Sofia de Sousa Sapateiro Claré Carapinha

Bachelor in Cellular and Molecular Biology

Microalgae cultivated in Swine Wastewater: Stimulation of Seed Growth and Biopesticide Potential

Dissertation to obtain the Master's Degree in
Nutritional Phytotechnology for Human Health

Supervisor: Luísa Maria Rodrigues Gouveia da Silva, Senior Investigator, National Laboratory of Energy and Geology, I.P. – Bioenergy Unit

Co-supervisor: Maria Fernanda Guedes Pessoa, Assistant Professor, Faculty of Science and Technology - NOVA University of Lisbon

Jury panel:

President: Fernando Henrique da Silva Reboredo (PhD), Assistant Professor of FCT/UNL

Defendant: Alberto Delgado dos Reis (PhD), Senior Investigator and Bioenergy Unit Coordinator of National Laboratory of Energy and Geology, I.P.

Members of the panel: Luísa Maria Rodrigues Gouveia da Silva (PhD), Senior Investigator

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Abstract

Humanity faces dramatic issues related to water scarcity and its contamination, as well as excessive use of chemical fertilizers and pesticides, to increase agriculture efficiency. Eutrophication, contamination and soil infertility threatens agricultural sustainability and public health, as well as the earth's ecosystems and biodiversity. Currently, microalgae are revealing themselves as promising on bioremediation of various wastewaters and as sustainable alternative on agriculture.

This dissertation pretends to ally bioremediation to agriculture: the microalgae *Chlorella prototheco*ides, *Chlorella vulgaris*, *Scenedesmus obliquus* e *Synechocystis* sp. were selected (after screening) for swine wastewater treatment. The resulting biomass of the swine wastewater treatment was tested as germination/growth stimulation of tomato, watercress, cucumber, soy, barley and wheat seeds, and as biopesticide against *Fusarium oxysporum*.

Regarding bioremediation, the four species reduced COD levels in 61-75%, total Kjeldahl nitrogen in 70-80%, ammonia nitrogen in 93-97% and phosphorus between 94-100%, especially *C. protothecoides* and *S. obliquus*. In general, the limits imposed by Decree Law 236/98 of Portuguese legislation for wastewater treatment were fulfilled and treated waters could be discharged or reused. The biochemical profiles of microalgae biomass presented protein contents between 34-47%, fatty acids (C12-C18) between 26-84%, and total sugars between 25-33%. The results for growth stimulation trials were positive for all microalgae depending on seed type and light conditions, *Synechocystis* sp. and *C. vulgaris* having the more relevant results. On biopesticide trials, *Synechocystis* sp. and *S. obliquus* obtained the best results as fungi growth inhibitors.

In summary, *S. obliquus* and *C. protothecoides* were the most efficient in the wastewater treatment, *S. obliquus* and *C. vulgaris*, on germination/growth stimulation, and *Synechocystis* and *S. obliquus*, for biopesticide potential.

Keywords: Swine wastewater, *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus*, *Synechocystis* sp.

Resumo

A humanidade depara-se com questões dramáticas relacionadas com a falta de água e sua contaminação, bem como o recurso excessivo a fertilizantes e pesticidas químicos, para aumento da eficiência agrícola. A eutrofização, contaminação e infertilidade dos solos ameaça a sustentabilidade agrícola e a saúde pública, bem como a biodiversidade e os ecossistemas terrestres. Actualmente, as microalgas revelam-se promissoras na biorremediação de efluentes vários e como alternativa sustentável na agricultura.

Esta dissertação pretende aliar a biorremediação à agricultura: as microalgas *Chlorella prototothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* e *Synechocystis* sp. foram seleccionadas (após *screening*) para o tratamento de efluente suinícola. A biomassa resultante do tratamento do efluente suinícola foi testada como estimulante na germinação/crescimento de sementes de tomate, agrião, pepino, soja, cevada e trigo, e como biopesticida contra o fungo *Fusarium oxysporum*.

Relativamente à biorremediação, as quatro espécies reduziram os níveis de CQO entre 61-75%, azoto Kjeldahl total em 70-80%, azoto amoniacal em 93-97% e fósforo entre 94-100%, destacando-se *C. protothecoides* e *S. obliquus*. Em geral, cumpriram-se os limites impostos pelo Decreto-Lei 236/98 da legislação Portuguesa para o tratamento de águas residuais, podendo as águas do tratamento serem descarregadas nos cursos de água ou reutilizadas. Os perfis bioquímicos da biomassa apresentaram teores de proteína entre 34-47%, ácidos gordos (C12-C18) entre 26-84%, e açúcares totais 25-33%. Nos ensaios para estimulação de crescimento de sementes os resultados foram positivos para as várias microalgas dependendo dos tipos de semente e das condições de luz, sendo os mais relevantes de *Synechocystis* sp. e *C. vulgaris*. Nos ensaios para potencial biopesticida, *Synechocystis* sp. e *S. obliquus* obtiveram os melhores resultados enquanto inibidores do crescimento do fungo.

Em suma, *S. obliquus* e *C. protothecoides* foram mais eficientes no tratamento do efluente, *S.* obliquus e *C. vulgaris*, na estimulação da germinação/crescimento, e *Synechocystis* e *S. obliquus*, no potencial biopesticida.

Palavras-chave: Efluente suinícola, *Chlorella prototothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus*, *Synechocystis* sp.

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Abbreviations and symbols

AFDW	Ash free dry weight
Al	Aluminium
As	Arsenic
BF_3	Boron trifluoride
BGA	Blue-green algae
Ca	Calcium
Cd	Cadmium
Co	Cobalt
CO_2	Carbon dioxide
COD	Chemical oxygen demand
Cl	Chloride
Cu	Copper
DW	Dry weight
FAMEs	Fatty acids methyl esters
Fe	Iron
GI	Germination index
GC	Gas chromatography
ISE	Ion-selective electrode
K	Potassium
LOD	Limit of detection
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
NaCl	Sodium chloride
NaOH	Sodium hidroxide
NH_3-N	Nitrogen as ammonia
NH_4-N	Nitrogen as ammonium
NI	Not identified
NPK	Nitrogen-phoshorus-potassium
NRE	Nutrient removal efficiency
OD	Optical density
P	Phosphorus
Pb	Lead

PBR	Photobioreactor
PO_4^{3-}	Phosphate or ortophosphate
P_2O_5	Phosphorus pentoxide
PO_4-P	Phosporus
PVK	Pikovskaya medium
RCF	Relative centrifugal force
S	Sulphur
Sn	Tin
SWW	Swine wastewater
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TP	Total phosphorus
WWT	Wastewater treatment
XRF	X-ray fluorescence
Zn	Zinc

λ	Wavelength
Ɛ	Length
Ø	Diameter

1. Introduction

Worldwide health is highly dependent on agricultural systems, being agriculture one of the world's essential food sources. Its practices have a direct impact on the quality and security of its products, its productivity and the fulfilment of the food necessities all around the globe. The present industrialized and automatized agricultural system aims to increase productivity and reduce costs through economical strategies. On the other hand, plant biotechnology searches for answers towards the environmental challenges. Current agricultural practices also raise their own challenges and concerns, which are explored among the scientific community. Some reports even defend that such practices are exceeding Earth's system ecological and environmental limits (Campbell et al., 2017). Clean water is an essential to human health and survival. Yet, water scarcity is a reality and access to water resources is currently threatened by various issues. This can all be related to its poor management, such as exploitation, pollution and climate change (Tundisi, 2008). The European Environment Agency (EEA) states that, although the quality of wastewater treatment is increasing across the European Union, wastewater treatment plants and agricultural runoff are main sources of water pollution (EEA, 2018). According to the World Wildlife Fund (WWF), by 2025 about two-thirds of the world's population can be under water shortages (WWF, 2019). The Food and Agriculture Organization of the United Nations (FAO) created 17 Sustainable Development Goals (SGDs), including good health and well-being, clean water and sanitation, responsible consumption and production and climate action and developed Agenda 2030, for transforming food and agriculture ways and achieve the SDGs (FAO, 2019).

1.1. Agriculture and the use of chemical fertilizers and pesticides

The "boom" of chemical fertilizers happened on the so called "Green Revolution", in 1960. Statistics on the world's consumption of nitrogen, phosphorus and potassium fertilizers by the International Fertilizer Association (IFA) reveal that it increased from ranges of 8 000-14 000k tons (t) to 36 000-110 000k t, from 1961 to 2017 (IFA, 2019). Nitrogen-rich synthetic fertilizers production started because most cultivated plant species are not capable of assimilating atmospheric nitrogen. The up-dose application of phosphorus- and nitrogen-rich fertilizers is still responsible for the eutrophication of many types of water systems (Nagendram, 2011). It promotes soil erosion and destroys the local ecosystems (meaning, the survival of microorganisms present on soil is compromised) and translates into an increased vulnerability towards invasive organisms and diseases, increased water needs and, consequently, diminished productivity. Furthermore, regarding nitrogen-rich fertilizers, there are several studies indicating that incorporation of nitrogen in plant biomass does not go much further than 50% (Campbell et al, 2017). This means that the remaining nitrogen will leach out and could be responsible for other contamination problems. Chemical fertilizers keep promoting, step by step, soil acidification, thus reducing soil fertility. Studies around synthetic pesticides have discussed their negative impacts on wildlife, as well as in human health. Although there are mechanisms for pesticide degradation in nature, there are limits for extent of that capability. The possible toxicity of such chemicals and transformation products may contribute to chronic diseases, and effects can strike several body systems (Nicolopoulou-Stamati et al., 2016; Fenner et al., 2013; Köhler & Triebskorn, 2013). Chemical fertilizers, synthetic pesticides and high yield crop varieties are, progressively, degrading the soil's chemical, physical and biological properties and water quality, contribute to the destruction of ozone layer and are responsible for GHG emission. In addition, their production depends on fossil fuels (Chatterjee et al., 2017).

Until today, these substances were improved to respect population health and continue to guarantee agricultural products' productivity and quality. For instance, India consumed almost 26 000 tons between 2016 and 2017, according to the 2017's statistic and economic report of the Indian Government's Department of Agriculture, Cooperation and Farmers Welfare (Bodh et al., 2017). Evidently, the misuse of chemical or synthetic fertilizers, pesticides and herbicides are part of the

unsustainable agricultural practices problematic. Current legislation restricts the use of these chemical products, since they indirectly represent a threat to the population health, as they accumulate on soils and edible crops, and compromise the future of agriculture. Coping with these calamities, solutions have been proposed, including organic fertilizers and biopesticides. The next plan will be a turn to methods and technologies that promote the plant natural development of adapting to growth conditions. Organic agricultural practices, which are based on traditional agriculture, have been gaining an important role as a sustainable alternative path to ensure crops health and further access to quality products. In a recent event, the European Parliament approved an amendment of regulations (EC) No. 1069/2009 and (EC) No. 1107/2009 for Fertilising Products Regulation (FPR). This novel version states that "certain substances, mixtures and micro-organisms, referred to as plant biostimulants, are not as such inputs of nutrients, but nevertheless stimulate plants' natural nutrition processes" and "act in addition to fertilisers, with the aim of optimising the efficiency of those fertilisers and reducing the nutrient application rates" (European Parliament and Council, 2019). The use of plant biostimulants in agriculture has been growing and scientific reviews keep collecting scientific evaluations (Rouphael & Colla, 2018; Yakhin et al., 2017; du Jardin, 2015; Calvo et al., 2014); regarding these products, algae have been revealed as a promising option (Dmytryk & Chojnacka, 2018).

1.2. The history of algae and microalgae in agriculture

The discovery of algae's diverse potentialities goes back to the 20th century, and its recognition has risen ever since, in different biotechnological areas. In chemical technology, the role of this group of organisms in refineries dates from the 1940s, when it was clear that algae can produce fuels or fuel precursors (Tentracoste et al., 2015). In the beginning of 1950s, its cultivation at large-scale production initiates in USA, Japan, Germany and Netherlands. The perception of algae agricultural abilities occurred with plant enhancement, and scientific investigation dwells on algae benefits on plant growth since the 1960s (Dmytryk & Chojnacka, 2018). Microalgae and cyanobacteria world production and commercialization, with feed and nutritional purposes, were triggered in 1980 by the Aquatic Species Program (ASP) of the U.S. Department of Energy (DOE) (Tentracoste et al., 2015).

In agriculture, algae have been becoming quite relevant, as evidences on its roles on soil fertility and reclamation, plant growth and development, and pest and disease control have been explored (Abdel-Raouf et al., 2012). In fact, algae-based fertilizers are interesting biofertilizers. These are biologic fertilizers, meaning they contain living microorganisms, such as bacteria, fungi, cyanobacteria and microalgae; their metabolites contribute to soil enhancement, crop growth and yield. In 1895, the first biofertilizer was commercialized, made of a laboratory grown Rhizobium culture. The bacteria Azotobacter and blue-green algae (BGA) were next to be found has potential biofertilizers. Among the various algae discovered, the mostly studied are brown and red macroalgae, BGA and Anabaena Azolla associations (Rouphael & Colla, 2018; Chatterjee et al., 2017; Kumar et al., 2017; Sahu et al., 2012). Initial studies aiming the growth promoting and fertilizing capacities on plants involved mainly macroalgae, particularly brown and red seaweeds. Extracts and concentrates were the principal formulas for practical applications (de Morais et al., 2015; Abdel-Raouf et al, 2012). Regarding soil reclamation, by 1993, green and blue-green algae were considered a way to fight desertification because of their role on water-retention and nutrient maintenance and slow release to the soil (Painter, 1993). Green microalgae and cyanobacteria also contribute to soil neogenesis. For example, Chlorella sorokiniana and Azospirillum brasilense were able to increase the organic matter, organic carbon and microorganism-produced carbon on eroded and dehydrated soil, after three applications (Trejo et al., 2012). Currently, brown seaweed extracts are widely applied on agriculture and microalgae are under study for better understanding of cultivation processes its applications in this field (Chiaiese et al., 2018; de Morais et al., 2015).

1.3. Microalgae: a sustainable solution

Microalgae belong to a group of photosynthetic and unicellular microorganisms, including eukaryotic protists and cyanobacteria, since they have physiological and photosynthetic similarities. Microalgae are today recognized as a sustainable and renewable way to improve crop performance (Chiaiese et al., 2018; Katiyar et al, 2016). The role of these versatile microorganisms on agriculture progress and sustainable profile improvement keeps being investigated and proved throughout the decades. Microalgae also were and continue to be distinguished in wastewater treatment for various types of wastewaters, e.g. swine wastewater (Nagarajan et al., 2019; Gonçalves et al., 2017; Gouveia et al., 2016; Alcántara et al., 2015; Renuka et al., 2015; Cai et al., 2013).

1.3.1. Microalgae in biofertilization, plant biostimulation, and antimicrobial activity

Green microalgae and cyanobacteria were the targets of several promising studies on its effects as source of biofertilizers and growth-promoting agents in different crops, such as rice, wheat and corn – three of the most important agricultural food sources worldwide. Together, these microorganisms form symbiotic associations. Both types of microorganisms possess properties as biocides, produce growth-promoting hormones, perform photosynthesis (therefore, CO2 fixation) and promote macro- and micronutrients solubilization. They can be applied as foliar spray and directly on soil, as formulations, liquid cultures or simple biomass. The use of microalgae-cyanobacteria based biofilms and bioflocs is a very beneficial approach. Regarding their effects on soil, improvements occur on stability, soil microorganisms' activity, macro- and micronutrient availability and carbon organic compounds (Renuka et al., 2018) By using microalgae-synthesized aminoacids as biofertilizers, the biologically produced nutrients become available to natural plant processes and stimulate the synthesis of biological active compounds (Sahu et al., 2012; Painter, 1993). On plants, both germination and growth are stimulated, as well as its productivity, water use efficiency and stoma conductivity. Green microalgae and cyanobacteria also increase fruits' and grains' nutritional richness. However, cyanobacteria's properties, effects and employment on plants are much well studied, compared with microalgae (Renuka et al., 2018).

According to the European Union Regulation 2019/1009 for EU fertilising products defines a plant biostimulant as a product that aims to "stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency; tolerance to abiotic stress, quality traits or availability of confined nutrients in the soil or rhizosphere". Microbial plant biostimulant is a subcategory of this group, consisting of "a micro-organism or a consortium of micro-organisms". According to The European Biostimulants Industry Council (EBIC), biostimulants have different mechanisms of action comparing to biofertilizers, and do not have a direct effect against pests and diseases (EBIC, 2019). The same directive explains that a fertiliser is a product which function "is to provide nutrients to plants or mushrooms". Although the term "biofertilizer" is not specifically stated and described on this directive, literature describes it as a substance containing microorganisms, able to improve the micronutrients availability to the plant when applied to the soil. (Saeid & Chojnacka, 2019; Singh *et al.*, 2019; Umesha *et al.*, 2018; du Jardin, 2015). Therefore, a biofertilizer can be considered a substance containing living microorganisms which provides nutrients to plants in crop production. EBIC also stated that "crop biostimulantion is thus complementary to crop nutrition and crop protection" (EBIC, 2019).

A study was conducted with the green microalgae *Acutodesmus dimorphus* in order to assess its influence on seed germination, plant growth and fruit production of *Solanum lycopersicum* var. Roma, a tomato variety (Garcia-Gonzalez & Sommerfeld, 2015). *A. dimorphus* culture, cellular extract and dry biomass were applied as biostimulant, foliar spray and biofertilizer, respectively. The results were positive for in-creased germination speed, plant growth and floral production. *S. lycopersicum* cvs. “Maxifort” and “Merlice” were also cultivated with different types of microalgae fertilizers: *Nannochloropsis* and microalgae-bacterial bioflocs fertilizers. The use of this microalgae-based biofertilizers enhanced carotenoids and sugar contents in the tomato fruits, as organic slow-release fertilizers (Coppens *et al*., 2015). *Chlorella vulgaris* was evaluated for biofertilizer potential on *Lactuca sativa* (lettuce plant) (Faheed & Fattah, 2008) and *Hibiscus esculentus* (okra) (Agwa *et al*., 2017). In the first study, the seeds of *L. sativa* were grown in culture medium and soil pots containing *C. vulgaris*, promoting seed growth and increased pigments content (Faheed & Fattah, 2008). In the second study, healthy *H. esculentus* seeds were inoculated with *C. vulgaris*, NPK fertilizer and poultry manure and promoted a faster germination, the highest pot yield being with combined seed and soil inoculation with the microalga (Agwa *et al*., 2017). More recently, *Chlorella fusca* was tested for biostimulant effect on Chinese chives (*Allium tuberosum*) and spinach (*Spinacia oliorera* L.), enhancing growth and mineral content. When it comes to the metabolites behind these abilities, fatty acids and carotenoids are examples of such compounds. Existing in both terrestrial plants and algae, are responsible for vital physiological functions: photosynthesis, respiration, and regulatory processes against stress and pathogen infections. Therefore, these two bioactive compounds have undeniable importance for plant growth and development, and thus of agricultural interest. Supercritical extracts of algae, with high concentrations of carotenoids and fatty acids, are application formulas (Dmytryk & Chojnacka, 2018). Phytohormones produced by microalgae are also responsible for numerous physiologic mechanisms in terrestrial plants and microalgae. These are under investigation as to how they can influence plant development, when applied for plant development (Lu & Xu, 2015). Microalgae are becoming remarkable due to their biologically active compounds, considered high-value products. Given this, microalgae’s metabolites can be important for plants and agriculture because of their role as biostimulants, (Yakhin *et al*., 2017; Katiyar *et al*., 2016).

Like cyanobacteria and green macroalgae, microalgae are likely to have a potential role on pathogen, pest and disease control (Renuka *et al*., 2018; Kulik, 1995). Regarding anti-microbial activity, the cyanobacteria are the more studied for its properties against pathogenic fungi and diseases in plants, due to biologically active metabolites (phenolic compounds, polyphenols, carbohydrates, protein, peptides, among others). *Spirulina platensis*, *Oscillatoria* sp. and *Synechocystis* sp. are examples of cyanobacteria with antibacterial activity. Species of *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Skeletonema*, were discovered for methanol extracts with anti-microbial activity and fatty acids as bioactive compounds against harmful microorganisms (Costa *et al*., 2019; Navarro *et al*., 2017). In a previously mentioned study, *C. fusca* was able to reduce disease impact of grey mold in the chives (Kim *et al*., 2018). Several microalgae species were also tested for antimicrobial effect against certain strains of Gram-positive and -negative bacteria. With *C. vulgaris*, for example, chlorellin was found as a bioactive compound capable of inhibiting the growth of Gram-negative and -positive bacteria, and phenolic compounds were responsible for antifungal activity (Hussein *et al*., 2008; Pratt *et al*., 1945). Scenedesmus sp. is also included, in studies where bioactive principles are carried by silver nanoparticles and in crude extract (Aremu *et al*., 2014; Jena et al., *2014*). *Chlorella sorokiniana* and *Coccomyxa onubensis* were also tested against Gram-negative and -positive bacteria and yeast strains, with positive results (Navarro *et al*., 2017). Results showed that four different extracts from the microalgae *C. vulgaris*, *Isochrysis galbana* and *Nannochloropsis gaditana* had inhibitory activity against two different Gram-positive bacteria (Ronga *et al*., 2019).

1.3.2. Microalgae cultivation in wastewater and bioremediation

Phytoremediation, or wastewater treatment (WWT) through plants, such as algae, and other microflora, began in the 1950s. Oswald and Gotaas (1955) were the first research team to explore phytoremediation (Renuka *et al*., 2015). For microalgae, the treatment of wastewaters is called phycoremediation. This type of bioremediation has led to a lot of investigation, because microalgae have several advantages.

