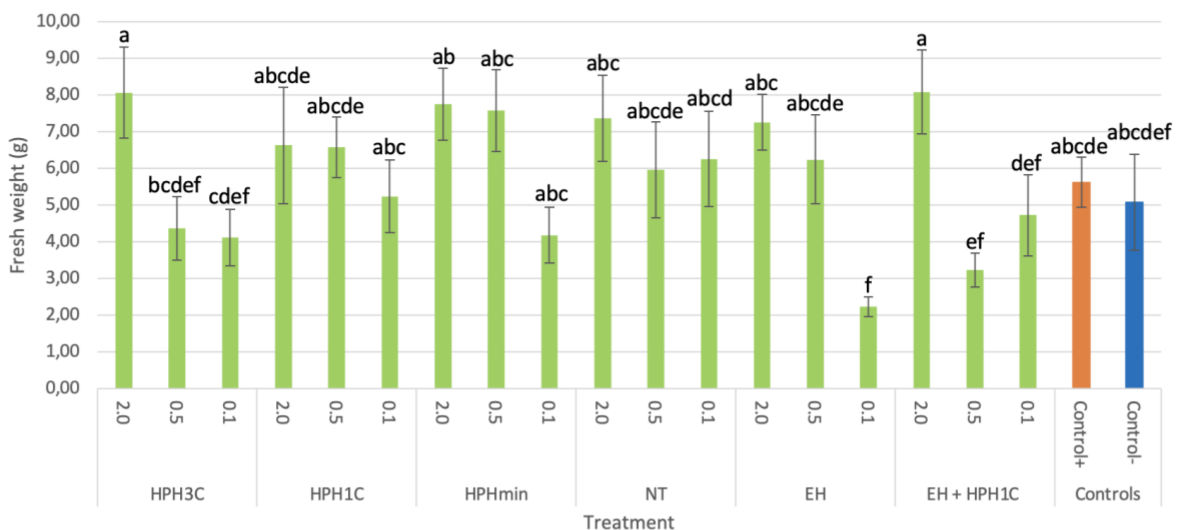


Figure 19:Fresh weight (g) of lettuce leaves obtained, when applying six treatments with the microalga C. vulgaris *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

It was shown in Figure 20 that there was no treatment with a higher result for the fresh weight of the leaves than the negative control, when treated with *T. obliquus*.

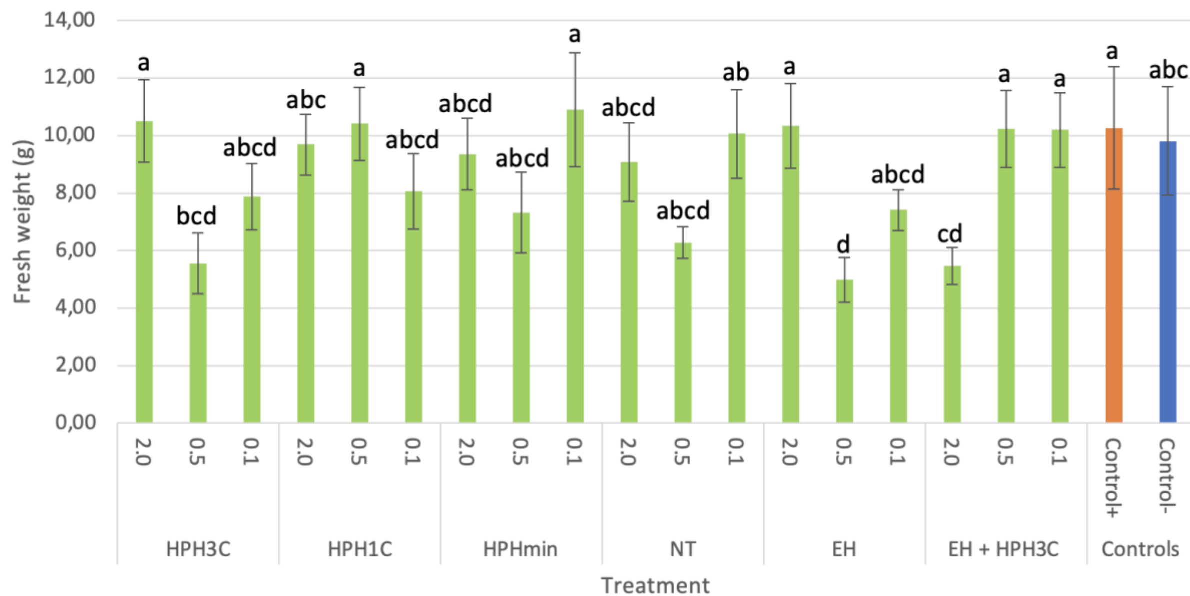


Figure 20: Fresh weight (g) of lettuce leaves obtained, when applying six treatments with the microalga T. obliquus *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH3C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

The productivity was determined, based on the fresh weight of the leaves, for both experiments with *C. vulgaris* and *T. obliquus*. In a study of Assefa et al. (2021), the lettuce was harvested 65 to 75 days after sowing four week old seedlings, while the harvest of the experiment with *C. vulgaris* and *T. obliquus* was carried out after five and four weeks respectively. Thus, the end of the crop cycles were not yet achieved, so the following numbers are a pre-estimation of the productivity.

For the experiment with *C. vulgaris*, 68% of the treatments with microalgal extracts showed a higher productivity than the negative control (7927 kg/ha). Treatments EH + HPH1C 2.0 g L^{-1}, HPH3C 2.0 g L^{-1} and HPHmin 2.0 g L^{-1} had the highest productivities, being 12617 kg/ha, 12598 kg/ and 12109 kg/ha respectively. In all treatments of *C. vulgaris*, the concentration of 2.0 g L^{-1} had a higher productivity than the negative control. Additionally, for eleven of the eighteen treaments with microalgal extracts, the productivity was higher than the positive control (8779 kg/ha), Algaman B, an already commercial biostimulant.

For the experiment with *T. obliquus*, only 32% of the treatments with microalgal extracts showed a higher productivity than the negative control (15328 kg/ha).

Treatments HPH3C 2.0 g L^{-1}, HPH1C 0.5 g L^{-1} and EH + HPH3C 0.5 g L^{-1} had the highest productivities, being 16418 kg/ha, 16270 kg/ha and 15979 kg/ha respectively. These treatments also resulted in a productivity higher and/or equal to the positive control (16040 kg/ha). In a study of Hasan et al. (2017), they obtained a final lettuce yield of 18650 kg/ha without applying any supplementary nitrogen fertilizer. Although the yields in the experiments with *C. vulgaris* and *T. obliquus* were lower, the final plant size had not yet been reached.