Compared with higher plants, microalgae are more effective in photosynthesis. Because microalgae can assimilate the effluent alkalinity and CO2 from oxidation of organic matter, high yields of microalgae are obtained; combined with the heterotrophic metabolism of microalgae and bacteria, a great potential for nutrient assimilation is generated (Muñoz and Guieysse, 2006). Being capable of growing in nutrient-rich medium, microalgae allow the recycle the inorganic and excessive nutrients present in wastewaters. They also perform carbon sequestration and metals, cleansing the contaminated waters. Additionally, algal biomass harvested from these treatments has many applications, including in agriculture (Ferreira *et al*., 2019; Figueroa *et al*., 2018; Gouveia *et al*., 2016; Renuka *et al*., 2016). Giving these particularities, microalgae's potential as microscopic biological remediating units is being investigated in different effluents, such as poultry, swine, cattle, brewery, dairy and urban waste waters (Ferreira *et al*., 2018). Different microalgae species were also grown in effluents. *Scenedesmus* and *Chlorella* are examples of robust strains used in effluents treatments. In fact, re-searchers hope that somewhere in the future, microalgae become a part of biorefineries in different industries, promoting the bio-circular economy. The main challenges for microalgae wastewater treatment are the high-cost production of microalgae, the different effects of different wastewaters and environmental and operational conditions. Therefore, studies are conducted in order to create strategies on the key points of the production process, to alleviate costs (Molinuevo-Salces *et al*., 2019; Figueroa *et al*., 2018; Khan *et al*., 2018; Gouveia *et al*., 2016; Reis & Gouveia, 2016; Gouveia, 2014;). This means that microalgae can become a part of non-polluting, more economical and renewable remediation system, allowing industries to reuse the water inform the effluents which go under treatment by microalgae (Khan *et al*., 2018; Gouveia, 2014).

1.3.3. *Chlorella protothecoides, Chlorella vulgaris, Scenedesmus obliquus* and *Synechocystis* sp.

Chlorella (*Auxenochlorella*) *protothecoides*, *Chlorella vulgaris* and *Scenedesmus obliquus* are green unicellular and eukaryotic microscopic green algae found in freshwaters, capable of performing different photosynthetic metabolisms (autotrophic, heterotrophic, mixotrophic and photoheterotrophic) (Guiry, 2019; Daliry *et al*., 2017; Ji *et al*., 2013). *Synechocystis* sp. is a unicellular cyanobacteria which shares the same photosynthetic versatility (Yu *et al*., 2013).

The four species have all been studied for WWT/nutrient removal from wastewaters, especially *C. vulgaris* and *S. obliquus* , coupled with different purposes, such as biofuels production (Trentin *et al*., 2019; Cuellar-Bermudez *et al*., 2017; Girard *et al*., 2017; Kho *et al*., 2017; Molinuevo-Salces *et al*., 2016; Pachacama *et al*., 2016; Mirzaie *et al*., 2015; Renuka *et al*., 2015; Sforza *et al*., 2014; Zhou *et al*., 2012; González *et al*., 1997). *C. vulgaris* and *S. obliquus* have been explored for its potential for agriculture, in plant growth stimulation and control of pathogens and invasive microorganisms (Ferreira *et al*., 2019; Win *et al*., 2018; Agwa *et al*., 2017; Renuka *et al*., 2016; Faheed & Fattah, 2008). In fact, a study was conducted very recently with *C. vulgaris* for testing its germination stimulant ability in tomato and cucumber seeds (Bumandalai & Tserennadmid, 2019). *C. protothecoides* and *Synechocystis* sp. are in need of more investigation for agricultural purposes.

1.4. Swine wastewater: characteristics and microalgae treatment

From 2012 to 2019, the worldwide pig production generated, 781.28 million of pigs, China being the leading country, and the global European pork production reached 24,300k t in 2018, according to Statista statistics on hog and pig farming (Statista, 2019).

The swine wastewater (SWW), or piggery wastewater, result from the cleaning of pig production facilities, consisting of a mixture of washing waters, animal urine and feces, among other substances. The SWW can be stored in lagoons, and sometimes it is applied to the lands for fertilization. Having a high content of nutrients and organic matters together with several contaminants, as other livestock wastewaters, SWW represents a source of pollution for the surrounding environment and a threat to public health. Therefore, it demands efficient, sustainable treatments (Zhu *et al*., 2013; Bradford *et al*., 2008).

The biological treatment through microalgae cultivation on SWW continues to be studied, applying different pre-treatments to the raw SWW. It has been reported the use of SWW pre-treated by sedimentation, filtration, autoclavation and dilution (Zhu *et al*., 2013), biologically-treated and filter sterilized SWW (Abou-Shanab *et al*., 2013), diluted SWW previously decanted (Wang *et al*., 2012), ultrafiltration of anaerobically digested SWW (Sandefur *et al*., 2016), diluted SWW previously centrifuged (García *et al*., 2018), fermented SWW (Kim *et al*., 2008) among others.

In case of the studied microalgae, *Scenedesmus* and *Chlorella* spp. are reported has very tolerant growing in pig farm effluents and are the most studied for the treatment of this type of wastewater (Figueroa *et al*., 2018; Mezzanotte *et al*., 2018; Ferreira *et al*., 2018, 2017). *C. vulgaris* cultivation with SWW has been explored (Nagarajan *et al*., 2019) and for various purposes, such as carbohydrate production (Wang *et al*., 2015) and biodiesel production (Nam *et al*., 2017). *S. obliquus* as also been explored for SWW treatment (Nagarajan *et al*., 2019), for example using SWW diluted with synthetic medium for its cultivation (Ji *et al*., 2013). *C. protothecoides* has been explored more for other types of wastewater, for instance municipal wastewater and brewery wastewater treatment (Pastore *et al*., 2018; Prakash & Babu, 2017; Nwoba *et al*., 2016; Darpito *et al*., 2015). *Synechocystis* sp. as also been reported on treatment of other wastewaters (Ashokkumar *et al*., 2019; Trentin *et al*., 2019), and regarding swine wastewater, is as been studied with *Chlorella* sp. (Pachacama *et al*., 2016), *Scenedesmus obliquus* (Ferreira *et al*., 2018) and *Scenedesmus almeriensis* (Acién *et al*., 2018, 2016), for instance.

1.5. Background and goals of this work

Water and agricultural feed crops are the most important source of nutrition all around the world and are being threatened by unsustainable ways of production, consumption and residue management. Therefore, it is important that the alternatives processes to obtain clean water and to sustainably produce food from agricultural crops are explored, to progressively improve their technology and access, and combine these processes (Khan *et al*., 2019; Mahapatra *et al*., 2018). Microalgae are studied today to be a part of new sustainable ways in various areas, as agriculture (Katiyar *et al*., 2016; Gouveia, 2014).

In this dissertation, the interest lies on the capabilities of microalgae for both bioremediation and agriculture purposes. Therefore, the developed work consisted of the following tasks and goals:

a) Characterization of the swine wastewater (SWW) for microalgae cultivation;

b) Screening trial of six microalgae by cultivation in SWW medium, in other to select the most robust strain(s);

c) Cultivation of the selected microalgae - green microalgae *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and cyanobacteria *Synechocystis* sp. - in swine wastewater (SWW), that simultaneously produce microalgae biomass and perform biological treatment of SWW;

d) Evaluation of the biological treatment performance by the four microalgae, according to the limits defined by Portuguese legislation (Decree Law 236/98);

e) Biochemical characterization of the resulting microalgae biomass;

f) Seed trials of various model crop plants – cucumber, barley, soy, tomato, watercress and wheat - for evaluation of the microalgae biomasses for seed germination and growth (biostimulant ability);

g) Trials with the fungi *Fusarium oxysporum* for evaluation of the microalgae biomasses for inhibition of fungi growth (biopesticide ability).

2. Materials and Methods

2.1. Swine wastewater (SWW) source, collection and characterization

The SWW was collected on May 13rd, from a lagoon containing SWW derived from all stages (swine sow, finisher and nursery) of pig production at Herdade do Pessegueiro, Salvaterra de Magos, Portugal. The raw SWW was left under room conditions during 3 days for settling of possible solids (decanted SWW). The decanted SWW was also stored at 4ºC and -18ºC and characterized three days later.

For the biochemical characterization of the decanted SWW, nitrogen and phosphorus contents and chemical oxygen demand (COD) were quantified, according to standard procedures for water characterization and analyses. The values of pH and conductivity were also measured. The decanted SWW was diluted to 5% (1:20), from all storage conditions. For these analyses, all samples consisted of 5%SWW, and were performed with replicates.

2.1.1. Chemical oxygen demand (COD)

The COD is the necessary amount of a specific oxidant (in this case, dichromate ion), which reacts with organic and inorganic oxidative compounds in a sample, allowing its quantification. The experiment was made through method 5520 B (APHA *et al,* 2017). The procedure has 2 stages:

- The first step is the digestion, for the oxidation of the inorganic and organic matter by the addition of potassium dichromate ($K_2Cr_2O_7$), which contains the dichromate ion ($Cr_2O_7^{2-}$). The reaction occurs in the presence of silver sulphate (Ag_2SO_4), the catalyst, and mercury sulphate ($HgSO_2$), the complexing agent.
- The final step was the titration, where the titrant is iron (II) and ammonium sulphate solution ($Fe(SO_4)•(NH_4)_2•6H_2O$). The addition of titrant will determine the excess of potassium dichromate ($K_2Cr_2O_7$). The titrant volume added to the sample allowed the determination of COD with the following equation:

$$\text{(1) COD (mg/L)} = 8000(V_0 - V_1) \times Tit/V$$

where V_0 is the volume (mL) of the titrant solution for blank titration, V_1 is the volume (mL) of the same solution for each sample titration, Tit is the title (N) of the solution and V is the volume (mL) used for each sample. The title (Tit) of the iron II and ammonium sulphate solution was checked after each procedure, and determined through the equation:

$$\text{(2) Tit} = \frac{V_{K2Cr2O7}}{V_{Fe(SO4)•(NH4)2•6H2O}} \times 0.25$$

where $V_{Fe(SO4)•(NH4)2•6H2O}$ (mL) is the volume of the titrant solution used to titrate the 0.25 N potassium dichromate solution, and $V_{K2Cr2O7}$ is the volume of potassium dichromate solution.

The COD method procedure consists of the following steps, steps 1 to 3 being for digestion, and 4 to 5 for titration:

1. The 20 mL samples of 5% SWW, were introduced in digestion tubes (P-Selecta, Spain). For the blank, a sample of 20 mL of distilled water was used.
2. For the reaction mixture, were added a full micro spatula of mercury sulphate, 10 mL of 0.25 N potassium dichromate and 25 mL of silver sulphate-sulfuric acid solution, to each sample. For lubrification, 5 mL of silver sulphate-sulfuric acid solution were spread across the tube entrance. In order to facilitate the reaction, 4 to 5 glass beads were added to each mixture.

3. The condensers were fitted in the tubes' entrances, and tubes were placed on the Bloc Digest 20 (P-Selecta, Spain) apparatus. The digestion was carried out at 150ºC, for 2h.
4. The condensers were washed with Millipore water and the tubes were left to cool down under room conditions.
5. After removing the condensers, distilled water was added to each tube until a final volume of approximately 400 mL was reached, as well as 5 drops of ferroin solution.
6. Each sample was titrated with 0.25 N iron (II) and ammonium sulphate solution, while on agitation, until it turned from green to a red tile colour. The added volumes were saved for calculations using equation 1.

2.1.2. Nitrogen content

Total Kjeldahl Nitrogen (TKN) represents ammonia (NH_3) and organic nitrogen contained in a sample. The experiment was made adapting the methods 4500-NH_{org} B and C (APHA *et al.*, 2017). The procedure has 3 stages:

- The first step is a digestion, where all forms of nitrogen (organic and inorganic) are converted into ammonium ion (NH_4^+) in the form of ammonium sulphate ($(NH_4)_2SO_4$), in the presence of sulphuric acid (H_2SO_4), potassium sulphate (K_2SO_4) and mercury sulphate ($HgSO_4$), which is the catalyst. The digestion reagent used for this stage is composed by all the previously compounds.
- The second step is a distillation, where ammonium ion (NH_4^+) is converted into ammonia (NH_3), in the presence of a strong base, in this case, sodium hydroxide-sodium thiosulphate ($NaOH\text{-}Na_2S_2O_3 \bullet 5H_2O$). The distillate is collected to a boric acid indicator solution (H_3BO_3).
- The third step is a titration, where the ammonia (NH_3) is titrated a strong acid, namely sulphuric acid (H_2SO_4). The titrant volume is then used to determine the concentration of TKN using the following equation:

$$\text{(3) TKN (mg N/L)} = (V_{tit} - V_{blank}) \times N \times 14 \times 1000 / V_{sample}$$

where V_{tit} is the standard solution volume for each sample titration, V_{blank} is the standard solution volume for the blank titration, 14 is the nitrogen milliequivalent weight (mg), N is the normality of the standard solution (0.02 N) and V_{sample} is the volume sample used for the procedure.

The TKN method procedure consists of the following steps, steps 1 to 4 being for digestion, 5 and 6 for distillation, and 7 for titration:

1. The 5 mL samples of 5% SWW were introduced in 800 mL Kjeldahl tubes (Büchi, Switzerland). For the blank was used a sample of 5 mL of distilled water.
2. For the reaction mixture, 50 mL of digestion reagent were added to each sample.
3. The tubes were placed on the Digestion Unit K-424 (Büchi, Switzerland) apparatus (previously turned on), inside the *hotte.* The fume extractor was adapted to the tubes' entrances and the vacuum tube was connected. The heat regulator was set up in position 8.
4. The digestion was carried out for 3-4h, under water vacuum. During the boiling, white fumes were released. The reaction was completed when most of fumes dissipated, the sample presented a colour slightly like straw colour, and visible water condensation on the tubes' walls. The tubes were left to cool down.

5. For the distillation mixture, 100mL of distilled water and 5 drops of 5% phenolphthalein. Right before each distillation, 50 mL of sodium hydroxide-sodium thiosulphate solution were added to each sample.
6. The distillation was carried out on the Distillation Unit K-350 (Büchi, Switzerland) apparatus. Each reaction took 6 min, and each distilled was collected to a 250 mL Erlenmeyer flask with 50mL of borate acid indicator solution.
7. Each sample was titrated with a 0.02 N sulfuric acid standard solution, while agitated, until the sample turned into a purple colour. The added volumes of titrant were saved for calculations using equation 3.

The ammonia nitrogen (NH_3-N) concentration was determined adapting methods 4500-NH_3 B and C (APHA *et al.,* 2017). The procedure consisted of a distillation and titration, as described for TKN determination, and the same steps were performed (5 to 7). In this case, samples consisted of 10 mL of 5% SWW and 10 mL of distilled water as the blank sample. As for TKN, equation 3 was used for calculations.

2.1.3. Phosphorus content

Phosphorus content was determined through ascorbic acid colorimetric method, in the form of phosphate (PO_4^{3-}). In this method, the combination of phosphate and molybdate in the presence of ascorbic acid makes the sample turn into a blue colour, in the presence of phosphate (Dabkowski & White, 2015). The measurement is done by spectrophotometry, at a certain wavelength (λ), and the results are given for phosphate, phosphate as phosphorus (PO_4^3-P) and phosphorus pentoxide (P_2O_5). The procedure was achieved using the PhosVer® 3 commercial kit (Hach, USA) and according to the following steps:

1. Each 25 mL sample of 5% SWW, previously diluted, was introduced in adequate glass flasks.
2. On the DR/2010 spectrophotometer (Hach, USA), the adequate programme was selected for spectrophotometric measurement of phosphate at λ = 890 nm. Each original sample was defined as its own blank in the spectrophotometer.
3. To each sample, a pill of PhosVer® 3 phosphate reagent for 25 mL (Permachem Reagents®, Hach, USA) was added. The mixture was then strongly shaken, and left to rest for 2 min.
4. Each sample was measured in the spectrophotometer and the concentration values (mg P/L) were given for the different forms of phosphorus.

2.1.4. Mineral content

The analysis of mineral content was conducted through x-ray fluorescence (XRF) spectroscopy. The samples were composed of freeze-dry concentrated SWW. The decanted SWW was centrifuged at 10 000*g (RCF) and 4ºC during 20-30 min. The pellet was then collected, frozen and freeze-dried on Heto PowerDry LL3000 freeze drier (Thermo Fisher Scientific, USA).

For XRF spectroscopy, a Niton™ XL3t XRF analyser (Thermo Fischer Scientific) was used and the data was collected and processed by the computer software, connected to the equipment (figure 2.1). The procedure was done according to Fidalgo (2018):

1. The freeze-dried biomass of each microalgae was placed on proper cuvettes.
2. The helium purge was connected to the analyser.
3. The samples were introduced in the XRF camera and the programme was set to 180s of radiation per reading.
4. The analysis consisted of 3 readings for each sample, performed under helium-rich atmosphere.

Figure 2.1 Set up for mineral analysis with the XRF Niton Analyser (Thermo Scientific), helium purge (on the right) and computer with reading software.

2.1.5. pH and conductivity

The measurement of pH and conductivity was also performed for the 5% SWW characterization. The pH and conductivity values were measured with Multimeter MM 41 (Crison-Hach, Spain), through 2 different and proper electrodes. These values were also measured for the decanted SWW.

2.2. Microalgae production

2.2.1. Microalgae source and selection

Initially, six microalgae species of LNEG's Bioenergy Unit stock were considered in this dissertation:

- *Neochloris oleoabundans* (UTEX 1185, University of Texas, USA);
- *Synechocystis* sp. (PCC 6803);
- *Scenedesmus obliquus* (ACOI 204/07, Algotec, Coimbra University, Portugal)
- *Chlorella vulgaris* (INETI 58);
- *Chlorella protothecoides,* also known as *Auxenochlorella protothecoides* (UTEX 25, University of Texas, USA);
- *Nostoc* sp. (Albufera) PCC 9202 (Institute de Bioquimica Vegetal y Fotosintesis, Sevilla, Spain).

In order to select the microalgae species which better adapted, can grow using SWW as a cultivation medium and treat the SWW, a *screening* trial was firstly made.

2.2.2. Culture conditions and photobioreactor operation

All microalgae inoculation was done under unsterilized conditions, since SWW used as medium naturally contained other microorganisms, namely bacteria. The cultivation medium was 5% SWW, prepared diluting SWW with tap water to a 1:20 dilution. Three different scales cultivations were conducted and, for each specie, duplicate inoculations in 5% SWW were prepared.

Regarding light and temperature conditions, the photobioreactor (PBR) operations described ahead were all performed under average room temperature and artificial light, provided by fluorescent lamps (Philips, TL-D, 18 and 36 W). To achieve similar and certain initial optical density/concentration, calculations were made depending on the optical density/concentration of previous cultures and the new cultures volume. For the up-scaling process, inoculum from the previous culture stage was kept for the next.

For the microalgae *screening*, 150 mL microalgae cultures were inoculated in 250 mL Erlenmeyer flasks (figure 2.2), on a G-25 incubator shaker (New Brunswick Scientific Co, USA), at 150 rpm (figure 2.3). Regarding light conditions, the microalgae cultures were under continuous light, with intensity of 41.2 µE/(m^2.s), provided by 3 fluorescent lamps (Philips TL-D, 18 W) (figure 2.3). The room temperature was ±24 ºC.

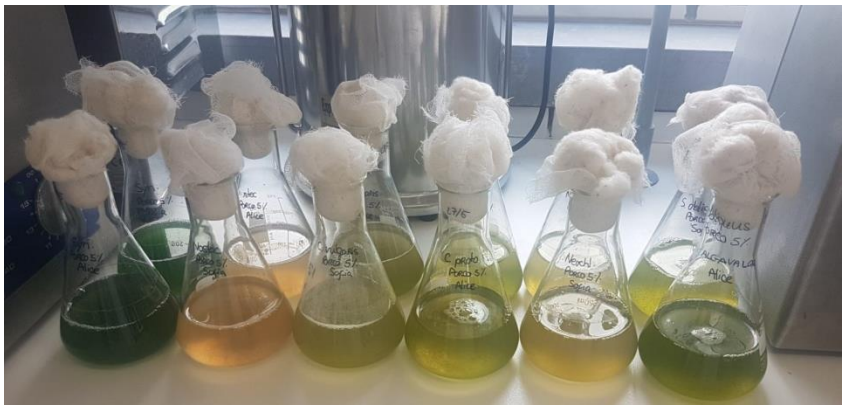


Figure 2.2 Microalgae cultures for *screening* trial: *Synechocystis* sp., *Nostoc* sp., *Chlorella vulgaris*, *Chlorella protothecoides*, *Neochloris oleoabundans* and *Scenedesmus obliquus* (from left to right).

Figure 2.3 Set up for screening trial on incubator shaker G-25 (New Brunswick Scientific Co., USA), around 24 ºC, at 150 rpm and at light intensity of 41.2 µE/(m^2.s).

The selected microalgae species from the *screening* trial (due to the better growth and better SWW treatment) were *Chlorella vulgaris*, *C. protothecoides, S. obliquus* and *Synechocystis* sp., and were inoculated in bubble column PBRs, of 1L capacity in duplicate (figure 2.4). The cultures were placed under aeration using aquarium air pumps with 0.6 vvm of airflow rate. In this case, the culture volume of 900 mL was maintained, adding the necessary volume of tap water. The cultures were under an average light intensity of 32.9 µE/(m^2.s), provided by 3 fluorescent lamps of 36 W and 6 fluorescent lamps of 18 W (Philips, TL-D). The room temperature was ±24 ºC.

Figure 2.4 Cultivation of microalgae in 1L bubble column photobioreactors (PBRs): *Chlorella protothecoides, Chlorella vulgaris, Scenedesmus obliquus* and *Synechocystis* sp. (from left to right).

The next 4L cultures were performed in bubble column photobioreactors (PBRs), of 5L capacity, in duplicate, using inoculum from previous bubble column cultures (figure 2.5). The cultures were also placed under aeration using aquarium air pumps, with 0.6 vvm of airflow rate. The cultures were provided an average light intensity of 52.6 µE/(m^2.s) from 6 fluorescent lamps (Philips TL-D, 36W/865). The room temperature was ±24 ºC.

Figure 2.5 Cultivation of microalgae in 5% SWW in 5L bubble column photobioreactors (PBRs): *Chlorella protothecoides, Chlorella vulgaris, Scenedesmus obliquus* and *Synechocystis* sp. (from left to right).

2.2.3. Culture monitoring

a) Microalgae growth

The optical density measurement was performed for all cultures for growth control, with a U-2000 spectrophotometer (Hitachi, Japan), at λ=540 nm. The samples were collected every 2-3 days during the cultivation period.

The dry weight (DW) and ash free dry weight (AFDW) were also determined. Samples were collected every 5-7 days, in duplicate. The applied procedure is described by the following steps:

1. Samples of 5 or 1 mL (V_{sample}) were collected from the different cultures for filtration.
2. Empty glass microfibre filters were placed on crucibles and incinerated in a LE 6/11 muffle furnace (Nabertherm, Germany), at 550ºC, for 1h. For 1 mL samples, ø 25 mm, Whatman GF/C glass microfibre filters (Sigma-Aldrich, Switzerland) were used, and Ø 47 mm, GF/C microfibre filters (Filter Lab, Spain) for 5 mL samples. After cooling in a desiccator, each filter was weighted (w_1).
3. The samples were the filtrated under vacuum with Büchner flask and fritted funnel, using air pump equipment.
4. The filters with the retained biomass were left to dry overnight at 100ºC, in a T-5028 lab oven (Hareaus, Germany), and weighted (w_2) after cooling in a desiccator.
5. The filters containing dry biomass were incinerated as previously described for the empty filters. After cooling in a desiccator, each was weighted again (w_3).
6. In order to obtain DW and AFDW results, the following equations were applied:

$$(4)\ \text{DW (g/L)} = \frac{(w_2 - w_1)}{V_{sample}} \times 1000$$

$$(5)\ \text{AFDW (g/L)} = \frac{(w_2 - w_3)}{V_{sample}} \times 1000$$

The biomass productivity ($P_{biomass}$) was also calculated with the following equation:

$$(6)\ P_{biomass}\ \text{(g/L/day)} = \frac{X - X_0}{t}$$

where X_0 is the initial concentration of biomass in g/L, X is the final concentration in g/L, based on AFDW, and t is the duration of cultivation in days.

To access if the inoculated species were growing without the contamination, a microscopic evaluation was made. From each culture, a sample of 20 µL was placed on glass slides, with a 20-200 µL micropipette, and placed the glass coverslip over the drop of culture. The sample was then observed through the microscope with 100x amplification. The observed microalgae cells were captured by photographs.

b) Nutrient consumption/removal

To evaluate the nutrient consumption by microalgae on *screening*, 1 L bubble column and 5 L bubble column PBRs cultivations stage, samples were collected from microalgae cultures and filtrated. The filtration was conducted as described for DW and AFDW procedure, with Ø 47 mm, GF/C glass microfibre filters (Filter Lab, Spain). The same procedures for COD, TKN and phosphorus contents in decanted SWW characterization were applied to supernatants (same volume samples, in duplicate), in order to evaluate their consumption rate of the nutrients provided by 5% SWW medium.

For nitrogen consumption, an analysis was conducted through ion-selective method, by an ion-selective electrode (ISE) attached to Multimeter MM 41 (Crison-Hach, Spain), to measure the NH_4^+ concentration. The procedure was done according to the following steps:

1. Each 10 mL sample of 5% SWW was placed in a 50 mL cup and 1 mL of ionic strength adjuster was added to the sample.
2. The ISE was immersed on the sample and measurement of NH_4^+ was made, under agitation. The concentration values were given in mg N/L.

The nutrient removal efficiency (NRE) was then calculated, for each nutrient, with the following equation:

$$(7)\ \text{NRE}\ (\%) = \frac{C_i - C_f}{C_i} \times 100$$

where C_i stands for the initial nutrient concentration and C_f stands for the final nutrient concentration.

c) pH and salinity

The cultures pH and salinity were measured for supernatants from culture filtration, with pH and conductivity electrodes and Multimeter MM 41 (Crison-Hach, Spain), respectively, for 5% SWW and original SWW.

2.2.4. Biomass harvesting and storage

For biomass harvesting, the cultures were left to settle down and supernatant was separated. The biomass was centrifuged at 4°C and 10 000*g (RCF) in Sigma 6-16KS centrifuge (Sigma-Aldrich, USA). The centrifugation time was different according to microalga necessity, varying from 10 to 30 min. Part of the collected fresh biomass was freeze-dried in Heto PowerDry LL3000 freeze drier (Thermo Fisher Scientific, USA). Both fresh and freeze-dried biomasses were kept at -18°C.

2.2.5. Microalgae biomass characterization

The procedures described ahead were conducted in duplicate, using freeze-dried biomass of *C. vulgaris*, *C. protothecoides*, *S. obliquus* and *Synechocystis* as samples.

a) Fatty acids content evaluation

Fatty acid content in microalgae biomass were determined through gas chromatography (GC), preceded by fatty acids methyl esters (FAMEs) extraction with boron trifluoride (BF_3), based on EN ISO 5509 (EN ISO 5509:2000). The BF_3 extraction procedure was done according to the following steps:

1. An amount of ±150 g of the different freeze-dried biomass was weighted for extraction.
2. The measured amount of biomass was introduced in a 50 mL boiling flask, followed by 10 glass beads and 4 mL of sodium hydroxide (NaOH) methanolic solution.
3. The boiling flask with the mixture was adjusted to a Soxlet extraction system and placed under bath in Precisterm equipment (P-Selecta, Spain) at 85ºC and gently agitated from time to time, for 20 min.
4. 5 mL of BF_3 were added to the mixture.
5. After 3 min, 3 mL of iso-octane solution were added. The boiling flask was removed from bath.
6. 20 mL of sodium chloride (NaCl) saturated solution vigorously rinsed through the refrigerant column.
7. With the same NaCl solution, the boiling flask volume was made up and agitated. The mixture was left to rest overnight, so the phase separation could occur (figure 2.6).
8. To remove the upper phase containing the extracted fatty acids, a 20-200 µL micropipette was used. The liquid was then pipetted into a Pasteur pipette, went through cotton impregnated with anhydrous sodium sulphate (Na_2SO_4) and was collected in a glass tube.

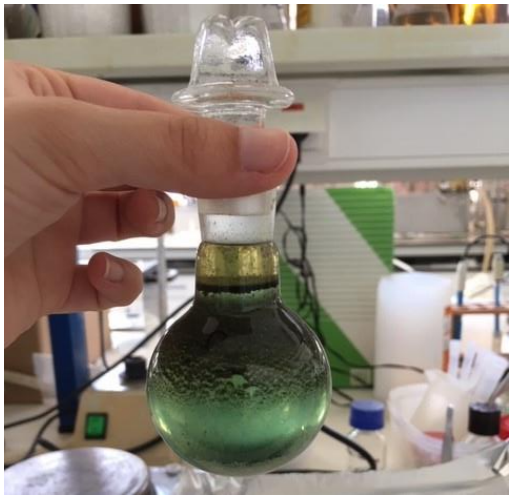


Figure 2.6 Separation of phases in fatty acids extraction for fatty acid content evaluation on microalgae biomass.

To perform the GC procedure, a sample of 1 mL of extraction liquid was used, for each microalgae biomass, in triplicate. The samples were placed on proper glass test tubes and the chromatography was conducted on CP-3800 gas chromatograph (Varian, USA) with a 30-m SUPELCOWAX 10 capillary column (film 0.32 µm). The carrier gas was helium. The method parameters are described in table 6.1 (Appendix A).

b) Protein content evaluation

The protein content in microalgae biomass was measured indirectly by the Kjeldahl method, applied before for SWW characterization (Person *et al.*, 2008). In this case, the samples consisted of 0.2 g of freeze-dried biomass, in duplicate, for each microalga. The following equation was applied for protein concentration determination in microalgae biomass:

$$(8)\ \text{Protein (\%w/w)} = \frac{(V_{tit} - V_{blank}) \times N \times 0.014}{M} \times 100 \times 5.95$$

where V_{tit} is the standard solution volume for each sample titration, V_{blank} is the standard solution volume for the blank titration, N is the normality of the standard solution (0.02 N), 0.014 is the nitrogen equivalent weight (g), and M is the sample weight (g). The value 5.95 is the nitrogen-to-protein conversion factor to obtain protein concentration in microalgae biomass (Waghmare *et al.*, 2016; López *et al*., 2010).

c) Glucose content evaluation

For glucose content in microalgae biomass, a quantitative acid hydrolysis for sugar extraction and measurement of sugar content through spectrophotometry (DuBois *et al.*, 1956). Its procedure is described ahead, and steps 1 to 4 are for the calibration curve construction.

1. A 1% glucose standard solution (100 mg/L) was diluted with Millipore water, in volumetric flasks, for different concentrations: 10, 20, 40, 60 and 80 mg/L.
2. For the reaction mixture, 1 mL of glucose solution, 1 mL of 5% phenol solution and 5 mL of 96 %(w/w) H_2SO_4 were placed in test tubes, for 10, 20, 40, 60, 80 and 100 mg/L glucose solutions, in triplicate. For the blank samples, 1 mL of Millipore water was used.
3. After 10 min, the test tubes were placed in bath at room temperature for cooling.
4. The absorbance of each sample was measured in quartz cuvettes with U-2000 spectrophotometer (Hitachi, Japan), at λ=490 nm.
5. In glass test tubes, ± 0.2 g of freeze-dried biomass was dissolved in 72 %(w/w) H_2SO_4.
6. The test tubes were placed under bath in Precisterm equipment (P-Selecta, Spain), at 30ºC, for 1h.
7. The samples were transferred to Schott flasks and 55 mL of Millipore water to each sample. The samples went under 120ºC, for 1h, in autoclave.
8. After cooling, the mixture was filtered with glass syringes (RUTHE®) and ø 3 mm and Ɛ=0.45 µm Acrodisc GHP filters (CHROMAFIL® Xtra).
9. Steps 2 to 4 were repeated, with 1 mL of Millipore water as blank and 1 mL of the filtered biomass mixture as sample, in triplicate, for each different biomass.

d) Mineral content evaluation

To evaluate mineral content, an elemental analysis was conducted through x-ray fluorescence (XRF) spectroscopy. Samples of freeze-dried biomass from the 4 microalgae were tested. For XRF spectroscopy, a Niton™ XL3t XRF analyser (Thermo Fischer Scientific, USA) was used and the adopted procedure was the same as for SWW samples. The analysis was performed under helium-rich atmosphere.

2.3. Microalgae for biostimulation: biomass effect on model crop seeds

2.3.1. Plant species selection

For seed trials, the following plant species were selected:

- *Glycine max* (common soybean);
- *Cucumis sativus* (common cucumber);
- *Licopersicon esculentum* (common tomato);
- *Hordeum vulgare* (common barley);
- *Triticum aestivum* (common wheat);
- *Nasturium officinale* (common watercress).

This plant species are model crop plants used for investigation on plant biology, biotechnology and agricultural development fields. Therefore, were found as the best suited for the trials.

2.3.2. Seed trials design and conditions

The selected seeds were displayed inside Petri glass plates with paper filters. For 10 days, all samples were placed under the same temperature and light conditions. For control, the seeds were watered with distilled water. For test samples, the seeds were watered with microalgae biomass suspensions. These suspensions were done using the freeze-dried biomass from the produced cultures. The concentration of the suspensions was 0.5 g/L. The same volume of distilled water and microalgae suspensions was applied to the different seeds everyday: 5 mL of water for control and 5 mL of biomass suspensions for the test samples. Control and test samples were done with 2 replicate plates, placing 8 seeds in each plate, for each 6 different seeds as presented on figure 2.7. Regarding temperature conditions, the samples were always under room temperature of ± 23ºC. This experiment was carried out under different light conditions:

- Sunlight, for 10 days;
- Dark/sunlight, 5 consecutive days in dark and 5 consecutive days in sunlight.

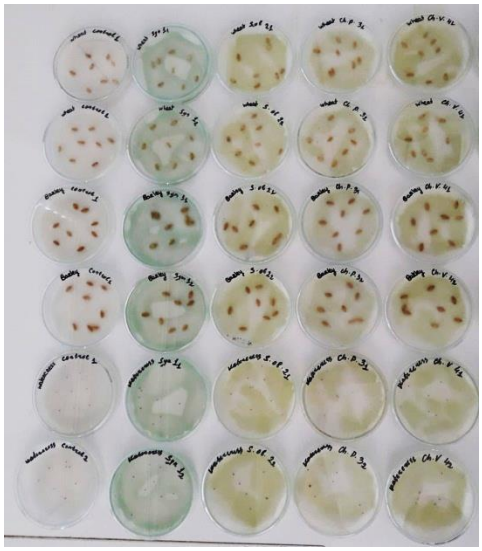


Figure 2.7 Example of seed trials design with wheat, barley and watercress seeds (from top to bottom).

2.3.3. Microalgae biomass performance evaluation

To evaluate microalgae biomass effect on germination and growth on the plant seeds, the germination index, as well as root, stem and sprout growth were determined. Chlorophyll *a* and *b* and carotenoids were also quantified to evaluate any effect on its production by microalgae biomass. The samples with microalgae biomass suspensions were compared to control samples (distilled water) for these parameters.

a) Germination index

The values needed for germination index (GI) were collected on growth days 3, 4, 5, 8 and 10. The GI was calculated for each sample with the following equation:

$$(9)\ GI\ (\%) = \frac{G \times L}{G_0 \times L_0} \times 100$$

where G represents the number of germinated seeds and L represents their length (cm), G_0 and L_0 represents the number of germinated seeds and their length (respectively) for control samples.

b) Root, stem and sprout growth

For all samples and respective germinated seeds, the main root and the lengths of sprouts' stems were measured, with rulers and the results were registered for comparison between microalgae strains.

c) Chlorophyll *a*, *b* and total carotenoids

For determination of chlorophyll *a*, *b* and carotenoids contents, a spectrophotometric analysis was conducted, preceded by an extraction with 80% acetone as solvent (Sumanta *et al*, 2014), for all samples. The procedure is described in the following steps:

1. The grown sprout leaves, from each plant seeds, were collected and grinded manually in 5 mL of 80% acetone as extraction solvent.
2. The mixture samples were homogenized for 2 min in vortex.
3. The sample mixture was centrifuged at 2364*g (RCF), for 20 min in a 2-6E centrifuge (Sigma, Switzerland).
4. The resulting supernatant was separate. A volume of 0.5 mL of supernatant mixed with 4.5 mL of 80% acetone.
5. The prepared solution absorbance was then measured by U2000 spectrophotometer (Hitachi, Japan).

The chlorophyll *a* (Ch-a), chlorophyll *b* (Ch-b) and total carotenoids (C_{x+c}) were calculated through the following equations (Sumanta *et al*, 2014):

$$(10)\ \text{Ch-a}\ (\mu g/mL) = 12.25 \times A_{663.2} - 279 \times A_{646.8}$$

$$(11)\ \text{Ch-b}\ (\mu g/mL) = 21.5 \times A_{646.8} - 5.1 \times A_{663.2}$$

$$(12)\ C_{x+c}\ (\mu g/mL) = (1000 \times A_{470} - 1.82 \times C_a - 85.02 \times C_b)/198$$

2.4. Microalgae as biopestice: biomass effect on *Fusarium oxysporum*

The microalgae biomass of *C. protothecoides, C. vulgaris, S. obliquus* and *Synechocystis* sp. was tested for inhibition of the fungi *Fusarium oxysporum* growth, to evaluate its biopesticide activity.

2.4.1. Trials design and conditions

The trials were conducted with solid Pikovskaya's agar (PVK) medium (recipe in table 6.2, Appendix B) and microalgae biomass suspensions, in sterilized glass Petri plates. For each microalga, the suspensions were prepared by suspending the biomass in sterilized water to a concentration of 0.5 g/L. A suspension of the fungi *F. oxysporium* was prepared and mixed with the PVK medium before solidification. The mixed medium was then placed on the Petri plates for solidification. For the introduction of microalgae suspensions, 4 circular holes were created in the solid medium, for each Petri plate. Once solidified, 3 mL of sterilie water were introduced in the holes, for control; for test samples; the same volume of microalgae suspension were introduced in each medium hole, as shown on figure 2.8. This procedure was reproduced to have duplicate samples: for each microalga suspension concentration, two Petri plates were prepared, exemplified on figure 2.8 for *Synechocystis* sp.

Regarding the trial conditions, all Petri places were wrapped in aluminium sheets and placed at 25ºC in a Memmert thermostat, in dark conditions, for 5 days.

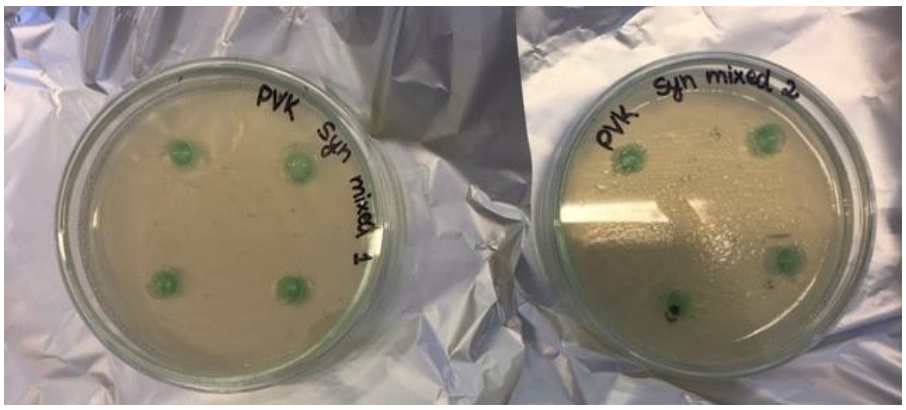


Figure 2.8 Example of biopesticide trial experimental design: Pikovskaya's agar (PVK) medium with mixed *Fusarium oxysporum* suspension. Inside the four medium holes.

2.4.2. Microalgae biomass performance evaluation

The ability of inhibition of *F. oxysporum* growth was evaluated by observation of an inhibition halo on PVK solid medium with mixed fungi suspension, around the microalgae suspension holes.

3. Results and discussion

3.1. SWW characterization

For characterization of decanted SWW, the pH, conductivity, chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), content of ammonium nitrogen and phosphorus are shown on table 3.1. Regarding the elemental analysis, results are shown on table 3.3.

Table 3.1 Chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), ammonia nitrogen (NH_3-N) and phosphorus (PO_4^{3-}, P_2O_5 and PO_4-P) quantification for biochemical characterization of decanted SWW from different storage conditions (average ± standard deviation for 2 to 3 replicates).

Decanted SWW	pH	Conductivity (mS/cm)	COD (mg O_2/L)	TKN (mg/L)	NH_3-N (mg/L)	PO_4^{3-} (mg/L)	P_2O_5 (mg/L)	PO_4-P (mg/L)
T_{amb}	7.72	22.3	10100±0	3360±560	1750±70	115	86.0	37.5
4ºC	7.71	22.4	16739±217	4200±840	1540±140	71.5	53.5	23.5
-18ºC	7.68	21.8	15625±240	2940±140	1610± 70	120	89.5	39.0

Regarding differences between the storage conditions, we can see variations between values which indicate there is some influence on the biochemical stability of the decanted SWW, especially for SWW under 4ºC.

Table 3.2 shows the literature results and the obtained values for COD and TKN are closer to the values reported by Barata *et al.* (2016); concerning NH_3-N, the obtained values are closer to those of Bradford *et al*. (2008) for swine finisher. Regarding phosphorus results, the values obtained for PO_4^{3-} are lower than the ones reported by Zhu *et al.* (2013). The PO_4-P obtained values are similar with those stated by Ji *et al.* (2013).

Table 3.2 Literature values for chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) or total nitrogen (TN), ammonia or ammonium nitrogen (NH_3-N/NH_4-N) and total phosphorus (TP) or phosphorus as phosphate (PO_4-P) (1- Swine sow; 2 – Swine finisher; 3 – Swine nursery).

Literature references	COD	Nitrogen content		Phosphorus content
		TKN/TN (mg /L)	NH_4-N/NH_3-N (mg /L)	TP/PO_4⁻P (mg/L)
Bradford et *al.*, 2008	-	129±15 [1]	944±23 [1]	264±5 [1]
		2430±40 [2]	1630±20 [2]	324±14 [2]
		2040±60 [3]	1370±90 [3]	368±35 [3]
Wang et *al.*, 2012	11000 mg/L	980	1388	158
Zhu et *al.*, 2013	3700±51 mg/L	162.0±8.0	-	209.0±5.5 mg PO_4^{3-}/L
Ji et *al.*, 2013	789±9 mg/L	1280±15	1197±6	42±3
Barata *et al.*, 2016	14160±1250 mg O_2/L	3171	2472.4±1.98	6.98±0.63

The results for pH measurement of the decanted SWW samples from the different storage conditions showed an average pH of 7.7±0.02. These values are and within the pH range of 6-9 found in literature (Cai *et al.*, 2013; Ji *et al.*, 2013; Zhu *et al.*, 2013; Wang *et al.*, 2012; Bradford *et al.*, 2008). For 5% SWW, the pH was 7.9±0.02, showing that the dilution did not alter significantly the pH. Regarding conductivity, there was not a significant difference between the different storage conditions, with an average conductivity of 22.2±0.26 mS/cm. This value is coherent with close to literature value

of 21.5±0.1 mS/cm, reported for SWW from swine nursery stage of pig production (Bradford *et al.*, 2008). For 5% SWW, the conductivity was 1735±12 µS/cm, which is explained by the diminishing of salts concentration due to the dilution.