4.1.4 Dry weight of leaves

Regarding the dry weight of the leaves, the highest value was observed for treatment EH + HPH1C 2.0 g L^{-1} of *C. vulgaris*, with 0.59 ± 0.08 g. This was the one treatment with a higher result compared to that of the negative control (Figure 21).

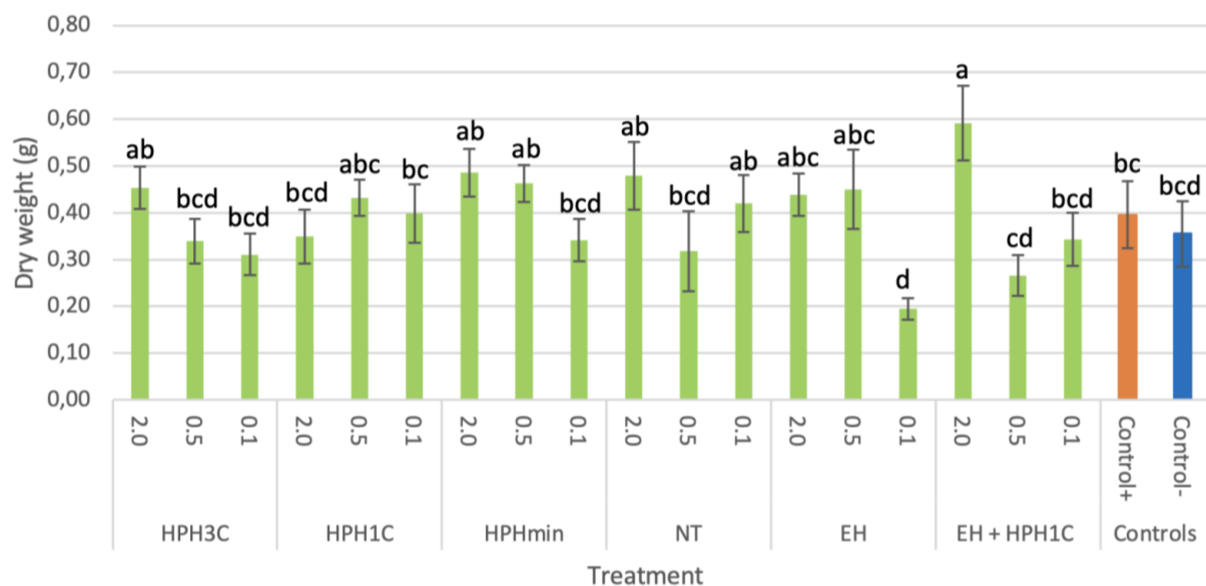


Figure 21: Dry weight (g) of lettuce leaves obtained, when applying six treatments with the microalga C. vulgaris *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

As mentioned before, there were no treatments with *T. obliquus* that had higher results than the negative control for the dry weight of the leaves (Figure 22).

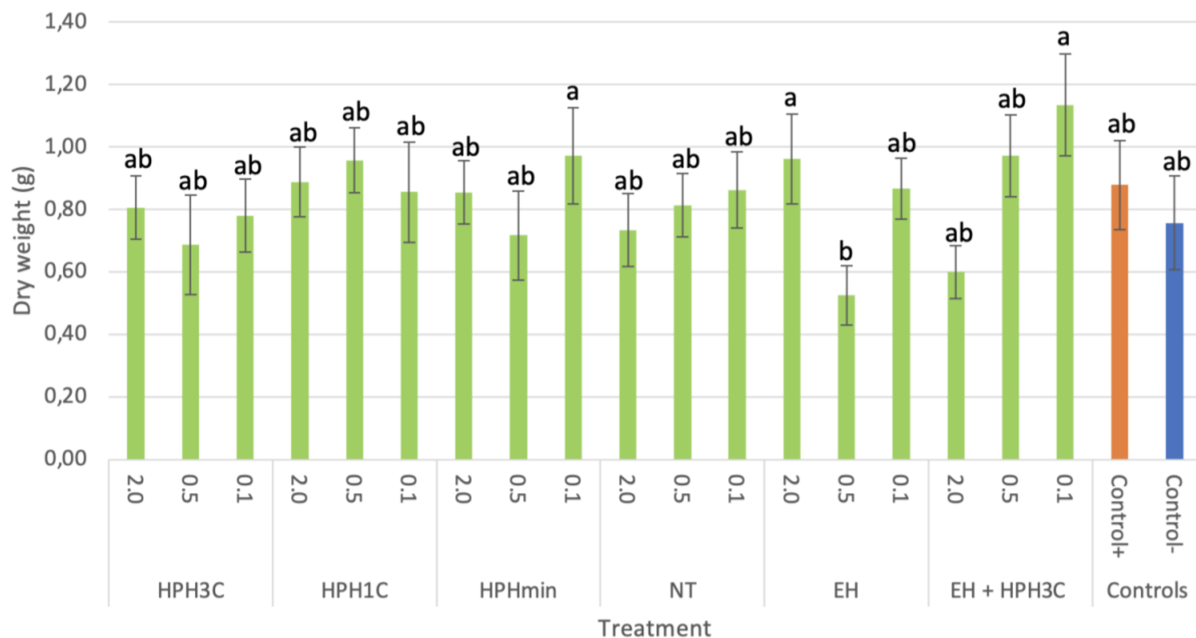


Figure 22: Dry weight (g) of lettuce leaves obtained, when applying six treatments with the microalga T. obliquus *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH3C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.1.5 Leaf surface area

The results of the leaf surface area are shown in Figure 23. Here, treatment EH + HPH1C 2.0 g L^{-1} of *C. vulgaris* presented the largest leaf area ($p<0.05$), with an area of 223.52 ± 20.62 cm^2. This treatment had a leaf surface area that is 119,73% larger than the negative control with a leaf surface area of 101.72 ± 6.49 cm^2. When comparing the length of the plants with treatment EH + HPH1C 2.0 g L^{-1}, shown in Figure 14, with the leaf surface area, it appears that the plants had invested more in leaf width than length. The smallest leaf area was observed in treatment EH 0.1 g L^{-1}, with an area of 74.35 ± 6.29 cm^2.