Typically, wastewater derived from swine production has high concentrations of urea, ammonium and organic acids (Hodaifa *et al.*, 2008), which was confirmed by the present work. The high COD, nitrogen and phosphorus content justifies the applied dilution for using the SWW as a culture medium for microalgae cultivation, as well the dark colour of the SWW. Both high nutrient content and dark colour can have a significant impact in reducing microalgal productivity (Muylaert *et al.*, 2015). Ammonium nitrogen and COD values can inhibit microalgae growth, depending on each microalgae tolerance (Cai *et al.*, 2013; Ji *et al.*, 2013; Zhu *et al.*, 2013; Wang *et al.*, 2012). For instance, some microalgae can be inhibited with by ammonia concentrations of 20 mg/L (Azov & Goldman, 1982). In addition, other studies have already reported that better results were always achieved by diluting the original SWW (Cheng *et al.*, 2018; García *et al.*, 2018; Kumar *et al.*, 2018; Nam *et al.*, 2017; Cristóvão, 2016; Prandini *et al.*, 2016; Wang *et al.*, 2015; Wang *et al.*, 2012; Mezzono *et al.,* 2010; Gantar *et al.*, 1991). For instance, García *et al.* (2018) used swine wastewater diluted to 15% (corresponding to 1375 mg/L of total organic carbon); Wang *et al.* (2015) centrifuged and filtered SWW was diluted to COD concentrations of 3665, 1864 and 1064 mg/L; Wang *et al.* (2012) used decanted swine wastewater and experimented with dilutions to COD concentrations of 250, 500, 750 and 1000 mg/L.

Regarding the presence of heavy metals, none was detected by the XRF elemental analysis. The copper, zinc, potassium, calcium, chlorine and sulphur high contents (table 3.3) may be explained by the animal diet and feed additives applied on swine industry (especially from organic and inorganic acids) to improve animal reproduction and growth (Trabue *et al.*, 2019; Liu *et al.*, 2018; Suiryanrayna & Ramana, 2015), which influence the composition of manure, thus influencing the composition of the SWW.

Table 3.3 Elemental analysis of decanted SWW through XRF for potassium (K), copper (cu), zinc (Zn), iron (Fe), manganese (Mn), molybdenum (Mo), phosphorus (P), chlorine (Cl), sulfur (S), calcium (Ca), cadmium (Cd), cobalt (Co), magnesium (Mg), aluminum (Al), lead (Pb), arsenic (As), tin (Sn) contents and not identified (NI) elements (LOD – limit of detection).

Element (ppm)					
K	$56.5x10^3$ ±391.9	**P**	$6.9x10^3$ ±144.5	**Mg**	<LOD
Cu	$1.2x10^3$ ±30.8	**Cl**	$17x10^3$ ±125.4	**Al**	<LOD
Zn	$1.9x10^3$ ±28.4	**S**	$22.7x10^3$ ±232.2	**Pb**	<LOD
Fe	$2.5x10^3$ ±64.8	**Ca**	$85.8x10^3$ ±658.9	**As**	<LOD
Mn	$1.1x10^3$ ±65.7	**Cd**	15±3	**Sn**	<LOD
Mo	26.1±1.4	**Co**	<LOD	**NI**	$800x10^4$ ±745.2

3.2. Microalgae production

To choose the best microalgae for the SWW treatment, it was done a *screening* of six microalgae - *C. protothecoides, C. vulgaris, N. oleoabundans, Nostoc* sp., *S. obliquus* and *Synechocystis* sp. The cultivation was interrupted after 23 days. The microalgae growth evolution based on optical density results is represented graphically in figure 3.1 Regarding pH, biomass dry weight (DW), ash free dry weight (AFDW) and biomass productivity ($P_{biomass}$) the results are displayed on table 3.4.

Regarding the next cultivation stage, in 1L bubble column photobioreactors (PBRs), the cultivation was interrupted after 15 days. The results from $OD_{\lambda=540\ nm}$ measurements are represented by growth curves on figure 3.2; DW, AFDW and $P_{biomass}$ results are displayed on table 3.5 and pH and conductivity on table 3.6.

The final cultivation stage, in 5L bubble column PBRs, was interrupted after 19 days of cultivation. Results are represented on figure 3.3 and table 3.6 for optical density, pH, conductivity, DW, AFDW and $P_{biomass}$.

3.2.1. Microalgae *screening*

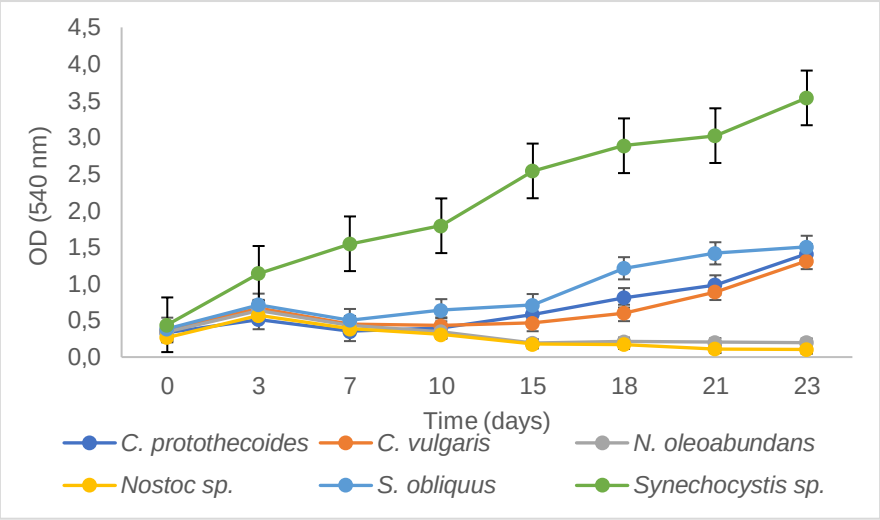


Figure 3.1 Growth curves of *Chlorella protothecoides*, *Chlorella vulgaris*, *Neochloris oleoabundans*, *Nostoc* sp., *Scenedesmus obliquus* and *Synechocystis* sp. based on average of optical density (OD) results, at λ=540 nm, for the screening of six microalgae (average ± standard deviation for 2 replicates).

The results of the growth curves on figure 3.1 show that *Nostoc* and *N. oleoabundans* reached OD values between 0.5 and 0.7 by day 3, but a decline in OD values occurred until the end of the trial. At the 15th day of cultivation, *N. oleoabundans* culture experienced the loss of the typical green colour (Rashidi & Trindade, 2018). *Nostoc* sp. was not forming the viscous, brown cells agglomerations, as it did in synthetic medium – *Nostoc* colonies are described as gelatinous and can have different colours, which in this case was dark brown (Laughinghouse IV *et al.*, 2019). Therefore, the high nutrient content of the SWW medium could be responsible for antagonizing its normal development. Both microalgae never adapted to the conditions until the interruption of cultivation period (23rd day).

For *C. protothecoides*, *C. vulgaris* and *S. obliquus*, there was a slightly fluctuation of OD values in the ranges of 0.3-0.5, 0.4-0.6 and 0.5-0.8, respectively, between the 3rd to 10th day, that could be due to the formation of small flocs, especially for the two *Chlorella* strains. From the 7th day to the 23rd day, the OD values increased from 0.351 to 0.784 for *C. protothecoides*, 0.449 to 1.313 for *C. vulgaris*, and 0.506 to 1.505 for *S. obliquus*. Previous studies pointed out the robustness of *S. obliquus*

which could explain the higher growths, compared with *C. vulgaris* and *C. protothecoides*. From the two *Chlorella* strains, *C. protothecoides* showed a decay of OD values from the 21st to 23rd day, which determined the interruption of the cultivation period. *Synechocystis* was without a doubt the most successful growth according to growth curves, starting with OD =0.369 and reaching OD =3.538 at the 23rd day, and no OD fluctuations were registered. This result was expected, since cyanobacteria have normally better adaptation capacity to different conditions and higher proliferation rates.

Table 3.4 pH values (2 replicates ± standard deviation), biomass dry weight (DW), ash free dry weight (AFDW) and biomass productivity ($P_{biomass}$) for *Chlorella protohecoides*, *Chlorella vulgaris*, *Neochloris oleoabundans*, *Nostoc* sp.. *Scenedesmus obliquus* and *Synechocystis* sp. for the *screening* stage, at 7th and 23rd days of cultivation (average value of 4 different samples ± standard deviation).

Specie	pH	DW (g/L)		AFDW (g/L)		$P_{biomass}$ (g/L/day)
		Day 7	Day 23	Day 7	Day 23	
C. protothecoides	9.82±0.06	0.26±0.02	0.70±0.07	0.22±0.02	0.53±0.18	0.02
C. vulgaris	8.66±0.12	0.34±0.02	0.55 ±0.11	0.28±0.02	0.45±0.05	0.01
N. oleoabundans	6.33±0.06	0.23±0.01	0.18±0.19	0.23±0.01	0.25±0.22	0.00
***Nostoc* sp.**	6.69±0.21	0.23±0.03	0.35 ±0.18	0.22±0.01	0.25±0.15	0.00
S. obliquus	10.1±0.11	0.34±0.03	0.95±0.18	0.33±0.03	0.93±0.23	0.04
***Synechocystis* sp.**	9.53±0.00	0.38±0.02	1.03±0.08	0.36±0.02	0.95±0.11	0.04

The final DW and AFDW results, support the evolution of growth curves: *Synechocystis* and *S. obliquus* had the same and higher biomass productivity, producing around 1 g of biomass per litre, and beginning with similar biomass concentrations, between 0.3 and 0.4 g/L. For *C. protothecoides* and *C. vulgaris*, biomass concentrations were between 0.4-0.55 g/L, lower biomass productivities, and with initial concentrations between 0.2 and 0.3 g/L. The results for *Nostoc* and *N. oleoabundans* evidently support the absence of growth showed by the respective growth curves, with null biomass productivities (the concentrations did not increase as for the other strains).

The pH results for *Nostoc and N. oleoabundans* cultures were below pH 7. Lower pH means lower consumption of dissolved carbon forms, which means both were not properly consuming CO_2 and had almost no photosynthesis activity. The remaining microalgae species showed higher pH scores, always above pH 8, meaning CO_2 was being consumed for photosynthesis. Considering all the results for growth curves, biomass production and pH, the 2 microalgae strains *Nostoc* sp. and *N. oleoabundans* did not proceed for the next trials.

3.2.2. 1L Bubble column PBRs cultivation

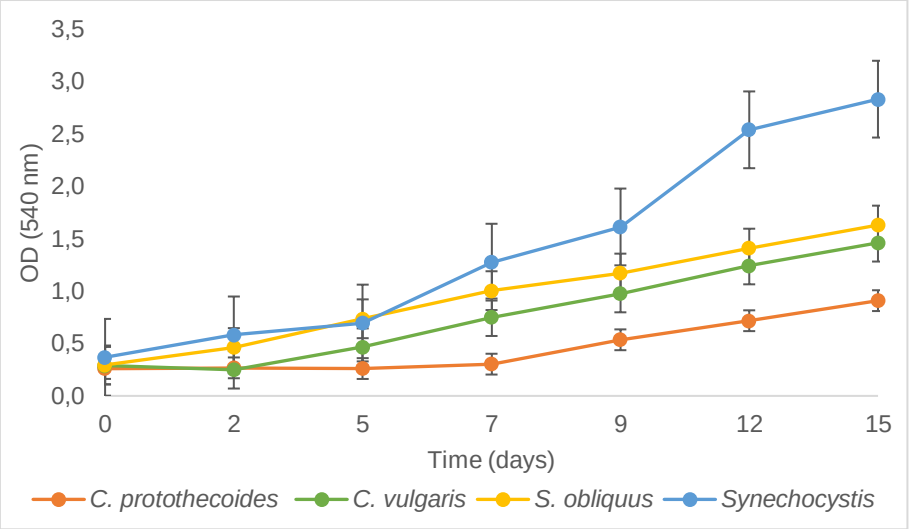


Figure 3.2 Growth curves of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. based on average optical density (OD) results at λ=540 nm, for 1L bubble columns photobioreactors cultivation stage (2 replicates ± standard deviation).

In following cultivation stage on bubble column PBRs, culture development based on growth curves (figure 3.2) shows that, once again, *Synechocystis*, had the highest OD values, from 0.369, on day 0, to 2.832, on the 15th day of cultivation. For *S. obliquus*, OD values increased in a gradual manner during the 15 days of cultivation, starting with OD=0.296 and reaching OD=1.631 (15th day). *C. vulgaris* reached similar OD values, starting with 0.289 at day 0 of cultivation and finishing with 1.461 at 15th day. For *C. protothecoides*, the OD values were around 0.262 at day 0 and showed a lag phase until the first 7th day of cultivation; after this day, the OD values raised gradually until reached 0.909 at the end of cultivation (15th day). It is important to notice that OD results got close to the ones from the *screening* up to 15 days of cultivation.

Table 3.5 Biomass dry weight (DW), ash free dry weight (AFDW) and biomass productivity (Pbiomass) for *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., from day 0 and 15th day of 1L bubble column PBR cultivation stage (average value of 4 different samples ± standard deviation).

Specie	DW (g/L)		AFDW (g/L)		$P_{biomass}$ (g/L/day)
	Day 0	Day 15	Day 0	Day 15	
C. protothecoides	0.16±0.01	0.87±0.13	0.17±0.01	0.92±0.15	0.05
C. vulgaris	0.16±0.02	0.75±0.09	0.16±0.00	0.67±0.13	0.03
S. obliquus	0.18±0.01	0.93±0.19	0.16±0.03	0.88±0.16	0.05
***Synechocystis* sp.**	0.16±0.02	0.68±0.04	0.18±0.02	0.70±0.07	0.03

However, looking at table 3.5, OD results are not always coherent with DW and AFDW ones. *C. protothecoides* had the highest AFDW, despite having the lowest OD values, followed by *S. obliquus*, *Synechocystis* and *C. vulgaris*, which had the lowest biomass concentration. *C. protothecoides* and *S. obliquus* revealed the same and highest biomass productivity, and so did *C. vulgaris* and *Synechocystis.* The latter performed the highest growth, which was not reflected on its biomass concentration, due to the lighter cells of this cyanobacterium compared to the other microalgae. Nonetheless, AFDW results are usually more reliable than OD results, because ashes are excluded. In the case of spectrophotometric analysis, ashes in the medium could have influenced the present results: OD reflects the ability of a sample to slow the light transmission velocity; so, the slower the light goes through the sample, the higher will be the given OD. Therefore, ashes in the sample culture can be responsible for the higher OD results.

The pH results (table 3.6) are within the range of 9 to 10.5, indicating that the microalgae development raised the pH values by consuming the CO_2, performing photosynthesis efficiently. The conductivity results are also much lower than the initial conductivity value for 5%SWW medium, with *S. obliquus* presenting the lowest conductivity value. These results can be translated to the decrease of salinity by uptake and regulation of inorganic salt ions dissolved in the medium, like Na^+ (sodium ion), Cl^- (chlorine ion) and Mg^{2+} (magnesium ion) (Vo *et al.*, 2019).

Table 3.6 pH and conductivity at the last day (15th) of cultivation in 1L bubble column photobioreactors for *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. (average ± standard deviation for 2 replicates).

Specie	pH	Conductivity (µS/cm)
5% SWW	7.9	1735±12
C. protothecoides	9.59±0.6	768±93
C. vulgaris	9.98±0.7	791±45
S. obliquus	10.4±0.2	665±8.6
***Synechocystis* sp.**	9.41±0.4	710±31

Regarding SWW treatment, Decree-Law 236/98 of the Portuguese legislation indicates that pH medium value can be comprised in the maximum range of 5.0 to 10.0 (table 6.3, Appendix C). *C. protothecoides, C. vulgaris* and *Synechocystis* presented pH values within that range, and even *S obliquus* value was not very far from this range.

3.2.3. 5L Bubble column PBRs cultivation

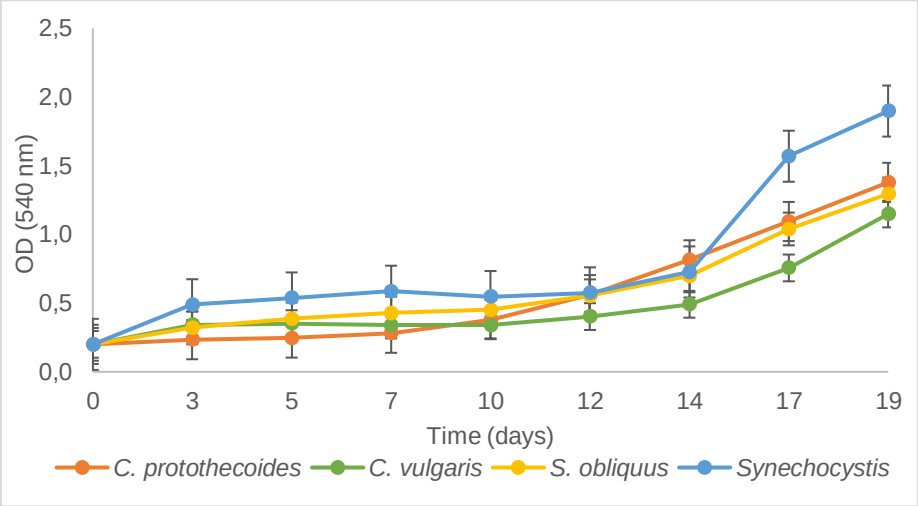


Figure 3.3 Growth curves of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., based on optical density (DO) results at λ=540 nm, for 5 L bubble column photobioreactors cultivation stage (2 replicates ± standard deviation).

Looking at the growth curves, during the first 3 days of cultivation, OD values did not increase significantly for *C. protothecoides, C. vulgaris and S. obliquus*, starting with OD= 0.2 and reaching the OD values of 0.234, 0.342 and 0.320, respectively, by the 3rd cultivation day. From the 3rd to the 7th day of cultivation, *C. protothecoides* maintained OD values between 0.2 and 0.3; the biggest increase on OD values occurred from the 10th day to the 19th period of cultivation, reaching OD=1.379 at the last day. From the 3rd to the 10th day, *C. vulgaris* maintained OD values between 0.3 and 0.4; the biggest increase was from the 14th (OD=0.492) to the last day of cultivation, reaching OD=1.149. From the 3rd to the 10th day, *S. obliquus* increased from 0.320 to 0.451, and the biggest increase started at

the 14th day (OD= 0.698); at the 19th day, an OD of 1.295 was reached. For *Synechocystis*, the OD values increased to 0.538 by day 7 but stayed between 0.5 and 0.6 until the 12th cultivation day. The biggest increase in OD values was from day 14 (OD=0.727) to day 19, where OD=1.898. Even though the inoculum used were from air lifts cultivation stage, supposedly habituated to the 5% SWW, in this next stage of cultivation the microalgae experienced stationary periods, where the OD values did not increase significantly. Still, *S. obliquus* seems to have had a more gradual increase of OD values throughout the cultivation period.

In order to test the influence of air flow rate, it was inoculated *C. vulgaris* and *C. protothecoides* in 5% SWW medium in 5L bubble column photobioreactors, providing twice the air flow (1.2 L/min), in the same light and temperature conditions as applied in this cultivation stage. It was observed that *C. protothecoides* was not developing as well as *C. vulgaris*. Other PBRs and operation conditions should be tested and to achieve higher biomass productivities.

Table 3.7 Biomass dry weight (DW) and ash free dry weight (AFDW) for *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. from the 5th day and 19th day of 5 L bubble column PBRs cultivation stage (average of 4 different samples ± standard deviation).

Specie	DW (g/L)		AFDW (g/L)		$P_{biomass}$ (g/L/day)
	Day 5	Day 19	Day 5	Day 19	
C. protothecoides	0.17±0.02	0.70±0.10	0.20±0.03	0.70±0.16	0.04
C. vulgaris	0.22±0.01	0.58±0.08	0.22±0.01	0.43±0.08	0.02
S. obliquus	0.18±0.01	0.55±0.09	0.21±0.01	0.60±0.07	0.03
***Synechocystis* sp.**	0.19±0.02	0.35±0.09	0.20±0.01	0.45±0.09	0.02

C. protothecoides had the highest biomass concentration/productivity (0.70 ±0.16 g/L), and the lowest ash contribution followed by *S. obliquus* (0.60 ±0.11 g/L). *Synechocystis* and *C. vulgaris* had similar results of 0.45±0.09 g/L and 0.43±0.08 g/L, respectively, and equal biomass productivity. *C. vulgaris* had the highest ash value.

Regarding pH results (table 3.8), the initial pH for 5% SWW was elevated from 7.9 to 9-10 score, due to the microalgae's photosynthetic metabolism. The conductivity values were greatly reduced, meaning that the salinity of the 5%SWW medium was diminished by the microalgae assimilation of inorganic ions. In this case, the best result is given by *C. vulgaris*.

Table 3.8 pH and conductivity at last day (19th) of cultivation in 5L bubble column photobioreactors for *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. (average ± standard deviation for 2 replicates).

Specie	pH	Conductivity (µS/cm)
5% SWW	7.9	1735±12
C. protothecoides	9.81±0.2	752±46
C. vulgaris	9.09±0.1	696±146
S. obliquus	9.01±0.1	741±9
***Synecocystis* sp.**	9.67±0.1	791±16

Regarding SWW treatment, Decree-Law 236/98 of the Portuguese legislation indicates that pH medium value can be comprised in the maximum range of 5.0 to 10.0 (table 6.3, Appendix C). All tested microalgae presented results that respect this range.

3.2.4. Microscope observations

The microscope observations are represented from figures 3.4 to 3.6. The species *C. protothecoides, C. vulgaris* and *S. obliquus* were easily identified. *Synechocystis* sp. was not so easy to see because of its smaller size.

The *Chlorella* species are both characterized with spherical or ellipsoid green cells, with different diameters: *C. protothecoides* cells and have around 3-6 µm and *C. vulgaris* cells normally have around 2-10 µm in diameter. Figure 3.4 represents both microalgae cells. *S. obliquus* cells (figure 3.5) also present a green colour and its morphology is characterized by fusiform shaped cells, with or without spines, that can form linear arrangements of 2 or more cells (Guiry, 2019; Akgül *et al.*, 2017; Lürling, 2003). *Synechocystis* sp. PCC 6803 (figure 3.6) is morphologically characterized as a small globed cell, with approximately 1.5 µm diameter (Van de Meen *et al.*, 2006), thus there was some difficulty in observing it under microscope, even at 100x amplification. No contamination was observed.