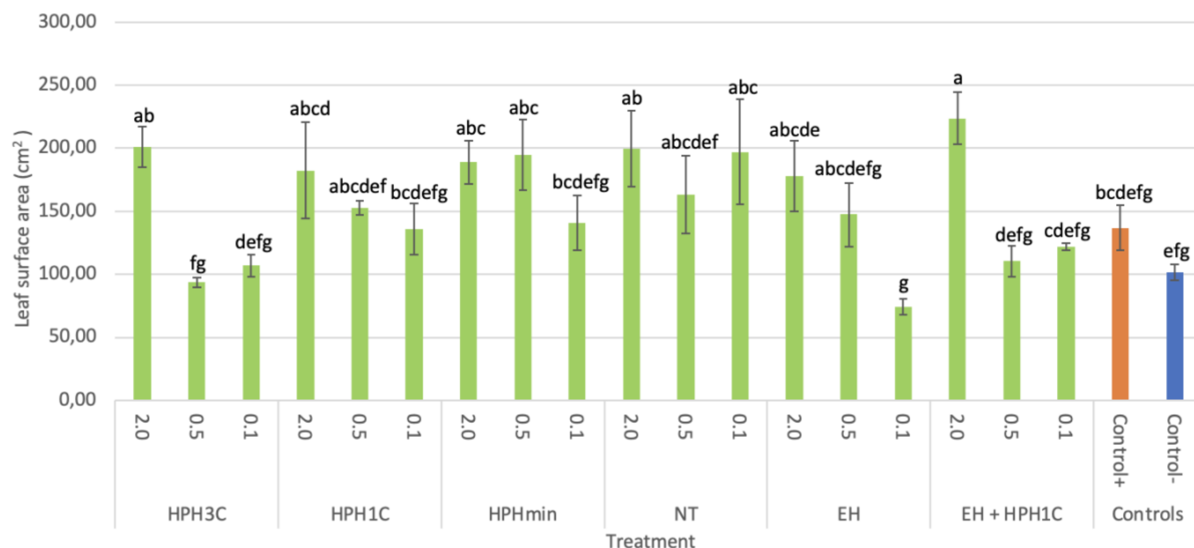


Figure 23: Leaf surface area (cm²) of lettuce leaves obtained when applying six treatments with the microalga C. vulgaris *on lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for p<0.05, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

Regarding the leaf surface area of the leaves from lettuce plants treated with *T. obliquus*, there were no higher results than the negative control (Figure 24).

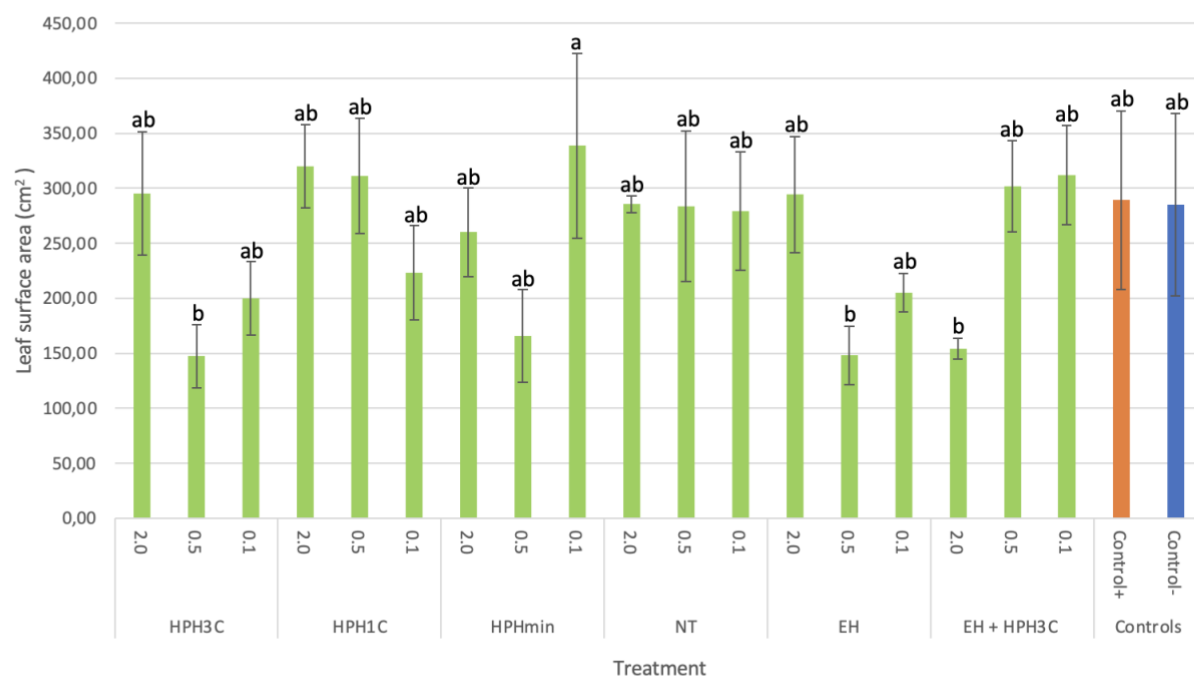


Figure 24: Leaf surface area (cm²) of lettuce leaves obtained when applying six treatments with the microalga T. obliquus *on lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH3C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The treatments containing the same letter do not present significant differences for p<0.05, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.1.6 SPAD analysis

A high SPAD value means a high amount of chlorophyll in the leaves. In Figure 25, the SPAD values can be observed for different treatments of *C. vulgaris*. There was no treatment with a higher SPAD value compared to that of the negative control.

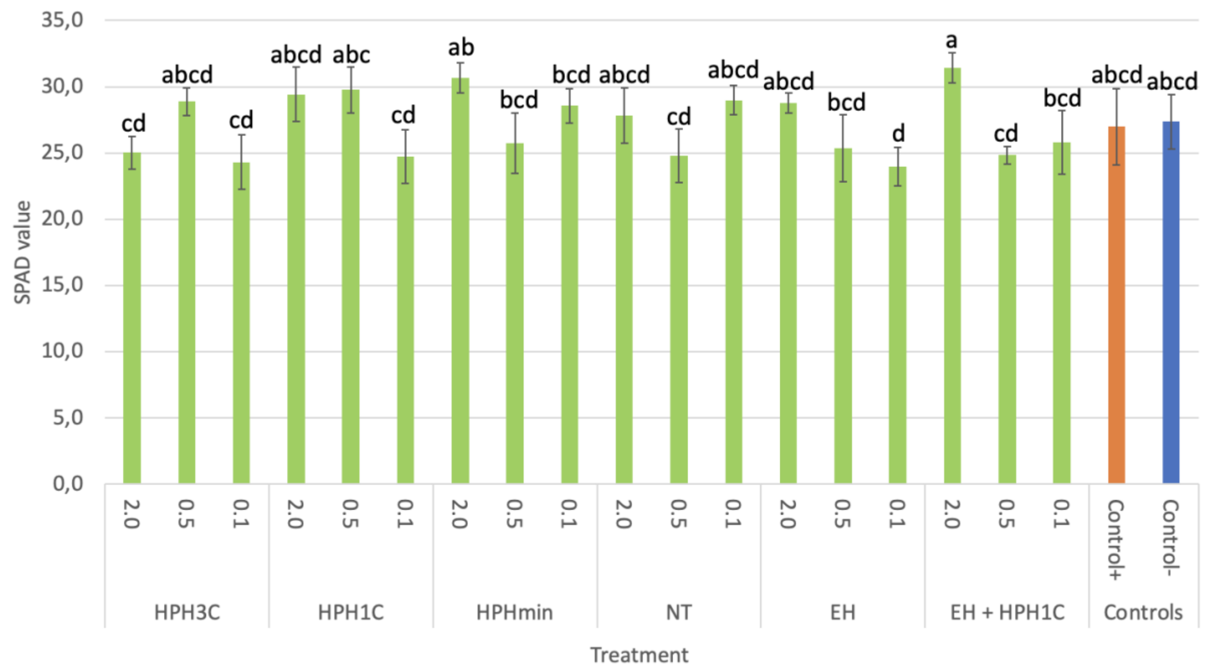


Figure 25: SPAD readings obtained throughout the assay, when applying six treatments with the microalga C. vulgaris *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}.The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test.*

For the SPAD values of the leaves of the treatments with *T. obliquus*, there were no higher values than the negative control. However, treatments HPH1C 2.0 g L^{-1}, HPH1C 0.5 g L^{-1} and EH + HPH3C 0.5 g L^{-1} resulted in higher SPAD values compared to that of the positive control (Figure 26). The positive control and treatment EH 0.5 g L^{-1} showed the lowest SPAD values.

Figure 26: SPAD readings obtained throughout the assay, when applying six treatments with the microalga T. obliquus *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH1C. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}.The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test.*

The experiments were carried out at a different time, which made it difficult to compare the results with each other. Other factors, such as temperature and photoperiod, may have caused differences in the lettuce development between both experiments. Additionally, at the end of the experiments the amount of water, coming from the irrigation system, was measured. In one of the three sprinklers, twice as much water came out compared to the other two. This had caused the standard deviations to increase. Therefore, optimization of the irrigation system is needed before starting a new trial.

Although it has already been proven that *C. vulgaris* provides a biostimulating effect in lettuce (La Bella et al., 2021), several treatments with *C. vulgaris* showed higher results than the negative control (Table 10). Particularly, treatment EH + HPH1C 2.0 g L^{-1} obtained higher values for the length, dry weight and leaf surface area than the negative control. Additionally, this treatment resulted in higher values for the dry weight and leaf surface area than the positive control Algaman B, which is an already commercial biostimulant.

Biostimulating effects had also been proven for *T. obliquus* in plants, such as tomato, soybean and barley (Ferreira et al., 2021). However, in this work there were no higher results obtained compared to the negative and positive control for treatments with *T. obliquus.*

Table 10: Overview of treatments of C. vulgaris *with higher results than the negative control. The order is descending, meaning that the treatment with the highest results is placed first in the row.*

Parameter	**Treatments of *C. vulgaris* with higher results than the negative control (in descending order)**
Length	HPH3C 2.0 g L^{-1}, HPHmin 0.5 g L^{-1}, HPHmin 2.0 g L^{-1}, NT 0.1 g L^{-1}, EH 2.0 g L^{-1}, **EH + HPH1C 2.0 g L^{-1}**
Number of leaves	NT 2.0 g L^{-1}
Fresh weight	/
Dry weight	**EH + HPH1C 2.0 g L^{-1}**
Leaf surface area	**EH + HPH1C 2.0 g L^{-1}**, HPH3C 2.0 g L^{-1}, NT 2.0 g L^{-1}, NT 0.1 g L^{-1}, HPHmin 0.5 g L^{-1}, HPHmin 2.0 g L^{-1}, HPH1C 2.0 g L^{-1}
SPAD	**/**

4.2 Substrate analysis

The substrate of all treatments was analyzed to verify that it had not affected the results of the plant growth. The pH, EC, moisture content and total mineral matter was measured. Additionally, for the initial substrate also the Kjeldahl nitrogen percentage was determined, being 1.11 ± 0.016 % dry weight.

4.2.1 Substrate pH

The pH of the substrate measured at the start of the experiment showed a lower value, being 5.94 ± 0.04, than the pH of the substrate of all other treatments with *C. vulgaris* (Figure 27). The same was found for the experiment with *T. obliquus*. The initial pH corresponded to the pH that was indicated on the packaging of the substrate (5.0 - 6.5) and was similar to the pH measured in a study of Cristina et al. (2020). According to Li et al. (2022), moderate alkaline substrate showed the highest potential response to biostimulant application. The pH of most microalgae treatments and the controls was around 7, indicating that the pH had significantly risen since the start of the experiment.

This can be explained by the more alkaline water (pH of 7.55 ± 0.05) from the greenhouse used for irrigation of the plants.

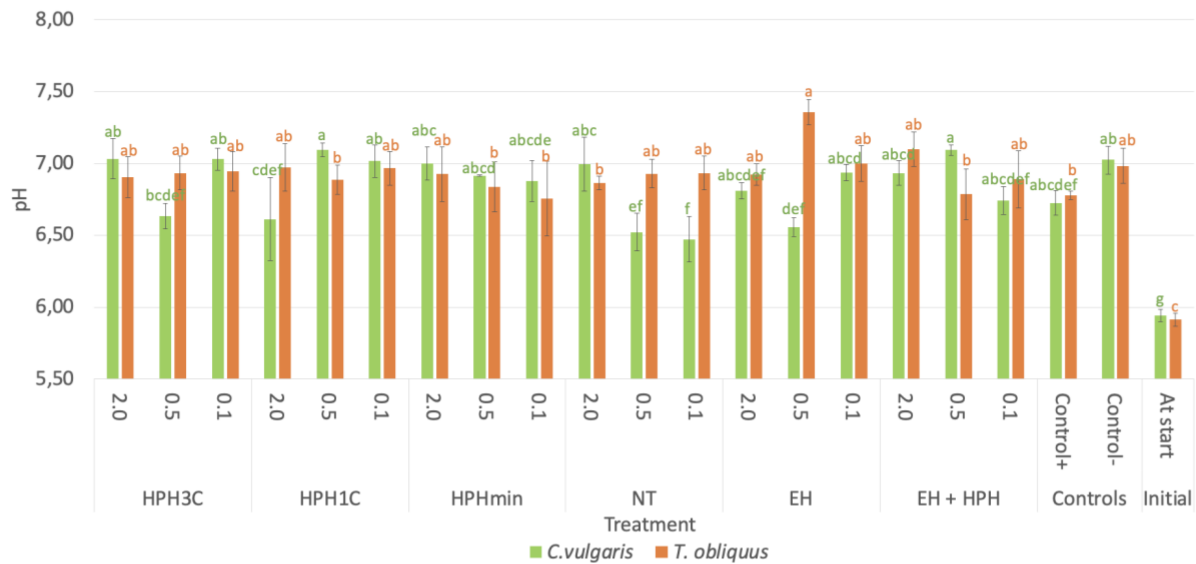


Figure 27: pH of substrate after applying six different treatments with the microalgae C. vulgaris *and* T. obliquus *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The pH of the substrate at the start of the experiment is indicated as initial substrate. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.2.2 Substrate EC