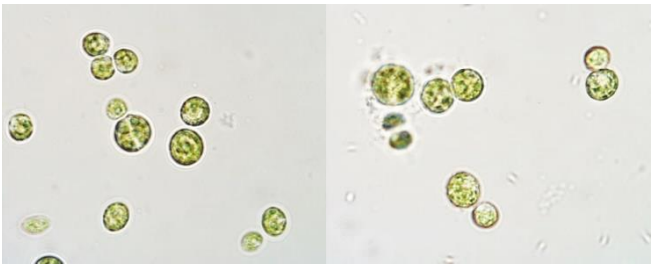


Figure 3.4 Representation of the *Chlorella protothecoides* (left) and *Chlorella vulgaris* cells (right) (100x amplification).

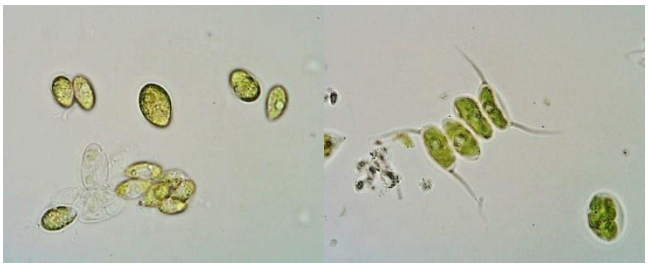


Figure 3.5 *Scenedesmus obliquus* cells, without spines, on the left, and with spines, on the right (100x amplification).

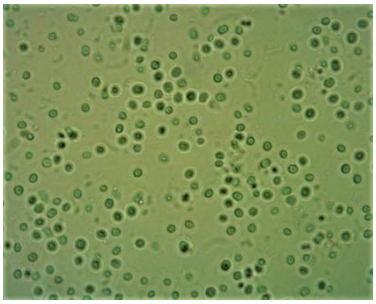


Figure 3.6 *Synechocystis* sp. cells (100x amplification).

3.3. Nutrient consumption/removal

Results corresponding to the initial COD value and after the cultivation for each microalgae and cultivation stage are represented on figure 3.7 and table 3.9.

Regarding nitrogen consumption, results for total Kjeldahl nitrogen (TKN) are showed on figure 3.5 and table 3.10, for all cultivation stages and microalgae strains. Results for ammonium nitrogen (NH_4^+) are represented for 1L and 5L bubble column PBRs cultivation stages, on figure 3.8 and table 3.8.

Phosphorus consumption by microalgae treatment is depicted on figure 3.9 and values are displayed on table 3.11.

3.3.1. COD consumption/removal

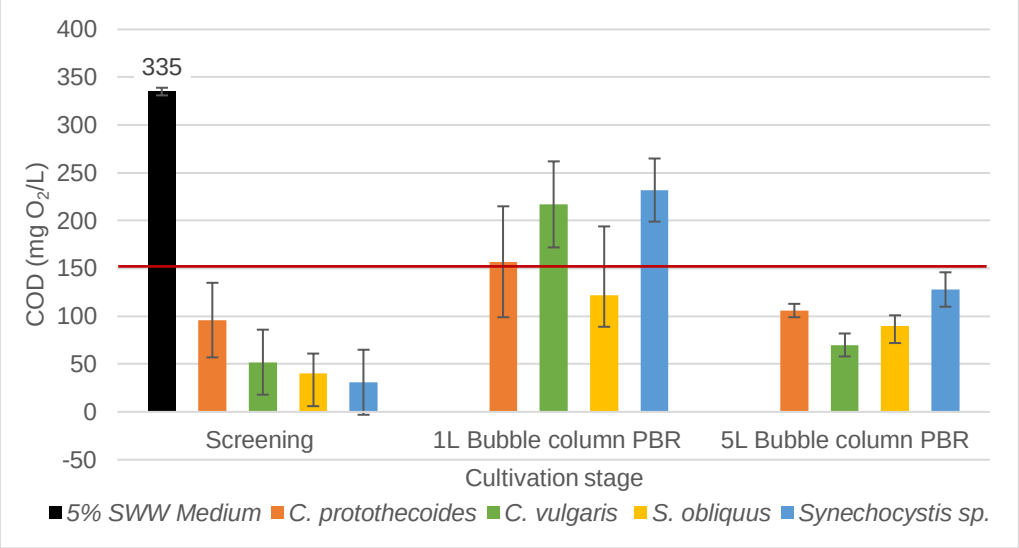


Figure 3.7 Initial chemical oxygen demand (COD) of 5% SWW medium (335±4 g O2/L) and after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., in all cultivation stages (average ± standard deviation for at least 3 replicates). The emission limit value according to Decree Law 236/98 of Portuguese Legislation is represented by the red line (150 mg/L).

Overall, the best results on COD removal were achieved on *screening* and 5L bubble column PBRs cultivation trial. *S. obliquus* and *C. protothecoides* performed a more coherent removal in all cultivation stages; considering all stages, *S. obliquus* could remove COD content between 64-88% and *C. protothecoides*, 53-71%, respectively. These results make sense because *S. obliquus* is considered a robust strain regarding different growth conditions and nutrient concentrations. *C. vulgaris* obtained better COD removal results on *screening* and 5L bubble column PBRs stages (84 and 79%, respectively), and reducing it in 1L bubble column PBR stage by only 35%. *Synechocystis* showed more differences in COD removal between all the stages: 91% removal in the *screening* stage; 31% in 1L bubble column PBRs stage; 62% in the 5L bubble column PBRs. These results indicate that cultivation conditions and mode of operation are influencing the ability of microalgae to uptake and removing nutrients from the 5% SWW, thus influencing their biomass growth.

Table 3.9 Chemical oxygen demand (COD) and nutrient removal efficiency (NRE) after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., for *screening*, 1L and 5L bubble column PBRs cultivation in 5% SWW medium from the last cultivation day (average ± standard deviation for at least 3 replicates).

Samples	COD (mg O_2/L)			NRE (%)		
	Screening	1L Bubble column	5L Bubble column	*Screening*	1L Bubble column	5L Bubble column
C. protothecoides	96±39	157±58	106±7.0	71	53	68
C. vulgaris	52±34	217±45	70±12	84	35	79
S. obliquus	40±21	122±72	90±11	88	64	73
***Synechocystis* sp.**	31±34	232±33	128±18	91	31	62

According to Decree Law 236/98 of Portuguese Legislation, the emission limit value (ELV) for COD in water and wastewater treatment is 150 mg O_2/L (table 6.3, Appendix C). In 1L bubble column PBR cultivation stage, only *S. obliquus* efficiently diminished COD concentration and satisfied the previous condition. This microalga has been described as very efficient on bioremediation from several effluents (Ferreira *et al.*, 2018, 2017; Batista *et al*., 2015). But, most importantly, in 5L bubble column PBRs cultivation stage, all COD results from biologically treated 5% SWW satisfied this condition (meaning better results with higher volumes).

3.3.2. Nitrogen consumption/removal

a) TKN

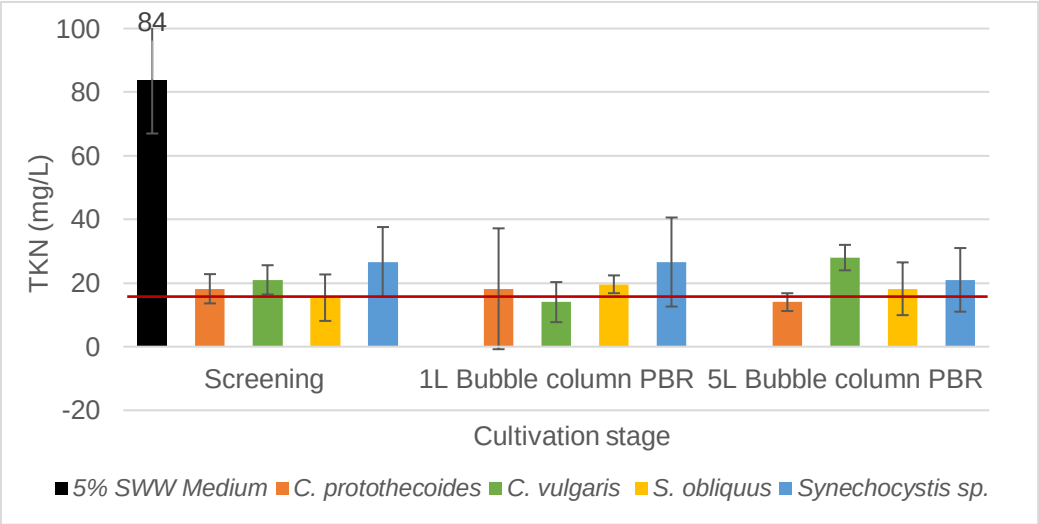


Figure 3.8 Initial total Kjeldahl nitrogen (TKN) of 5% SWW medium (84±0.0 mg/L) and after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., for all cultivation stages (average ± standard deviation for 4 replicates). The emission limit value according to Decree Law 236/98 of Portuguese Legislation is represented by the red line (15 mg/L).

Overall, the four microalgae reduced in great amounts the TKN concentrations of the initial TKN of 5% SWW.

Table 3.10 Total Kjeldahl nitrogen (TKN) and nutrient removal efficiency (NRE) after biological treatment of 5% SWW medium by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., for screening, 1L and 5L bubble column PBRs cultivation (average ± standard deviation for 4 replicates).

Samples	TKN (mg/L)			NRE (%)		
	Screening	**1L Bubble column**	**5L Bubble column**	***Screening***	**1L Bubble column**	**5L Bubble column**
C. protothecoides	18.2±4.6	18.2±19	14±2.8	78	78	83
C. vulgaris	21±4.6	14±6.3	28±4.0	83	75	67
S. obliquus	15.4±7.3	19.6±2.8	18.2±8.3	77	82	78
***Synechocystis* sp.**	26.6±11	26.6±14	21±10	68	68	75

Considering the present TKN results, all microalgae strains seemed to have greatly reduced the initial TKN of 5% SWW (84±17 mg/L). For *C. protothecoides* and *S. obliquus*, TKN concentration was reduced between 77-83%. The former achieved better results in 5L bubble column cultivation, which is consistent with previous OD and DW/AFDW results, and the latter showed better results on the 1L bubble column PBRs. For *C. vulgaris*, TKN concentrations were reduced around 67-83%, having achieved its best result on *screening*. *Synechocystis* was able to reduce TKN concentrations up to 75%.

The Decree Law 236/98 from Portuguese Legislation places the maximum allowed of total nitrogen concentration for wastewater treatment on 15 mg/L (table 6.3, Appendix C). *C. vulgaris* and *C. protothecoides* accomplished that condition in 1L and 5L bubble column PBRs stage of cultivation. Nonetheless, it is important to notice that TKN values are very close to fulfil this condition.

b) Ammonium nitrogen ($N\text{-}NH_4^+$)

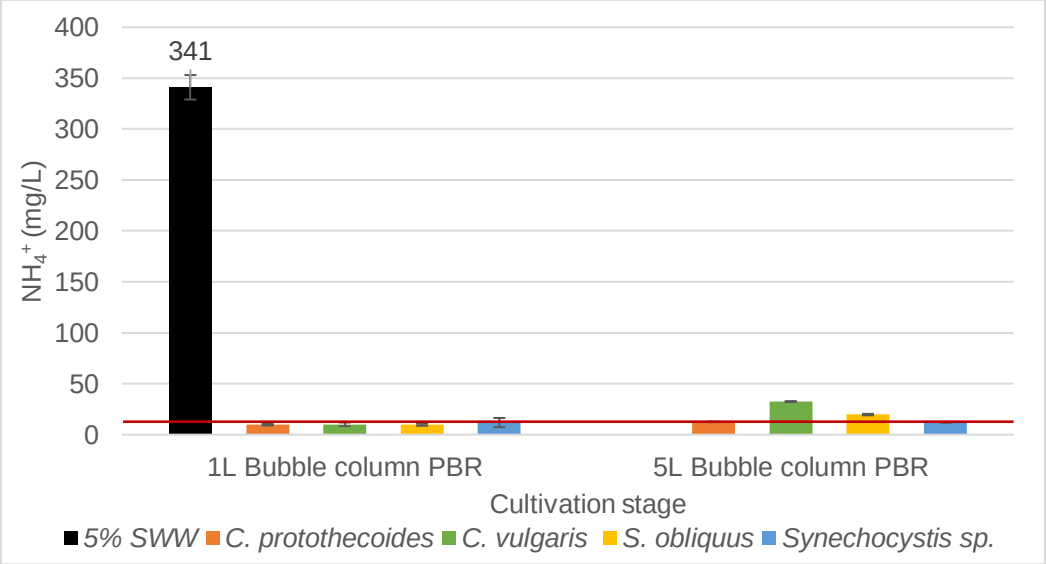


Figure 3.9 Ammonium nitrogen (NH4+) for 5% SWW medium (341±12 mg/L) after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., for 1L and 5L bubble column PBRs cultivation (average ± standard deviation for at least 2 replicates). The emission limit value according to Decree Law 236/98 of Portuguese Legislation is represented by the red line (10 mg/L).

Table 3.11 Ammonium nitrogen and nutrient removal efficiency (NRE) after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. for 1L and 5L bubble column PBRs cultivation (average ± standard deviation for at least 2 replicates).

Samples	$N\text{-}NH_4^+$ (mg/L)		NRE (%)	
	1L Bubble column	5L Bubble column	1L Bubble Column	5L Bubble column
C. protothecoides	9.75±0.8	12.6±0.3	97	96
C. vulgaris	10.2±1.8	32.5±0.4	97	90
S. obliquus	9.83±1.1	19.7±0.7	97	94
***Synechocystis* sp.**	11.9±4.5	12.0±0.2	97	96

Although biomass concentrations were low, all microalgae were able to greatly reduce nitrogen in the form of ammonium, achieving removal over 90%. Microalgae can perform nitrogen removal from the medium by two different mechanisms: directly, by assimilating the nitrogen on inorganic forms, especially as ammonium, and indirectly, by inducing ammonia stripping through pH and temperature elevation (Cai et *al*, 2013). The occurrence of ammonia stripping is a plausible explanation for the low biomass concentrations and the efficient reduction of TKN and ammonium/ammonia nitrogen by the microalgae.

According to Decree/Law 236/98 of Portuguese legislation for water and wastewater treatment, the ammonia nitrogen levels must be below 10 mg/L (table 6.3, Appendix C). *C. protothecoides* and *S. obliquus* were able to reduce NH_4^+ levels to satisfy this condition. Although this condition was not always satisfied, concentrations were still greatly reduced from the initial concentration, and optimized treatment conditions can help to reduced it even more.

3.3.3. Phosphorus consumption/removal

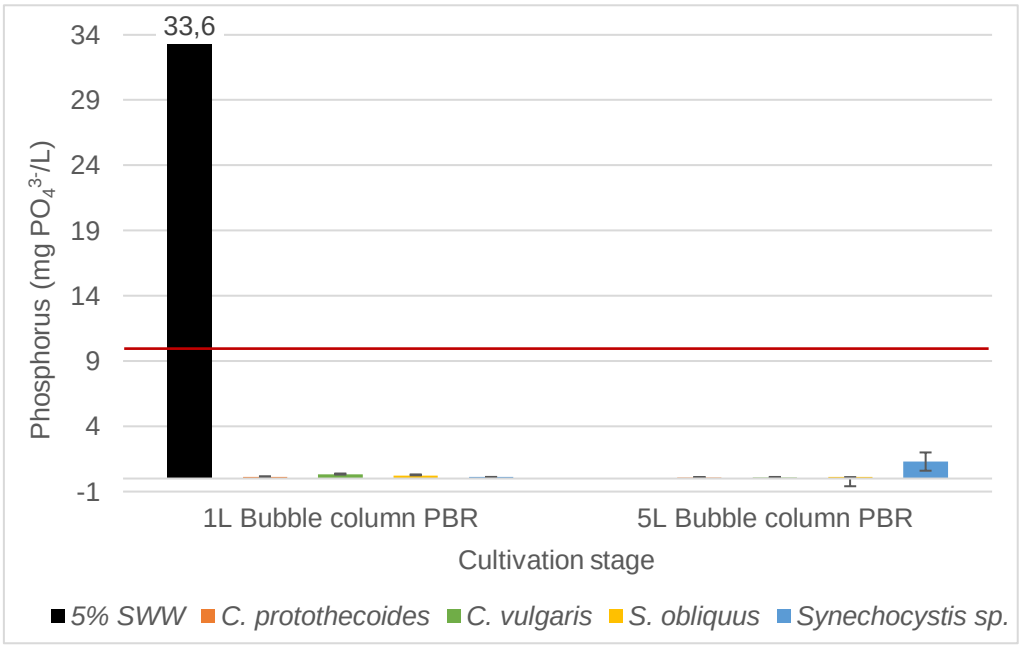


Figure 3.10 Phosphorus as phosphate (PO_4^{3-}) concentration of 5% SWW medium (33.6±0.4 mg/L) and after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., for 1L and 5L bubble column PBRs cultivation (average ± standard deviation for at least 2 replicates). The emission limit value according to Decree Law 236/98 of Portuguese Legislation is represented by the red line (10 mg/L).

Phosphorus concentration results show that removal by biological treatment with the mentioned microalgae was above 97%. In case of 1L bubble column PBR stage, the PO_4^{3-} concentrations represented on table 3.12 were achieved on the 8th day of cultivation; for 5L PBR stage, the concentrations were reached at the 14th day of cultivation. Phosphorus concentrations can be diminished directly by incorporation of inorganic phosphates by the microalgae cells, or by precipitation as result of the increase of pH and dissolved oxygen concentration caused by the microalgae growth. The high removal rates achieved could be due to the combination of both mechanisms for phosphorus consumption (Cai *et al.*, 2013).

Table 3.12 Phosphorus as phosphate (PO_4^{3-}) concentration and nutrient removal efficiency (NRE) after biological treatment by *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. for 1L and 5L bubble column PBRs cultivation (average ± standard deviation for at least 2 replicates).

Sample	PO_4^{3-} (mg/L)		NRE (%)	
	1L Bubble column	5L Bubble column	1L Bubble column	5L Bubble column
C. protothecoides	0.13±0.05	0.10±0.00	99	99
C. vulgaris	0.35±0.04	0.10±0.02	99	99
S. obliquus	0.25±0.07	0.11±0.00	99	98
***Synechocystis* sp.**	0.12±0.00	1.3±0.7	99	98

According to Decree/Law 236/998 of Portuguese legislation for water and wastewater treatment, the total phosphorus levels must be below 10 mg/L (table 6.3, Appendix C). All microalgae were able to decrease phosphorus levels below this value.

3.4. Microalgae biomass characterization

Results for fatty acids content are represented in figure 3.11 and table 3.13; for protein in figure 3.12 and for total sugar content, results are represented in figure 3.13.

Results for mineral nutrients from the elemental analysis are represented on figure 3.14, for macro- and microelements for the different biomasses. No heavy metals concentrations were detected; therefore, its representation can be discarded.

3.4.1. Fatty acids distribution

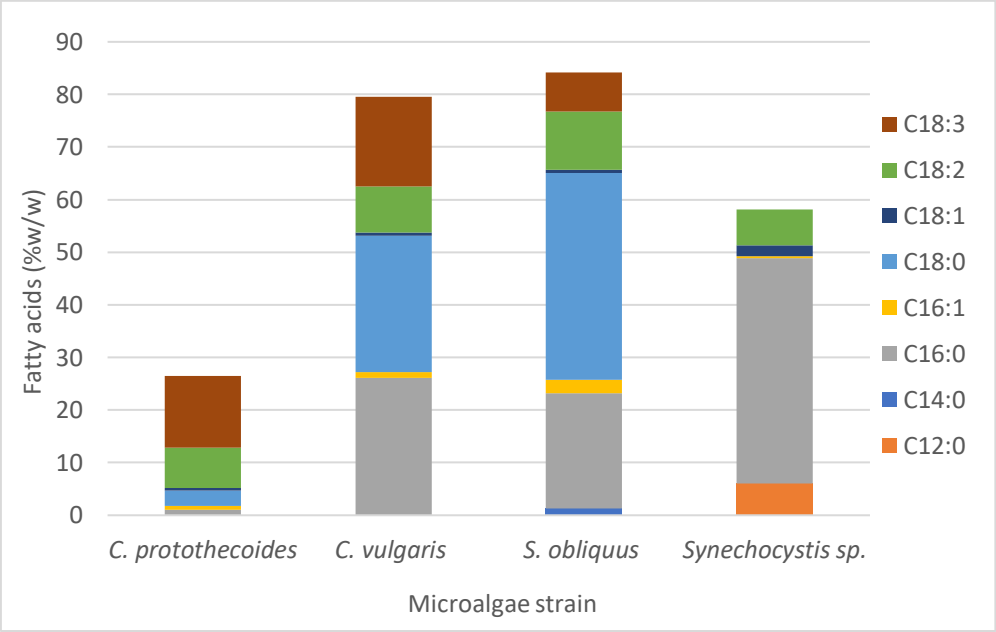


Figure 3.11 Relative fatty acid distribution of total lipids for biomass of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., produced after cultivation/SWW treatment on 5L bubble column PBRs (C18:3 - α-linoleic acid; C18:2 - linoleic acid; C18:1 - oleic acid; C18:0 – stearic acid; C16:1- palmitoleic acid; C16:0 – palmitic acid; C14:0 – myristic acid; C12:0 – lauric acid).

Overall, observing figure 3.11, the microalga *S. obliquus* had the highest fatty acid distribution regarding the identified fatty acids (approximately 85%w/w), followed by *C. vulgaris* (approximately 80%w/w).

The depicted distribution shows that *C. vulgaris* biomass could have the highest content on unsaturated fatty acids (27.5 %w/w), followed by *C. protothecoides* (22.4 %w/w); *S. obliquus* could have the highest biomass content for saturated fatty acids (62.4 %w/w), followed by *C. vulgaris* (52.1 %w/w), and for FAMEs (8.69 %w/w), followed by *C. protothecoides* (7.55 %w/w). The dominant fatty acids were α-linolenic acid (C18:3) for *C. protothecoides*, palmitic acid (C 16:0) for *C. vulgaris*, stearic acid (C18:0) for *S. obliquus*, and palmitic acid (C16:0) for *Synechocystis* sp. Further results are depicted on table 3.13.