The EC of the substrate at the start of the experiment was higher, being 0.63 ± 0.03 ms cm^{-1}, than the EC of the substrate of all other treatments (Figure 28). This value was similar to the EC of peat measured in a study of Cristina et al. (2020). The difference in EC between substrate of treatments and initial substrate can be explained by the fact that the irrigation of the plants leached large amounts of minerals present in the substrate. Additionally, the plants absorbed nutrients and minerals from the substrate to ensure maximum growth. The EC of the substrates treated with microalgae had the same order of magnitude as measured in a study of Alvarenga et al. (2023). According to Albornoz and Lieth (2015), a high concentration of nutrients (EC of 6 to 10 dS m^{-1}) in the root zone reduced lettuce yield as a consequence of a combination of decreased stomatal conductance and leaf area. As illustrated in Figure 28, no such high EC was reached.

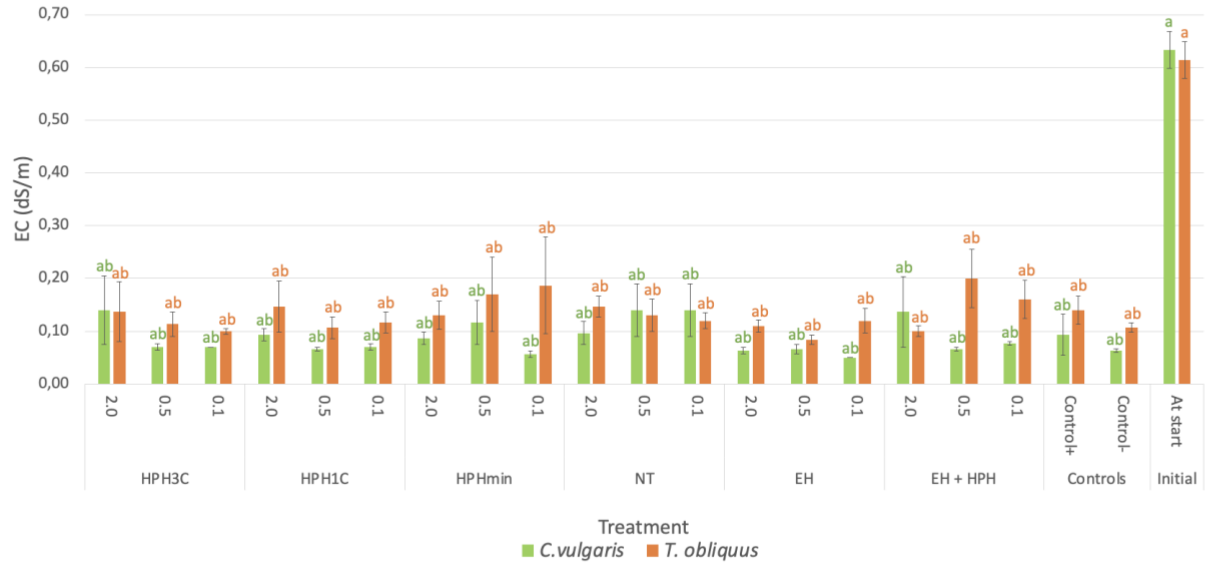


Figure 28: EC of substrate after applying six different treatments with the microalgae C. vulgaris *and* T. obliquus *in lettuce plants, displayed by HPH3C, HPH1C, HPHmin, NT, EH, EH + HPH. Each treatment with microalgal extracts was applied in three concentrations 2.0, 0.5 and 0.1 g L^{-1}. The EC of the substrate at the start of the experiment is indicated as initial substrate. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.2.3 Moisture content of substrate

Overall, the moisture content of the substrates was very similar between treatments and microalgae. The initial substrate that had not been irrigated, obviously contained the lowest moisture content (Figure 29).

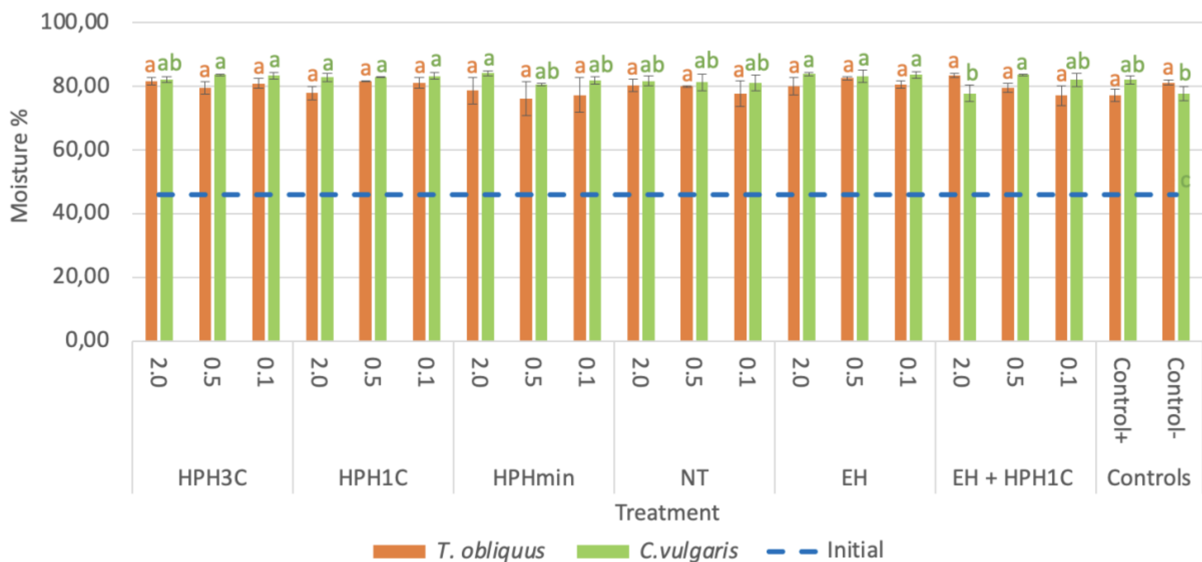


Figure 29: Moisture content of the substrate of both experiments with C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The initial moisture content at the start of both experiments is indicated with the blue dotted line. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.2.4 Mineral matter of substrate

On top of the similar moisture content of the substrates, Figure 30 showed that the mineral matter percentage did not differ between treatments.

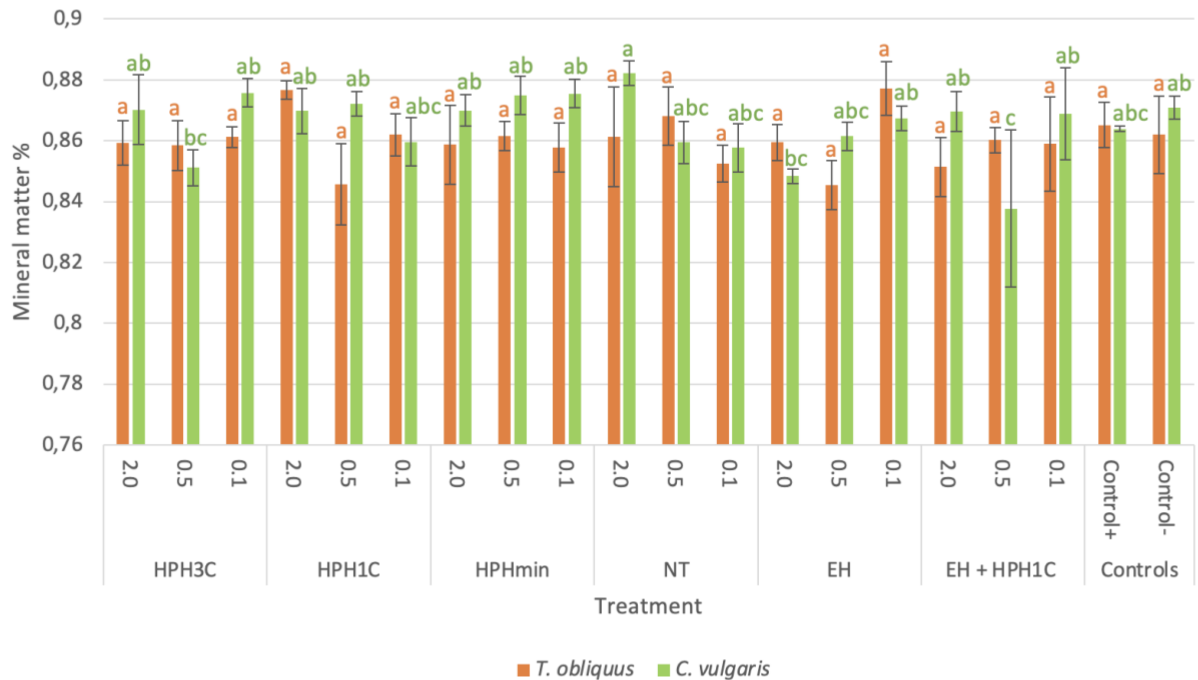


Figure 30: Mineral matter of the substrate of both experiments with C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for* $p<0.05$*, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.3 Microalgae suspensions

4.3.1 pH and EC

For *C. vulgaris* suspensions, Figure 31 showed that in all treatments, except for treatment HPH3C, the highest concentration of 2.0 g L^{-1} led to a lower pH than concentrations 0.5 and 0.1 g L^{-1}. Additionally, the pH of the positive control was higher than that of treatments with microalgal extracts. For *T. obliquus* extracts, there was no difference between concentrations of a treatment.

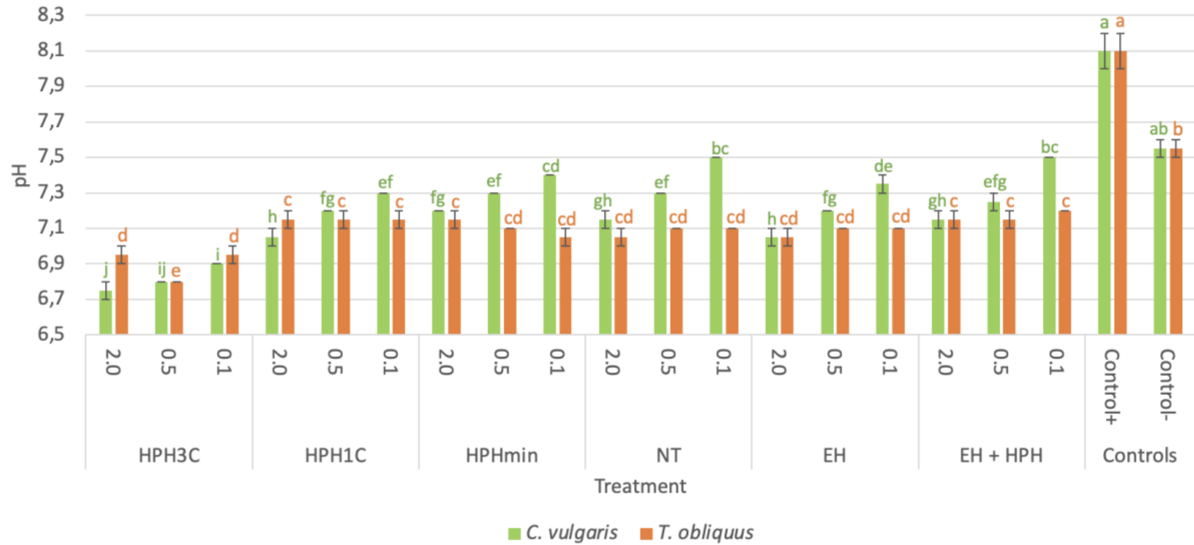


Figure 31: pH of microalgae suspensions with C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for p<0.05, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

As illustrated in Figure 32, the EC of the highest concentration 2.0 g L^{-1} showed a higher result compared to concentrations 0.5 and 0.1 g L^{-1} in all treatments and for both microalgae. Furthermore, the enzymatic hydrolysis (EH) treatments resulted in higher EC's compared to other treatments with microalgal extracts. The possible reason for this can be assigned to the usage of acid and base solutions, added during the EH for pH adjustments.