Table 3.13 Fatty acid distribution (of total lipid composition) of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. biomasses, produced after cultivation/SWW treatment on 5L bubble column PBRs.(FAMEs: fatty acids methyl esters).

Fatty acid	Fatty acid content %(w/w)			
	C. protothecoides	*C. vulgaris*	*S. obliquus*	*Synechocystis* sp.
Lauric acid (C12:0)	-	-	-	6.01
Myristic acid (C14:0)	-	-	1.30	-
Palmitic acid (C16:0)	1.04	26.1	21.8	42.8
Palmitoleic acid (C16:1)	0.73	1.04	2.63	0.46
Stearic acid (C18:0)	2.93	25.9	39.3	-
Oleic acid (C18:1)	0.38	0.63	0.66	2.03
Linoleic acid (C18:2)	7.68	8.81	11.1	6.75
α-linolenic acid (C18:3)	13.6	17.0	7.40	-
Total (C12-C18)	26.4	79.5	84.2	58.1
Saturated	4.02	52.1	62.4	48.8
Non-saturated	22.4	27.5	21.8	9.25
Not identified	73.5	20.4	15.8	41.9
FAMEs (%)	7.55	3.80	8.69	1.78

Regarding biodiesel production, most common FAMEs are C16:0 and the C18 group (Hoekman *et al*., 2012). The most interesting seems to be *S. obliquus*, with the highest FAMEs content, and distribution of 82.9% for C16-C18 fatty acids (from total lipids).

Regarding human/animal food/feed supplementation, PUFAs (polyunsaturated fatty acids) are a group of fatty acids that are essential to human health, which include omega-3 polyunsaturated fatty acids, such as α-linolenic acid (C18:3), and omega-6 polyunsaturated fatty acids, such as linoleic acid (C18:2). Microalgae are already referred as an important alternative source of PUFAs (Koyande *et al.*, 2019; Kumar *et al*., 2019). *C. protothecoides* and *C. vulgaris* had the highest content on both referred PUFAs (21.3% and 25.8% of total lipids, respectively), and so both microalgae seem to be interesting in this context.

3.4.2. Protein content

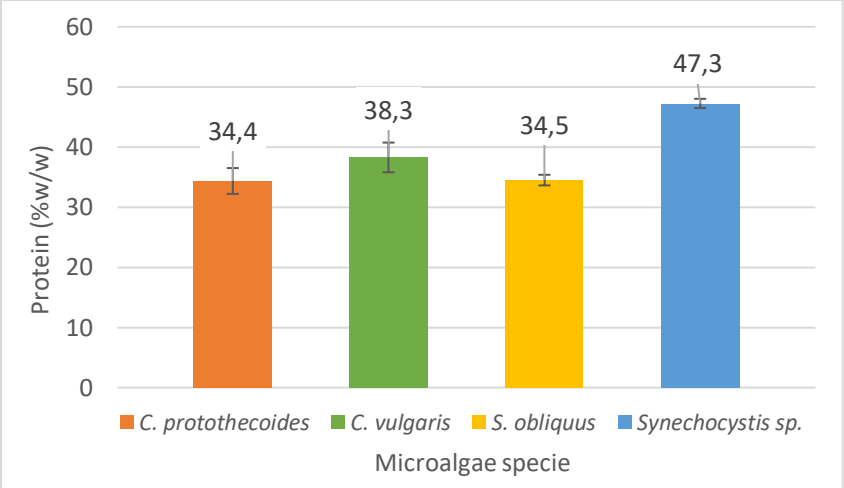


Figure 3.12 Protein content for *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. biomasses produced after cultivation/SWW treatment on 5L bubble column PBRs (average ± standard deviation for at least 3 replicates).

The results for protein content on the different microalgae biomass demonstrate that *Synechocystis* sp. has the higher content of protein (47.3±2.8 %w/w), followed by *C. vulgaris* (38.3±0.9 %w/w), *S. obliquus* (34.5±2.1 %w/w) and *C. protothecoides* (34.4±0.8 %w/w), which have the same protein content.

C. vulgaris and *S. obliquus* protein content were under the general reported range of protein of 51-58% and 50-56% of dry matter, respectively (Becker, 2007). Different values for *Synechocystis* sp. strains were reported, depending on culture conditions, ranging from 32 to 73% of dry biomass content (Zavřel, *et al.*, 2017; Touloupakis *et al.*, 2015).

Having the highest protein content, *Synechocystis* sp. can be the most interesting as a source of protein for human/animal food/feed supplementation, as other cyanobacteria e.g. *Arthrospira platensis*, also known as Spirulina (Koyande *et al.*, 2019).

3.4.3. Total sugars content

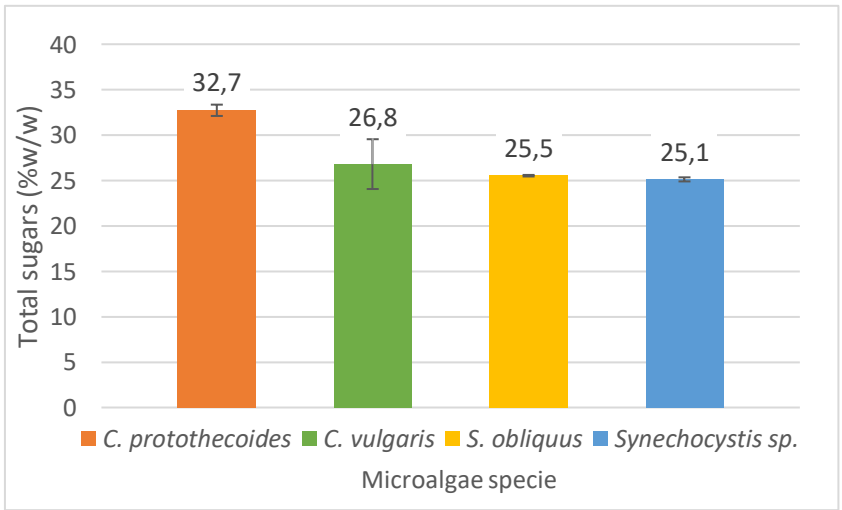


Figure 3.13 Total sugars content for *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. biomasses produced after cultivation/SWW treatment on 5L bubble column PBRs (average ± standard deviation for 3 replicates).

Regarding total sugar content, *C. protothecoides* had a higher biomass content (32.7±0.6 %w/w), followed by *C. vulgaris* (26.8±2.7 %w/w), *S. obliquus* (25.5±0.1 %w/w) and *Synechocystis* sp. (25.1±0.2), the two of which had the same total sugars content.

C. vulgaris and *S. obliquus* had superior contents of total sugars comparing to the reported general content of 12-17% and 10-17% of dry matter, respectively (Becker, 2007). Regarding *Synechocystis* sp., reported total sugar contents depend on strain and conditions, being the maximum 40% of dry weight (Zavřel *et al.*, 2017).

Regarding applications, carbohydrates (or sugars) are important as substrates for biofuels production, namely bioethanol or biohydrogen (Markou *et al.*, 2012). In this context, *C. protothecoides* appears interesting because of its highest total sugar content; also *C. vulgaris* and *S. obliquus* also are potential sources of carbohydrates, since they add up to its general carbohydrate production.

3.4.4. Mineral content

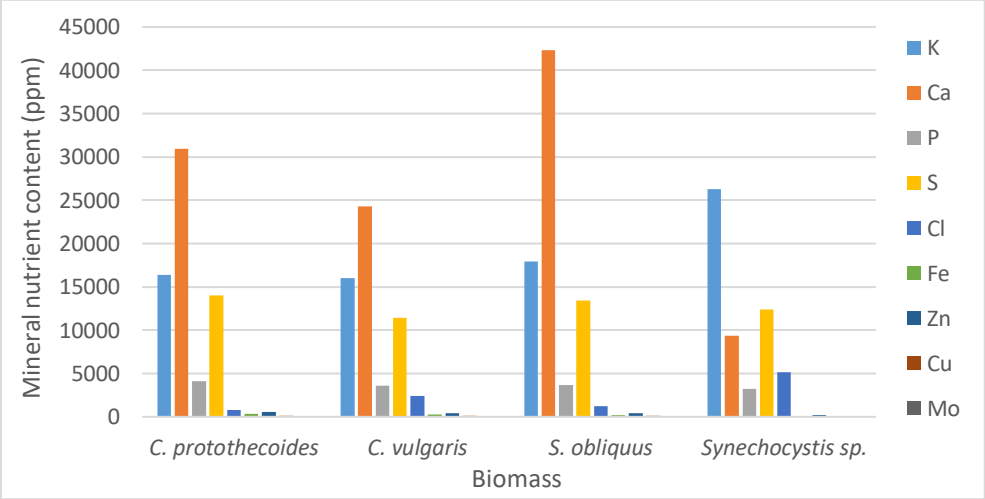


Figure 3.14 Mineral macro- (K, Ca, Mg, P, S) and microelements (Cl, Fe, Zn, Cu, Mo) concentration on biomass of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp. Produced after cultivation/SWW treatment on 5L bubble column PBRs (potassium – K; calcium – Ca; magnesium – Mg; phosphorus – P; sulphur – S; chlorine – Cl; iron – Fe; zinc – Zn; copper -Cu; molybdenum Mo).

In figure 3.14, it is possible to see that for all the microalgae (except *Synechocystis*) calcium (Ca) had the highest contents, especially for *S. obliquus*, with 4.2% of biomass weight (42303.0±404.5 ppm), followed by *C. protothecoides*, with 3.1% of biomass weight (30901.0±337.6 ppm). The next is potassium (K), ranging from 1.6-1.8% of biomass weight (16007±284.7 to 26249.1±251.7 ppm) for the three microalgae. Regarding *Synechocystis* sp., the highest content is for potassium, with 2.6% of biomass weight (26249.1±251.7 ppm), followed by sulphur (S), with 1.2% (12414.7±143.6 ppm), and calcium (Ca), with 0.9% (9331.2±227.4 ppm).

In these analyses, significant concentrations of magnesium (Mg), manganese (Mn) and nickel (Ni) were not detected, seeing its values were below the limit of detection of the apparatus. The concentrations for molybdenum (Mo) concentrations are also not significant, giving the high error. Further results are displayed on tables 3.14, for microelements and macroelements.

Table 3.14 Microelements (Cl, Fe, Zn, Cu) and macroelements (K, Ca, P, S) from XRF analyses for *Chlorella protothecoides* (C.p.), *Chlorella vulgaris* (C.v.), *Scenendesmus obliquus* (S.o.) and *Synechocystis* sp. (Syn. Sp.) biomasses, produced after cultivation/SWW treatment on 5L bubble column PBRs (chlorine – Cl; iron – Fe; copper – Cu; potassium - K; calcium - Ca; phosphorus – P; sulphur – S). Average ± standard deviation for 3 readings.

Specie	**Elements (ppm)**			
	Cl	**Fe**	**Zn**	**Cu**
C.p.	780.9±27.0	349.7±28.4	560.6±12.5	134.9±13.0
C.v.	273.0±28.3	273.0±28.3	447.0±13.6	119.9±13.3
S.o.	221.9±26.8	221.9±26.8	434.4±11.5	123.8±13.1
***Syn.* sp.**	74.8±250	74.8±25.0	187.9±8.4	78.5±12.8
	K	**Ca**	**P**	**S**
C.p.	$16.4x10^3$ ±185.3	$30.9x10^3$ ±337.6	$4.1x10^3$ ±89.9	$14x10^3$ ±145.7
C.v.	$16x10^3$ ±284.7	$24.3x10^3$ ±461.4	$3.6x10^3$ ±99.5	$11.4x10^3$ ±193.1
S.o.	$17.9x10^3$ ±199.1	$42.3x10^3$ ±404.5	$3.6x10^3$ ±90.9	$13.4x10^3$ ±148.3
***Syn.* sp.**	$26.2x10^3$ ±251.7	933.1±227.4	$3.2x10^3$ ±88.7	$12.4x10^3$ ±143.6

Overall, all microalgae biomass analyses revealed higher concentrations for:

- Potassium (K), the highest content value for *Synechocystis* sp.;
- Calcium (Ca) and sulphur (S), the highest content value for *S. obliquus*;
- Phosphorus (P), the highest content value for *C. protothecoides*.

All the listed minerals macro- and microelements are essential nutrients for plant physiology and development, being part of several cellular mechanisms, like ion fluxes, osmosis, salt tolerance and even as co-factors for enzymes. Macronutrients are normally found in plants within a range of 1000 to 15000 ppm and micronutrients concentrations 100 to 10000 times lower (Buchanan *et al*, 2015).

3.5. Microalgae biostimulant effect on seed growth

3.5.1. Germination index

The germination index results are displayed from figure 3.15 to 3.20.

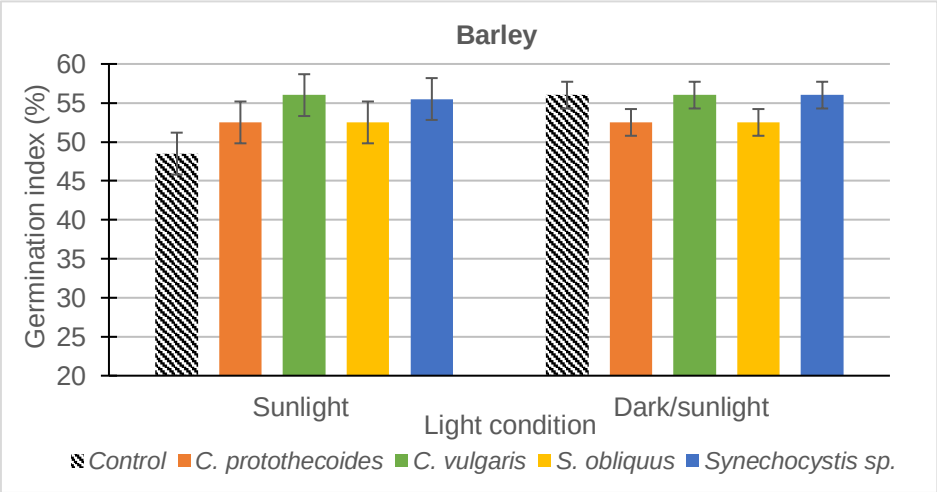


Figure 3.15 Germination index results for barley seeds grown with distilled water (control) and with microalgae biomass suspensions of *Chlorella prototheocoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

Regarding barley seeds, in sunlight conditions, *C. vulgaris* (56%) and *Synechocystis* sp. (55.5%) increased germination index comparing with control, with a difference of 7.5% and 7%, respectively. *C. prototheocoides* (52.5%) and *S. obliquus* (52.5%) also obtained higher values comparing to control, with a difference of 4% comparing to control. In dark/sunlight conditions, *Synechocystis* sp. and *C. vulgaris* had the same germination index as control (56%).

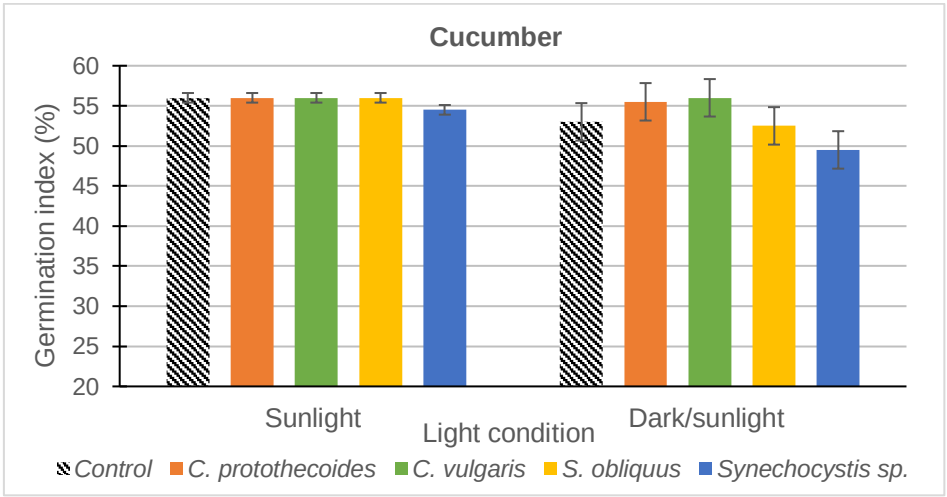


Figure 3.16 Germination index results for cucumber seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheocoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

For cucumber seeds, in sunlight conditions, both *Chlorella* species and *S. obliquus* had the same germination index as control for cucumber seeds (56%). Nevertheless, an increase of 2.5 and 3% were observed for *C. prototheocoides* (55.5%) and *C. vulgaris* (56%) germination index, respectively, in dark/sunlight conditions when compared to control (53%).

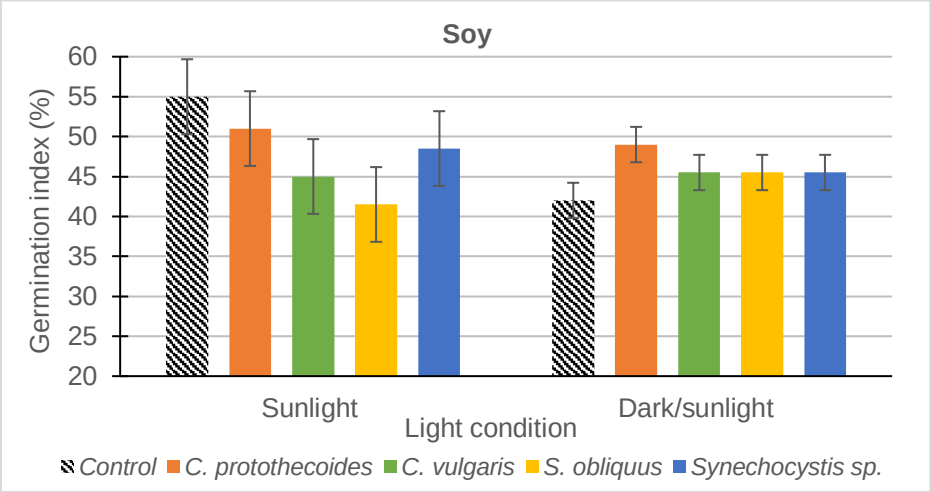


Figure 3.17 Germination index results soy seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

The soy seed trials, under sunlight, the results for the four microalgae were all below the control. However, under dark/sunlight conditions, was revealed an enhanced germination index for all microalgae, especially for *C. protothecoides* (49%). For the remaining microalgae, all increased germination to 45.5%, differing 3.5% from control germination index.

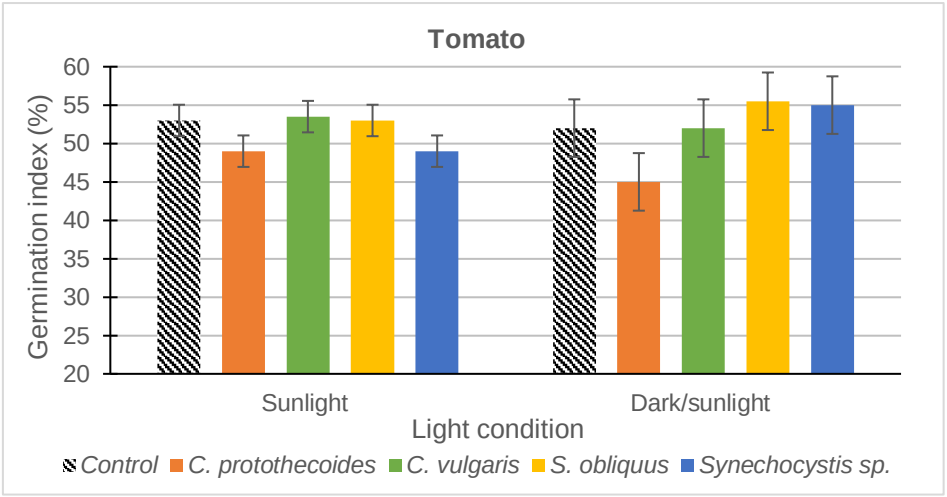


Figure 3.18 Germination index results for tomato seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

Tomato seeds did not have significant differences between germination indexes on sunlight conditions, although *C. vulgaris* had a germination index close to control (53.5%) and *S. obliquus* had the same germination index as control for these seeds (53%). On dark/sunlight conditions, there was an increase of 3.5 and 3% with *S. obliquus* (55.5%) and *Synechochystis* sp. (55%) on germination index, respectively.

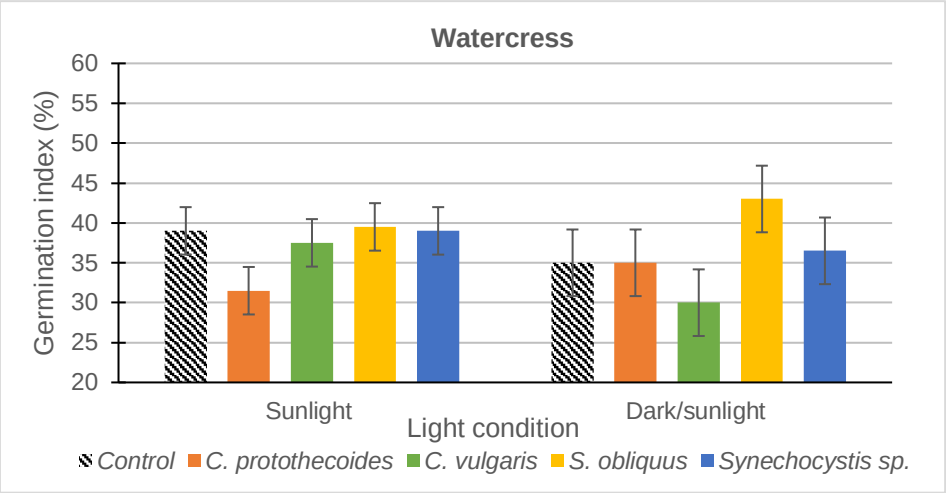


Figure 3.19 Germination index results for watercress seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheco ides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

For watercress seeds, under sunlight conditions, *S. obliquus* (39.5%) and *Synechocystis* sp. (39%) had germination indexes close to/equal to control. Under dark/sunlight was detected a positive difference from the control, with *S. obliquus* (43%) and *Synechocystis* sp. (36.5%), increasing the germination index in 8 and 1.5%, respectively; *C. prototheco ides* had equal germination index to the control's (35%).

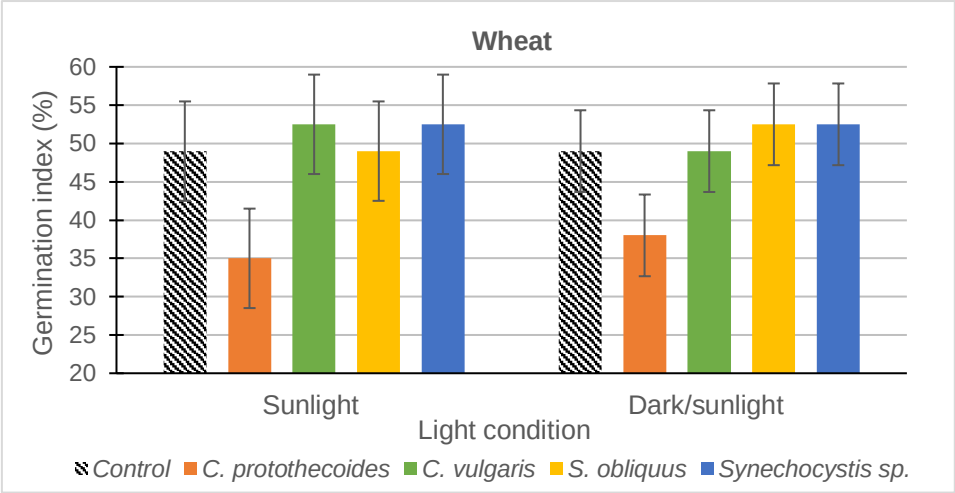


Figure 3.20 Germination index results for wheat seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheco ides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

Wheat seeds had positive results on both conditions. With *Synechocystis* sp., the germination index was 52.5% for both sunlight and dark/sunlight conditions, meaning it was 3.5% above the control. The same result was obtained for *C. vulgaris* at sunlight conditions and for *S. obliquus*, in dark/sunlight conditions. Both had the same germination index as control in the dark/sunlight (*C. vulgaris*) and light conditions (*S. obliquus*). On the other hand, *C. prototheco ides* did not have a positive effect on the germination index, in either the light conditions.

3.5.2. Sprout and root growth

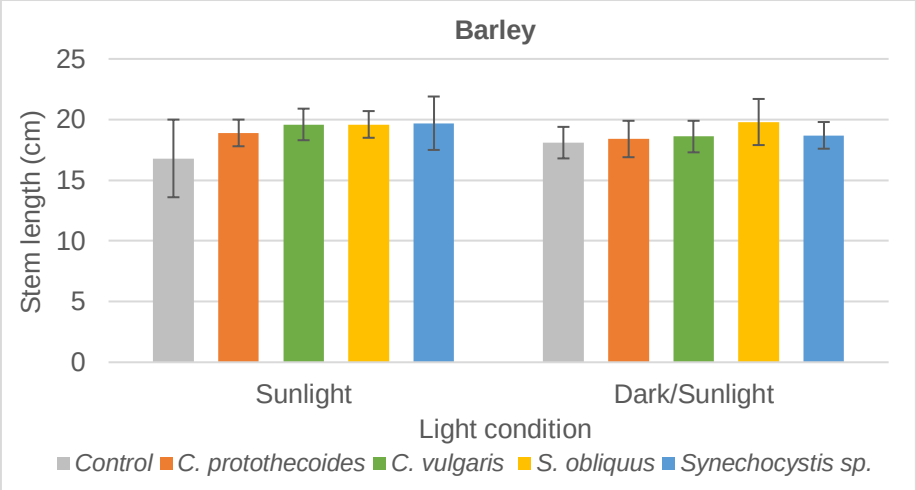


Figure 3.21 Average length of the sprout stem for barley seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

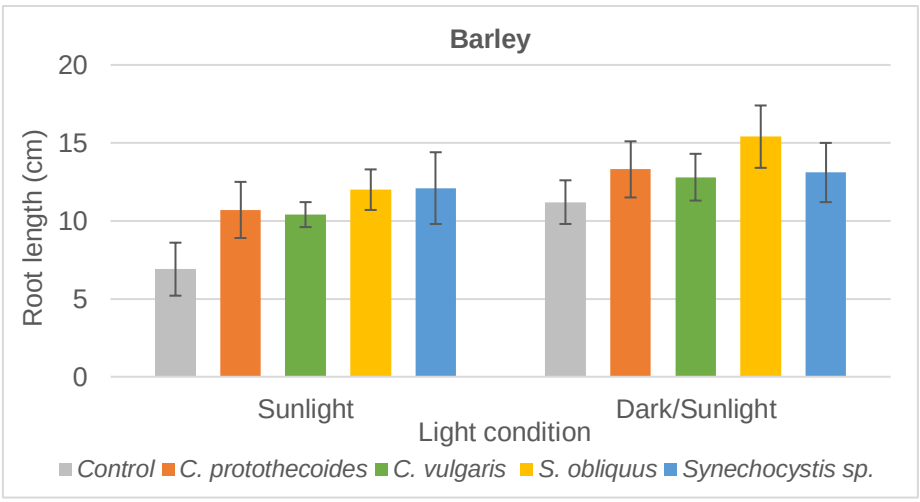


Figure 3.22 Average length of the main root for barley seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

For barley, the main roots length demonstrated to be more affected by light conditions, having achieved higher values under dark/sunlight conditions. This might be because the previous light condition resembles the light conditions in soil. However, the lengths of the sprout stem and main roots both showed positive results with microalgae biomass suspensions, in both conditions. On sunlight conditions, root length results for *S. obliquus* and *Synechocystis* were almost the double comparing to control, followed by both *Chlorella* species and stem lengths had better results for *C.* vulgaris, *S. obliquus* and Synechocystis sp. Regarding dark/sunlight conditions, *S. obliquus* corresponds to the most significant difference in stem length, followed by *Synechocystis* sp., and for root lengths also, followed by *C. protothecoides*.

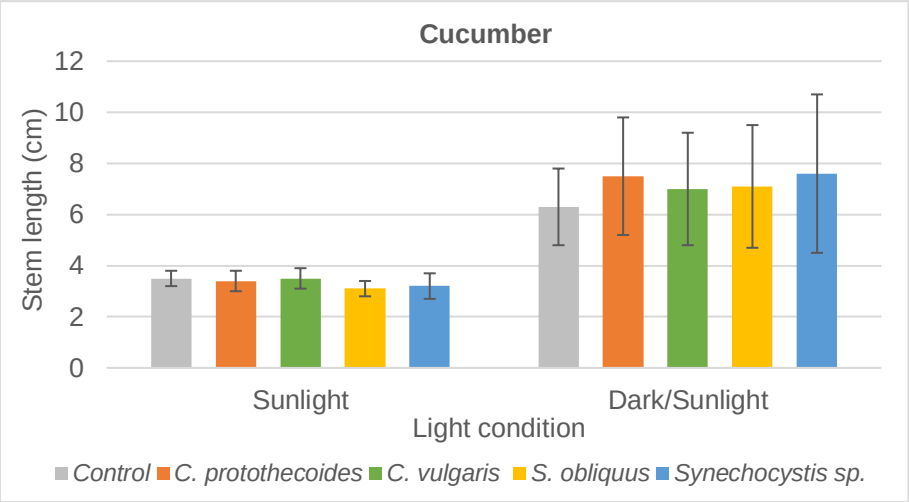


Figure 3.23 Average length of the sprout stem for cucumber seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

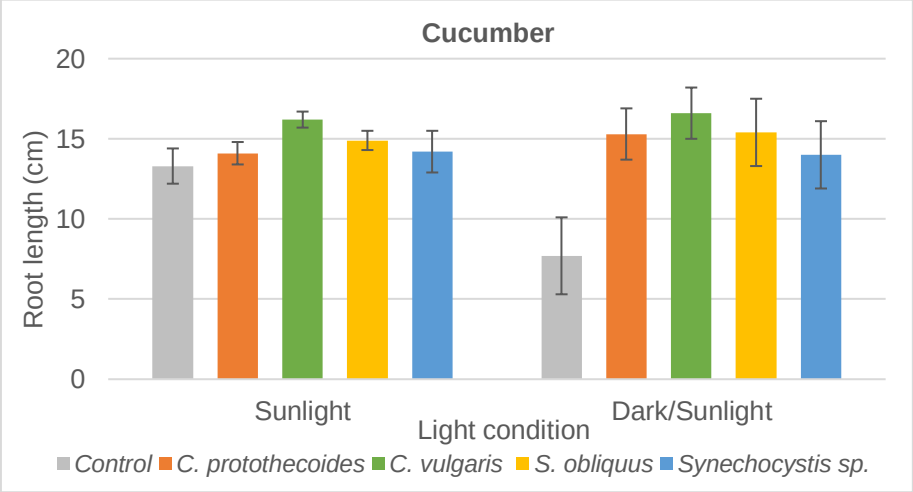


Figure 3.24 Average length of the main root for cucumber seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

Regarding the stem lengths of cucumber, in sunlight conditions, the presence of microalgae biomass suspensions seems to not have an impact comparing to control. Their growth seems to be affected in a positive way by the absence of light, since better results were obtained in dark/sunlight conditions. Highest values were obtained for seeds with all microalgae comparing to control, especially with *Synechocystis* sp., followed by *C. protothecoides.* Regarding root length, in sunlight conditions, seeds had positive response to the presence of microalgae biomass (higher results comparing to control). *C. vulgaris* had the better results for the main root length, followed by *S. obliquus*. However, results were more remarkable under dark/light conditions, the best results with *C. vulgaris*, followed by *C. protothecoides* and *S. obliquus*, with values that are close to the double of those obtained for control. In recent and similar work, *C. vulgaris* also revealed positive results regarding root and shoot lengths of cucumber germinated seeds (Bumandalai & Tserennadmid, 2019).

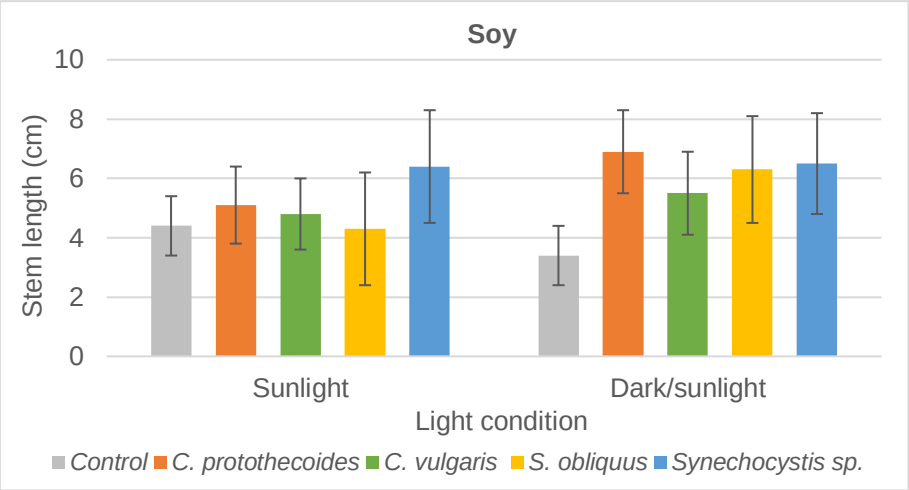


Figure 3.25 Average length of the sprout stem for soy seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheco ides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

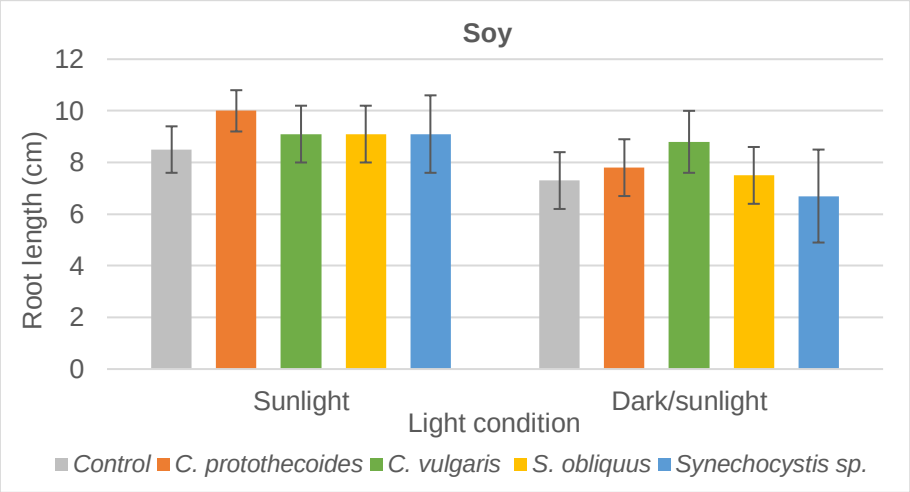


Figure 3.26 Average length of the main root for soy seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheco ides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

Regarding soy stem length, under sunlight conditions, the most significant difference towards control result, was for *Synechocystis* sp. Under dark/sunlight conditions, better results were obtained with microalgae species comparing to control, especially for *C. prototheco ides*, followed by *Synechocystis* sp and.*S. obliquus*. Overall, for soy roots, sunlight appears as beneficial for its lengths. *C. prototheco ides* provided better results in this condition, followed by the remaining microalgae, which did not distinguish much from each other. Under dark/sunlight conditions, roots lengths values were lower, in general. However, *C. vulgaris* provided the better results comparing to control, which are close to those achieved under sunlight, followed by *C. prototheco ides*. This suggests that the absence or presence of light might not affect the contribution of *C. vulgaris* biomass to the root growth.

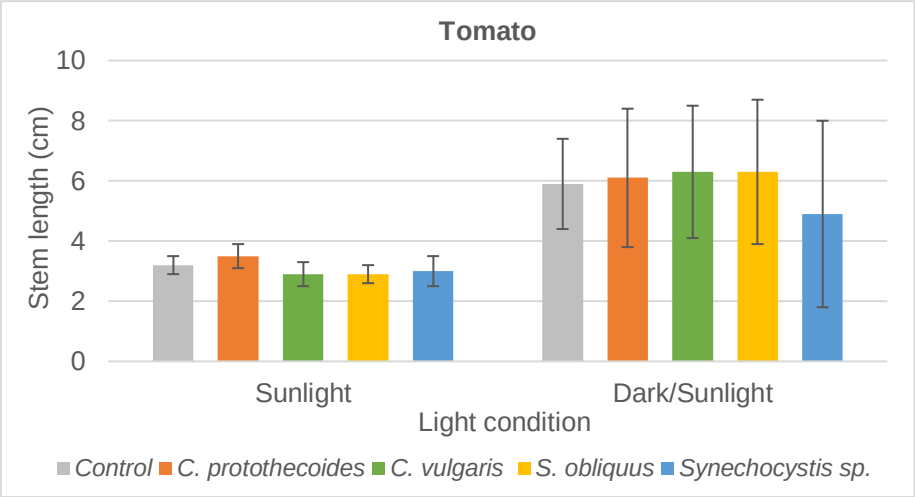


Figure 3.27 Average length of the sprout stem for tomato seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

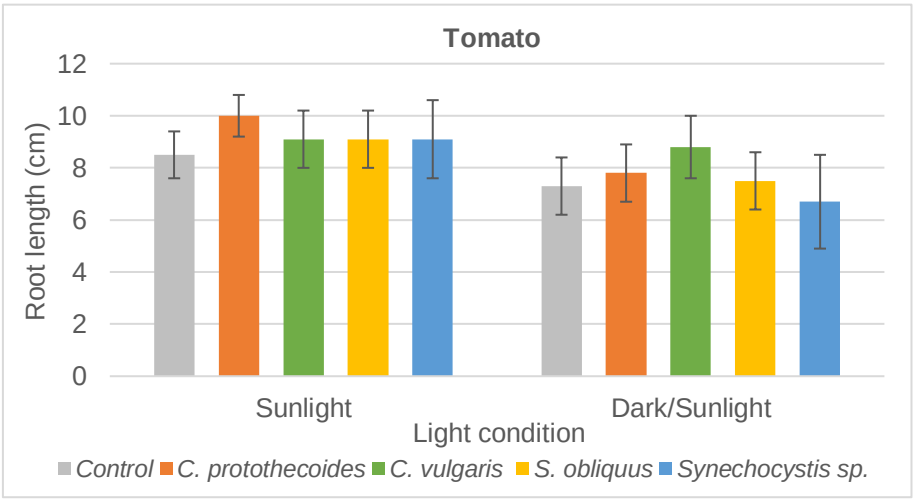


Figure 3.28 Average length of the main root for tomato seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

For tomato, the light conditions show different influences on sprout stem and root lengths, having more effect on the first. Sunlight conditions affected negatively the stem growth. Under dark/solar light conditions, *Synechocystis* sp. was not beneficial. The results were better for control and *C. protothecoides, C. vulgaris* and *S. obliquus*. However, these microalgae did not seem to have a better effect comparing to control, independently of the conditions. In terms of root growth, *C. protothecoides* had a significant effect on root length under solar light conditions, facing the control results. In dark/sunlight conditions, *C. vulgaris* had better feedbacks on root growth than control, followed by *C. protothecoides*. On the other hand, *Synechocystis* sp. was not beneficial. In recent and similar work, *C. vulgaris* also revealed positive results regarding shoot lengths of tomato germinated seeds (Bumandalai & Tserennadmid, 2019).

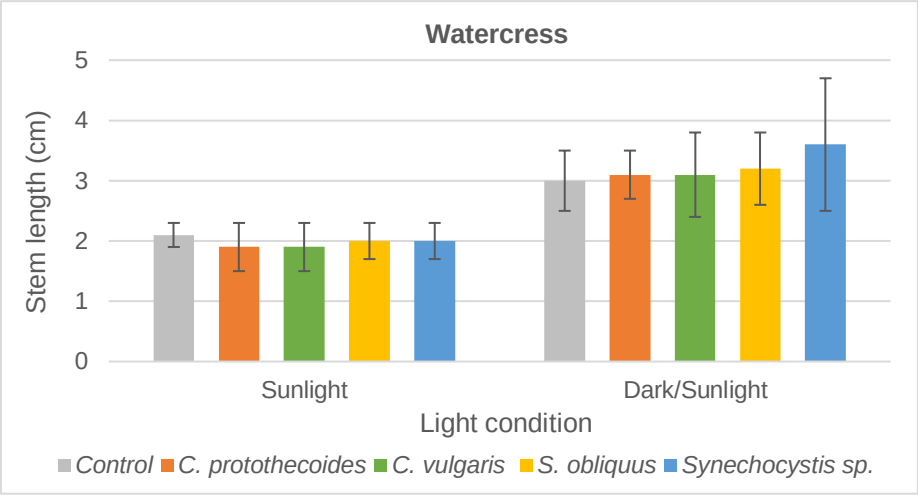


Figure 3.29 Average length of the sprout stem for watercress seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheocoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

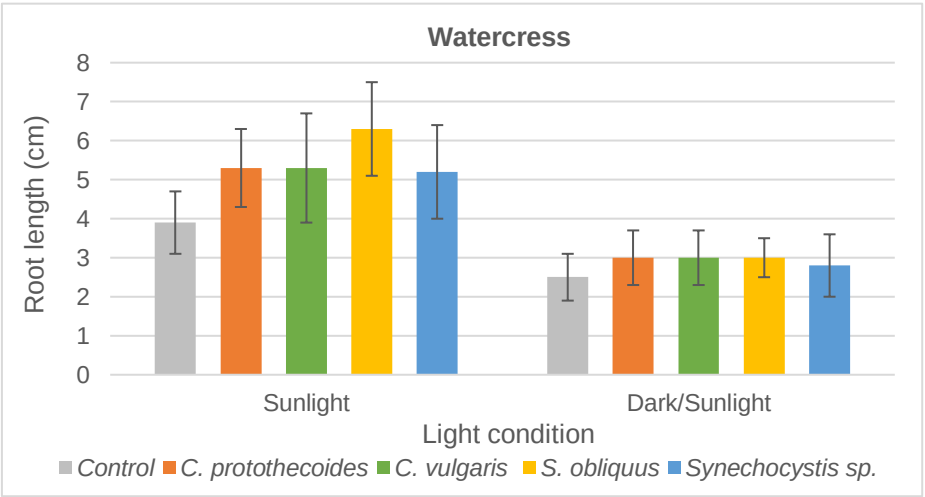


Figure 3.30 Average length of the main root for watercress seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototheocoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

For watercress, the absence of light had positive effects on stem length, in generally. No significant difference occurred between controls and the presence of microalgae biomass suspensions, except for *Synechocystis* sp., under dark/sunlight conditions.

Regarding watercress root lengths, there's a clear difference in sunlight conditions, with positive results for the presence of microalgae biomass comparing to the control, especially for *S. obliquus*. For the rest of the microalgae, a positive impact on root length was also achieved. In dark/sunlight conditions, the results root lengths were lower, and the differences between control and microalgae were not so significant.