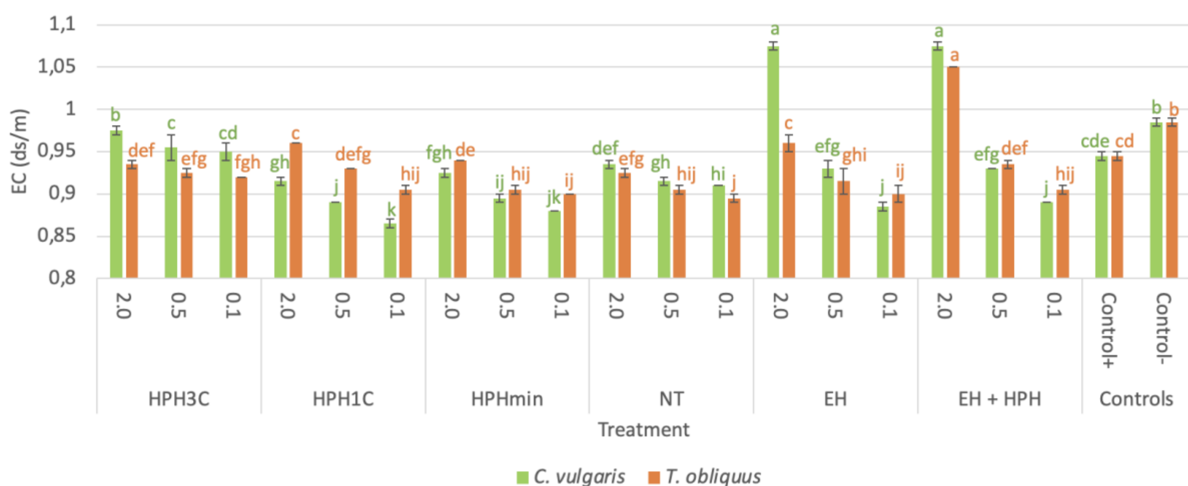


Figure 32: EC of microalgae suspensions with C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for p<0.05, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.4 Microalgal biomass characterization

Within the limited time frame, only the free amino acid content, the reducing sugar content and total Kjeldahl nitrogen percentage was determined for the microalgal biomasses. If more time, equipment and budget had been available, it would have been interesting to look into what kind of amino acids were present in the microalgae, the presence of phytohormones, polysaccharides, vitamins and study the elemental composition.

4.4.1 Free amino acid content

Amino acids are basic building blocks of protein and are therefore important in plant growth, development and metabolite sythesis. As shown in Figure 33, the free amino acid content was higher for the EH and EH + HPH1C disruption methods of *C. vulgaris* and for the EH + HPH3C of *T. obliquus,* compared to the other disruption methods. The enzymatic treatment should have increased the total amino acid content since in all of them proteases were used, except in the EH of *T. obliquus*. Additionally, high pressure homogenization (HPH) increased the free amino acid content in *C. vulgaris* more than for *T. obliquus*. The higher free amino acid content of disruption method EH + HPH1C of *C. vulgaris* could have possibly resulted in the higher plant growth values (length, dry weight and leaf surface area), but in a subsequent study it would be interesting to study which amino acids were present in this microalga (Andreeva et al., 2021; Popko et al., 2018). According to Templeton and Laurens (2015), free amino acids in non-treated algae can account for a significant 3 - 12% fraction of the algal dry weight. These percentages correspond to the values of *T. obliquus*, except for EH + HPH3C. However, the values of *C. vulgaris* were higher, ranging from 12% for HPHmin to 54% for EH + HPH1C. According to Hempel et al. (2012), *Chlorella sp.* can have an amino acid content above 40% (dry weight), which is in line with the values found for disruption methods EH and EH + HPH1C of *C. vulgaris*.

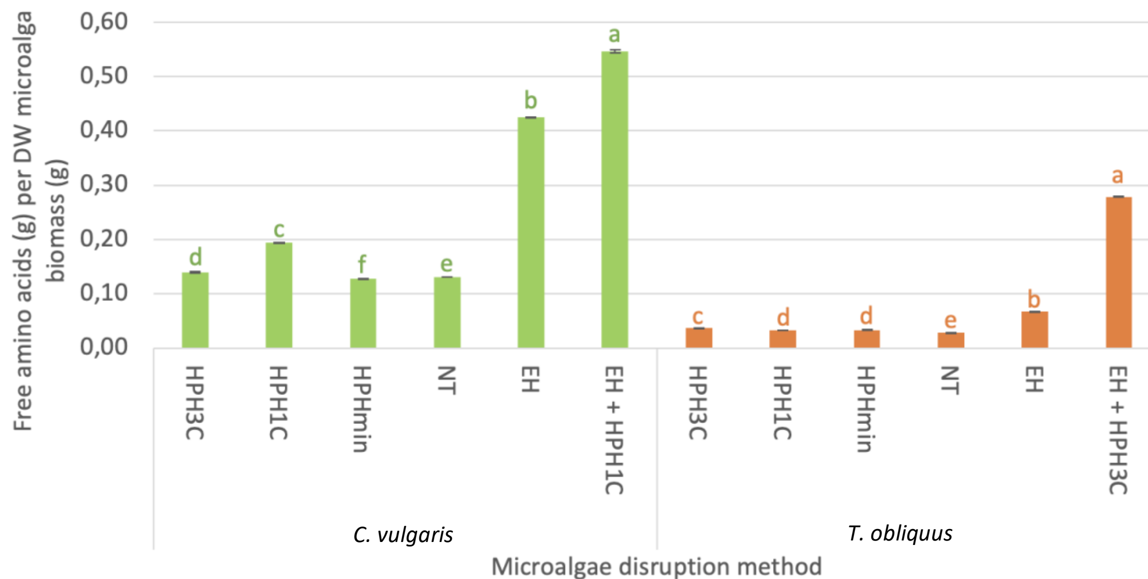


Figure 33: Free amino acids content (g) per DW of microalga biomass (g) for different treatments of C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for p<0.05, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.4.2 Reducing sugar content

As illustrated in Figure 34, there was a higher reducing sugar content for *T. obliquus*. The reason is that the enzymatic hydrolysis (EH) of *T. obliquus* was done with carbohydrases, whereas for *C. vulgaris* this was done with a protease. Both EH + HPH treatments were carried out with a carbohydrase and protease cocktail. However, the cell wall of *T. obliquus* was more susceptible for carbohydrases. For *C. vulgaris*, the carbohydrase and protease cocktail consisted of a greater amount of protease compared to carbyhydrase, which explained the lower reducing sugar content of this microalga. The values obtained for both microalgae disrupted with HPH, ranging from 0.025 g per gram biomass for HPH3C of *C. vulgaris* to 0.035 g per gram biomass for HPH3C of *T. obliquus,* were similar to values found in literature (Shene et al., 2016).