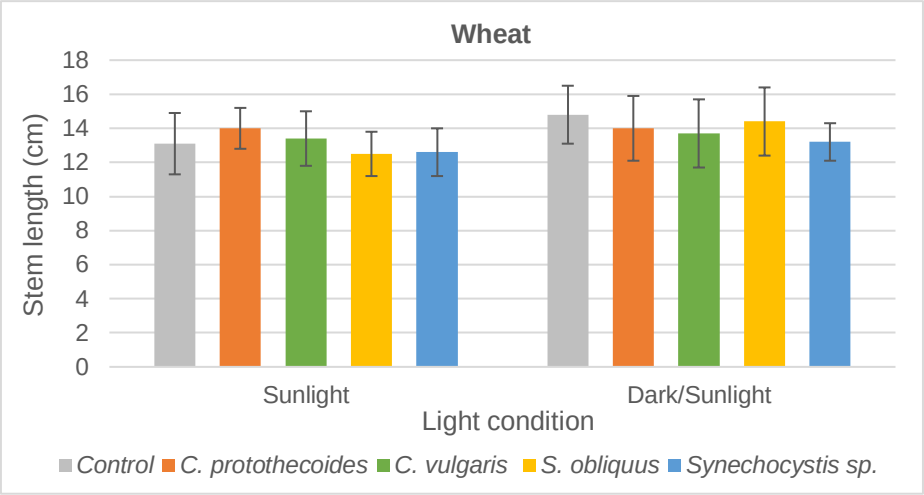


Figure 3.31 Average length of the sprout stem for wheat seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

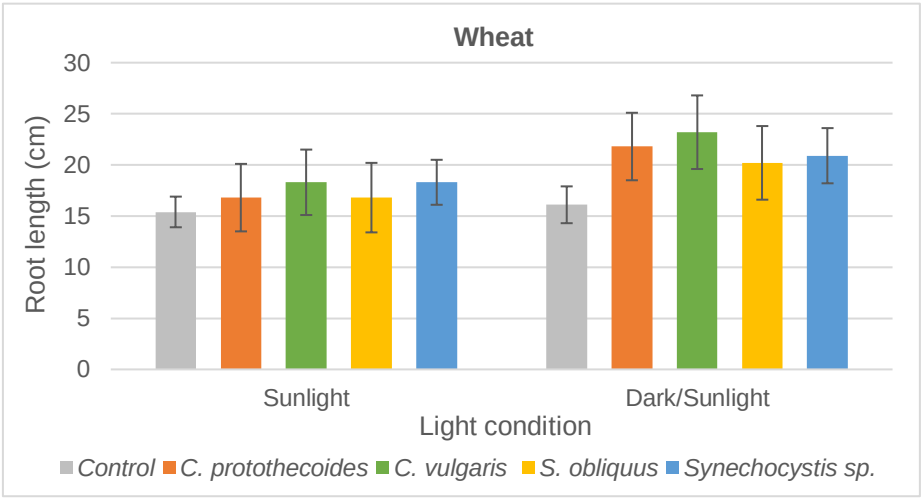


Figure 3.32 Average length of the main root for wheat seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

Regarding the sprout stem lengths in wheat, the different conditions did not greatly affect their growth, and neither did the presence of microalgae biomass suspensions. For root growth, not much difference was noticed from control to the presence of microalgae biomass, in sunlight conditions. Results for *Synechocystis* sp. were close to *C. vulgaris*, as *C. protothecoides* results were close to *S. obliquus* ones. On the other hand, the presence of algae biomass suspensions did positively affect roots lengths in dark/sunlight conditions, where *C. vulgaris* had the better results, followed by *C. protothecoides* and *Synechocystis* sp.

3.5.3. Chlorophyll *a*, chlorophyll *b* and total carotenoids contents

Overall, for chlorophyll *b* and total carotenoids contents, there were not significant differences with the presence of microalgae biomass. Significant differences were seen in chlorophyll *a* concentration. As expected, the chlorophylls had higher values in the presence of light.

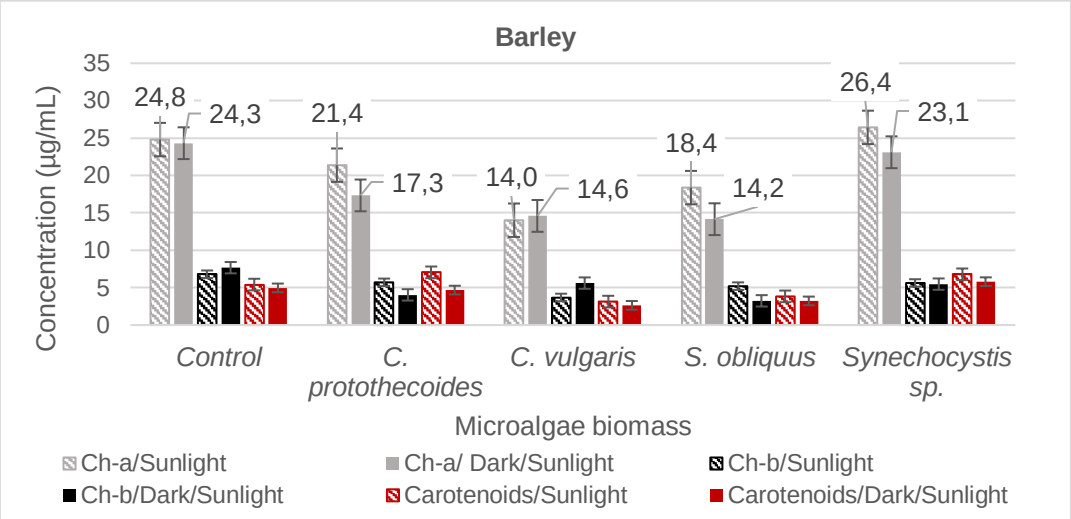


Figure 3.33 Results of chlorophyll *a*, chlorophyll *b* and total carotenoids for barley seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

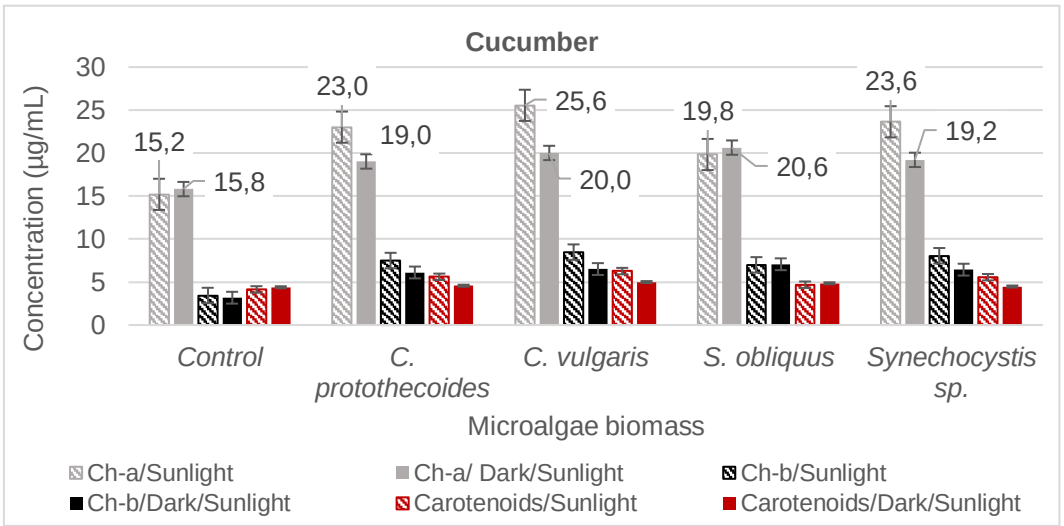


Figure 3.34 Results of chlorophyll *a*, chlorophyll *b* and total carotenoids for cucumber seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

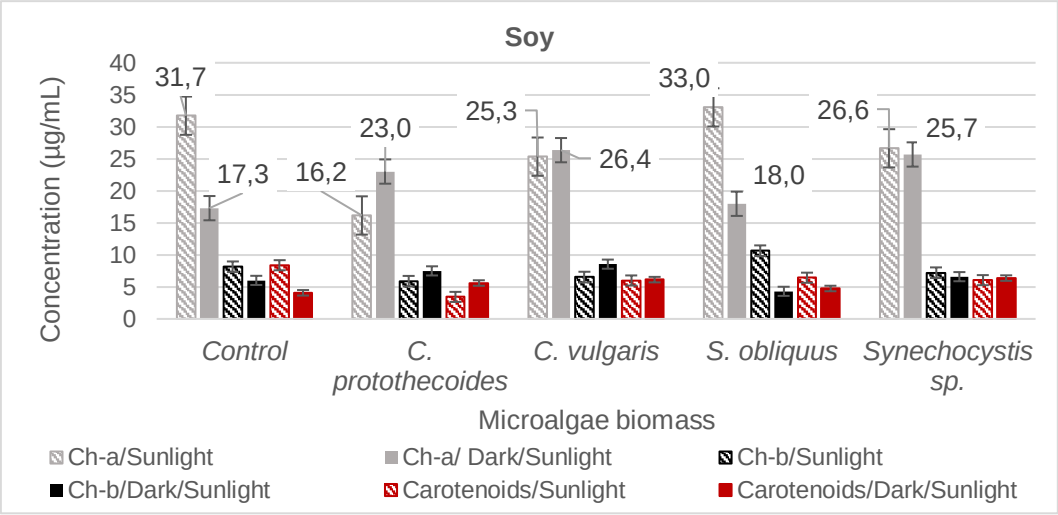


Figure 3.35 Results of chlorophyll *a*, chlorophyll *b* and total carotenoids for soy seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

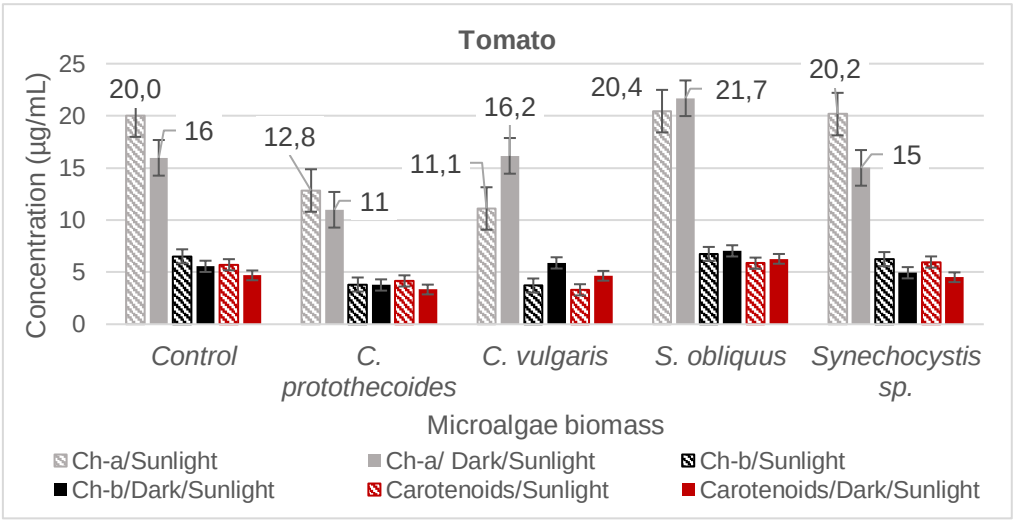


Figure 3.36 Results of chlorophyll *a*, chlorophyll *b* and total carotenoids for tomato seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

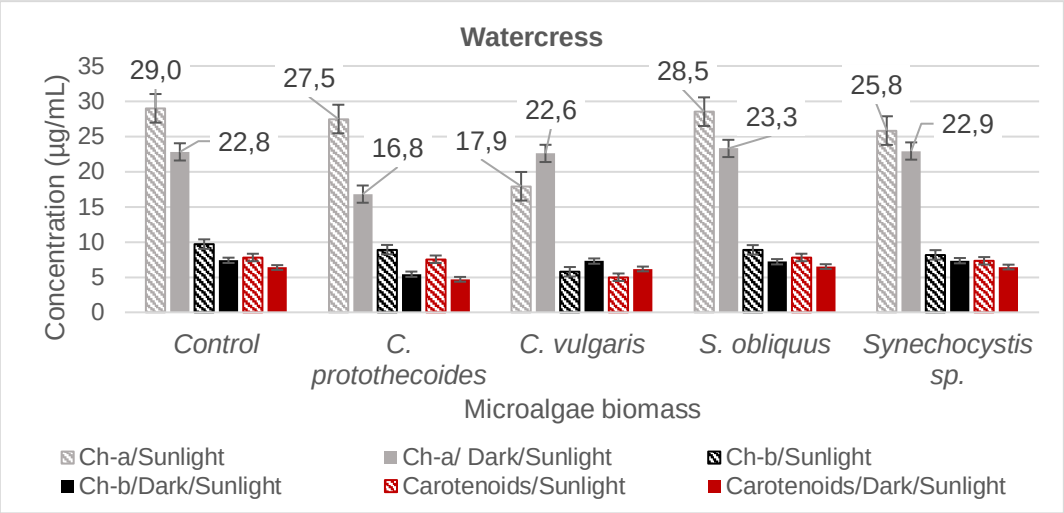


Figure 3.37 Results of chlorophyll *a*, chlorophyll *b* and total carotenoids for watercress seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototthecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

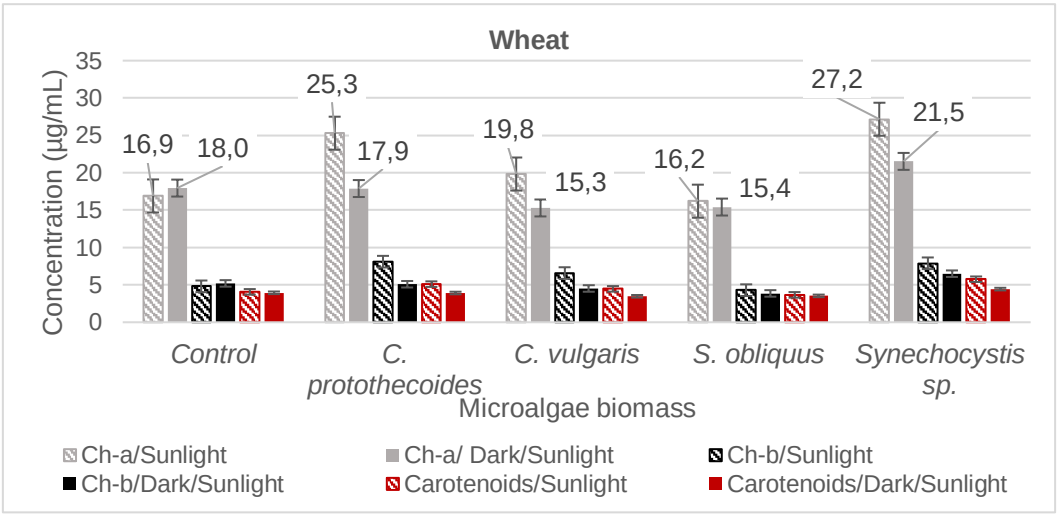


Figure 3.38 Results of chlorophyll *a*, chlorophyll *b* and total carotenoids for wheat seeds grown with distilled water (control) and microalgae biomass suspensions of *Chlorella prototthecoides*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Synechocystis* sp., under sunlight and dark/sunlight conditions.

For barley, under sunlight conditions, the presence of microalgae biomass has no association to significant higher contents of chlorophyll *a* in its leaves, and the same goes for soy and tomato. Under dark/sunlight conditions, there were positive differences for:

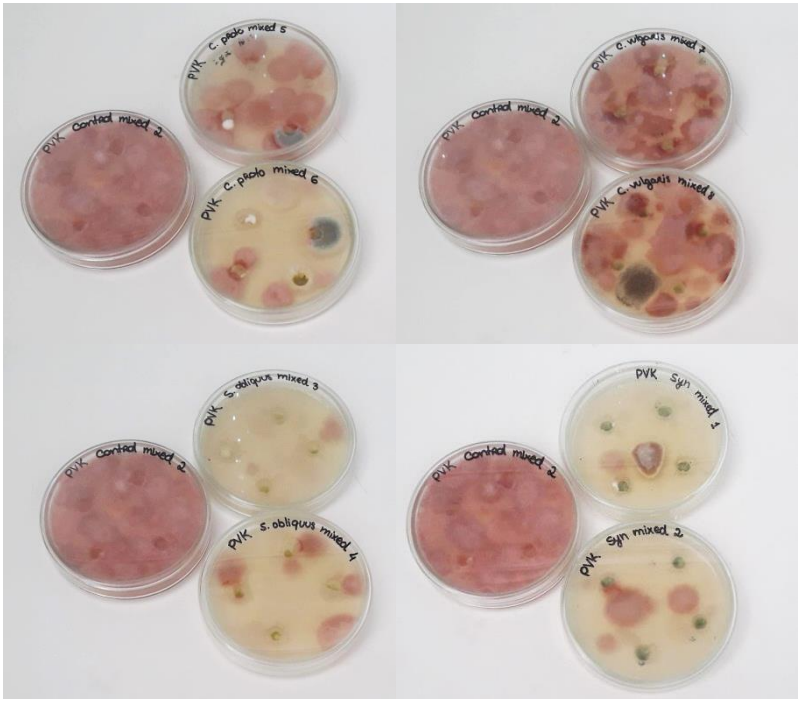
- Tomato, especially with *S. obliquus*, followed by *C. vulgaris* and *Synechocystis* sp.;
- Soy, particularly for *C. vulgaris*, followed by *Synechocystis* sp. and *C. prototthecoides*.

For cucumber and wheat, the following differences must be referred:

- Cucumber had better results for chlorophyll *a* concentration with all microalgae - under sunlight conditions for *C. vulgaris*, followed by *C. prototthecoides* and *Synechocystis* sp., and under dark/sunlight conditions, with S*. obliquus*, followed by *C. vulgaris* and *C. prototthecoides*;
- Wheat had better results under dark/sunlight conditions only with *Synechocystis* sp. and, under sunlight conditions, chlorophyll concentrations were superior for *Synechocystis* sp., followed by *C. prototthecoides* and *C. vulgaris*.

3.6. Microalgae biopesticide effect on *Fusarium oxysporum*

Results for biopesticide trails with *F. oxysporum* are represented on figure 3.39.



Looking at the pictures, *C. prototheco*ides and *C. vulgaris* did not show evidence of anti-fungal activity against *F. oxysporum*. This is especially evident for *C. vulgaris*, since it spread across all the medium and microalga suspension holes. Although it did not grow as much, the fungi still grew in microalgae suspension holes in case of *C. prototheco*ides. The best observed results are for *Synechocystis* sp. and *S. obliquus*, which were able to inhibit the fungi growth, since it did not grow on the holes containing its microalgae biomass suspensions.

4. Conclusions and future work

Nowadays, the water scarcity and pollution, as well as the overuse of chemical fertilizers and pesticides in agriculture to increase productivity efficiency, compromise human health, and secure and nutritional food access.

Microalgae are being increasingly recognized for its valuable characteristics and products in different fields. Among these, wastewater microalgae-based treatment and agricultural microalgae-based products and how microalgae such as biostimulants and biopesticides should be explored in order to reach a renewable and sustainable development of our societies.

The present dissertation aims to ally microalgae's ability for swine wastewater (SWW) treatment, a source of pollutants for both water systems and land, and plant stimulant and anti-microbial potentials from the obtained biomass. The SWW treatment ability was done by cultivation of the four microalgae *Chlorella vulgaris, Chlorella protothecoides, Scenedesmus obliquus* and *Synechocystis* sp. PCC 6803 in SWW medium, for its biological treatment. For testing biostimulant effect on germination and growth of roots and stems on germinated seeds, biomass suspensions were developed and applied in seed trials (barley, cucumber, soy, tomato watercress and wheat seeds). For biopesticide effect, trials were conducted with the fungus *Fusarium oxysporum*, applying the microalgae suspensions for fungi growth inhibition.

For SWW biological treatment, *S. obliquus* and *C. protothecoides* were the most successful, with removal efficiencies over 60%; nonetheless, all microalgae had very satisfying nutrient removal efficiencies. In order to improve the removal efficiencies/nutrient consumption and higher biomass productivity/biomass growth, it is mandatory that cultivation conditions will be optimized. It is also important to investigate new strategies to make the SWW more suitable for microalgae growth, since the raw SWW has very high concentrations of TKN, ammonia and COD which certainly inhibit microalgae growth, besides the dark coloration, to be possible to diminish the applied dilution.

Regarding the growth stimulation on seed trials, positive effects were observed with all microalgae, although *C. vulgaris* and *S. obliquus* did stand out. The germination index had increases between 0.5% and 8%. For root and stem growths, wheat and cucumber roots benefited especially with the application of microalgae biomass suspensions, increasing its length more the 4 cm. Wheat and cucumber also had the more significant increases in chlorophyll *a* concentration, increasing up to 10.4 µg/mL. To continue the study of the biostimulation effect by these microalgae, it is important to study each model crop plant and its germination and growth mechanisms, as well the microalgae biomass, to establish a correlation between both and understand which are the metabolites and bioactive compounds present in the microalgae biomass that stimulate their development. Furthermore, it is important to change to a more realistic scenario, doing soil trials with these plants and the microalgae biomass as source of nutrients.

Regarding biopesticide effect, *S. obliquus* and *Synechocystis* sp. were the ones that showed an inhibition of *F. oxysporum* growth. Nonetheless, new trials must be done in order to optimize biomass concentrations and bioactive compounds related to the inhibition.

5. References

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6. Appendix

A. Gas-chromatography parameters

Table 6.1 Description of operating parameters for GC CP-3800 (Varian, USA).

GC Method Parameters	
Flow rate (carrier gas)	3.5 mL/min
Injector temperature	250ºC
Detector temperature	280ºC
Split ratio	1:50 (first 5 min) - 1:10
Column temperature programme	200ºC (first 8 min) – 240ºC (16 min) Increase rate: 4ºC/min

B. Pikovskaya's Agar (PVK) medium

Table 6.2 Pikovskaya's agar (PVK) medium recipe (Pikovskaya, 1948).

Ingredients	**Quantity (g/L)**
Agar	15
Dextrose	10
Yeast extract	0.5
Calcium phosphate	5.0
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Ferrous sulphate	0.0001

C. Decree Law 236/98 of Portuguese Legislation: Emission Limit Values (ELVs) and pH range for wastewater discharge

Table 6.3 Emission Limit Values (ELVs) for chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) and ammonium nitrogen (NH_4^+) and allowed pH range for wastewater discharge according to Decree Law 236/98 of Portuguese Legislation.

ELVs (mg/L)				**pH**
COD	**TN**	**TP**	**NH_4^+**	
150	15	10	10	6 - 9 (max. 5-10)