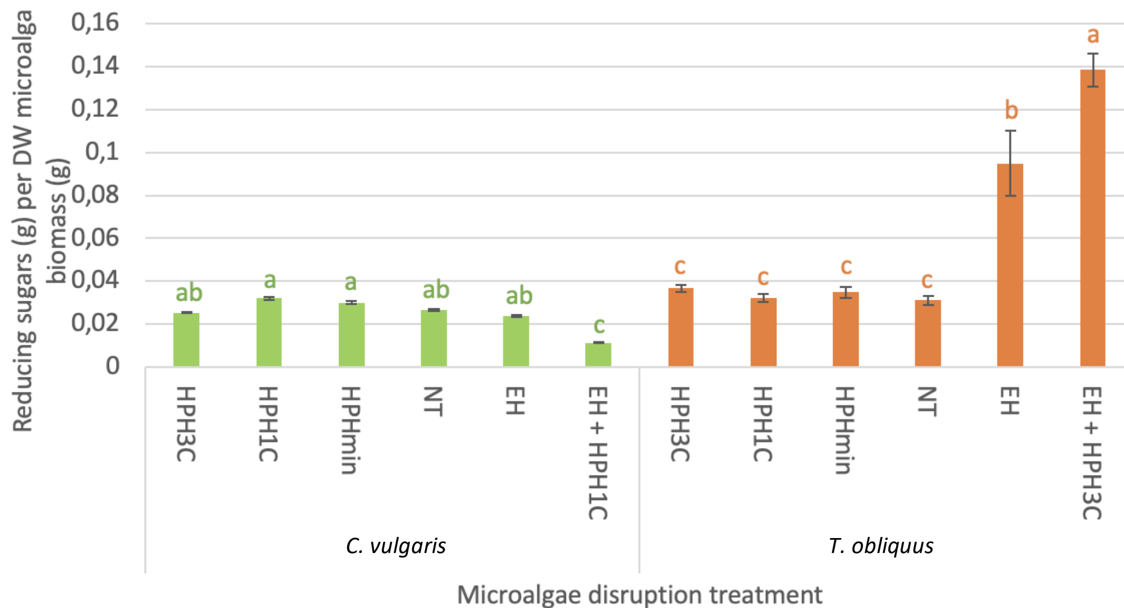


Figure 34: Reducing sugar content (g) per DW of microalga biomass (g) for different treatments of C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for p<0.05, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

4.4.3 Total Kjeldahl nitrogen %

As shown in Figure 35, the dry biomass of *C. vulgaris* contained a larger amount of total Kjeldahl nitrogen compared to *T. obliquus*. Since total Kjeldahl nitrogen consists of ammonia and organic nitrogen compounds, it is bioavailable for the lettuce plants. However for plant growth, nitrate and ammonium commonly serve as the primary sources of nitrogen (Craine et al., 2015; Domini et al., 2009). When comparing these percentages with the growth measurements of the plants of different treatments, no clear correlation can be made.

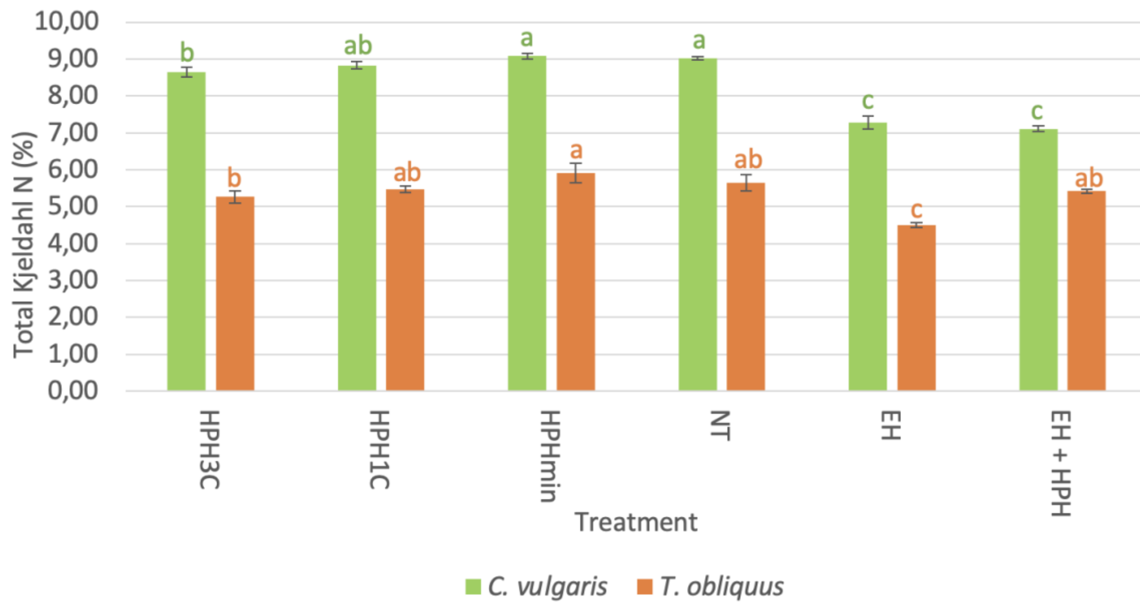


Figure 35: The percentage of total Kjeldahl nitrogen present in dry biomass of C. vulgaris*, indicated with the green color, and* T. obliquus*, indicated with the orange color. The green and orange letters on top of the error bars indicate the statistical analysis carried out for* C. vulgaris *and* T. obliquus *respectively. The treatments containing the same letter do not present significant differences for $p<0.05$, using a Duncan's multiple range test. Data points are shown as mean ± standard error. Standard errors are represented by error bars.*

5. Conclusion

The application of *C. vulgaris* suspensions on the substrate is to be considered a promising and innovative agricultural technique, since it has the potential to reduce to amount of synthetic fertilizer while maintaining the needed yield. New alternatives such as microalgae are much needed, as the EU Green deal foresees to reduce the use of synthetic fertilizers with 20% by 2030. Microalgae are safe to the environment and can improve agricultural sustainability.

Taking all results in consideration, from the two microalgae, processed at GreenCoLab, only *C. vulgaris* had a biostimulant effect on the lettuce crops. *C. vulgaris* disrupted by EH + HPH1C 2.0 g L^{-1} led to a higher lettuce productivity than that of the negative control, enhancing the yield and growth of the plant under trial. Furthermore, this treatment obtained better results for the dry weight and leaf surface area than those of the positive control, Algaman B, an already commercial biostimulant product. Besides, for almost all disruption treatments, the highest concentration of 2.0 g L^{-1} of *C. vulgaris* resulted in higher values for the length, dry weight and leaf surface area of the lettuce plants. From all treatments with *C. vulgaris*, disruption method EH + HPH1C can be referred to as the most optimal cell disruption method. However, lettuce plants treated with *T. obliquus* did not show any higher results than those of the negative control. Therefore, no optimal concentration and cell disruption method can be indicated as the most optimal for this microalga.

For further studies, it would be recommended to perform both experiments on the same time and therefore excluding the influence of the temperature and photoperiod difference. In addition, it would be interesting to investigate the effect of both microalgae in different crops and throughout the crop cycle to study the real effect of the algae on the productivity. Finally, it would be interesting to study the bioactive compounds, such as phytohormones, vitamins, polysaccharides, amino acids among others, in more detail to understand their role in the overall biostimulant activity.

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