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Assessment of microalgal biomass as potential feedstock for sustainable, eco-friendly biostimulants and biopesticides in plant production

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Declaração de autoria de trabalho

Assessment of microalgal biomass as a potential feedstock for sustainable, eco-friendly biostimulants and biopesticides for plant production

Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Benjamin Schmid

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Resumo

O uso excessivo e contínuo de agroquímicos, fertilizantes sintéticos e pesticidas, levou à poluição antropogénica de nutrientes, tendo causado um grande número de degradações ambientais. Além disso, os agroquímicos são poluentes ambientais que podem causar graves problemas de saúde humana. A expansão global de "zonas mortas" nos oceanos, nas quais os baixos níveis de oxigénio ameaçam a vida marinha, é apenas um dos muitos sinais de alerta de que medidas contrárias são necessárias com urgência. Os bioestimulantes e biopesticidas à base de microalgas representam uma alternativa promissora para alcançar uma maior sustentabilidade na agricultura moderna. A biomassa de microalgas contém numerosos aminoácidos e fitohormonas que promovem o crescimento das plantas, podendo aumentar a produtividade das culturas, estimulando o crescimento da raiz e da canópia. Além disso, sabe-se que as microalgas inibem o crescimento de vários agentes fitopatogénicos, devido às suas propriedades antimicrobianas, podendo ser uma alternativa sustentável aos pesticidas sintéticos no setor agrícola. Neste estudo, focámo-nos na aplicação de extratos aquosos de microalgas como fungicidas contra *Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea* e *Alternaria alternata*. Esses fungos são agentes causais de doenças frequentes na agricultura, ameaçando a segurança alimentar global. As informações disponíveis, relacionadas com a utilização de microalgas na proteção de plantas e bioestimulação, são ainda escassas, embora as microalgas possam desempenhar um papel importante no desenvolvimento da agricultura sustentável. Secundariamente, sabendo-se que o uso agrícola de compostos de resíduos orgânicos apresenta vários benefícios relacionados com a fertilidade do solo e a resistência das plantas a algumas doenças, avaliou-se o efeito daquelas microalgas na compostagem de uma mistura de resíduos agrícolas comuns na região, devido à possibilidade de as microalgas poderem apresentar alguma influência na atividade microbiana responsável pela compostagem.

O principal objetivo do presente estudo foi determinar as propriedades bioestimulantes e biofungicidas de microalgas e a sua capacidade de melhorar o processo de compostagem de resíduos orgânicos para um objetivo final de tornar a agricultura mais sustentável através do uso destes microrganismos fotossintéticos, nomeadamente *Scenedesmus* sp., *Chlorella vulgaris*, *Nannochloropsis* sp., *Arthrospira* (Spirulina) sp. e *Phaeodactylum tricornutum*. Para atingir o objetivo supracitado, os objetivos específicos desta dissertação são: (1) avaliar

o controle de doenças de plantas com extratos aquosos de microalgas *in vitro*, e (2) avaliar e caracterizar processos de compostagem enriquecidos com microalgas.

O Capítulo II descreve a aplicação promissora de extratos aquosos de *Nannochloropsis* sp., *Phaeodactylum tricornutum, Scenedesmus obliquus* e Spirulina sp. *in vitro* para o desenvolvimento de antifúngicos de origem algal. A supressão do crescimento por estes extratos foi observada nos fungos fitopatogénicos *Sclerotium rolfsii*, *Rhizoctonia solani* e *Botrytis cinerea*. De facto, as espécies de microalgas são uma fonte promissora de agentes antifúngicos não prejudiciais ao meio ambiente que podem reduzir o uso de fungicidas sintéticos e limitar o impacto ecológico do setor agrícola. Uma vez que a maioria dos estudos se foca nas propriedades antifúngicas de cianobactérias procarióticas, o presente estudo visou preencher a lacuna de conhecimento sobre o uso de microalgas eucarióticas como agentes antifúngicos. Para evitar métodos complexos de extração e etapas de purificação, que aumentam os custos e restringem as aplicações em larga escala de fungicidas à base de algas, foi usada uma extração simples à base de água. Assim, foram investigadas as propriedades de extratos aquosos de microalgas eucarióticas (*Nannochloropsis* sp., *Phaeodactylum tricornutum, Scenedesmus obliquus* e *Chlorella vulgaris*) e procarióticas (*Spirulina* sp.) *in vitro* quanto à sua atividade inibidora em relação aos fungos fitopatogénicos *Sclerotium rolfsii* e *Alternaria alternata*. A análise estatística revelou que *Scenedesmus obliquus* apresentou a maior atividade antifúngica de todas as estirpes de microalgas contra *Sclerotium rolfsii,* com inibições de crescimento de até 32,01 ± 4,82%. *Nannochloropsis* sp. mitigou *Sclerotium rolfsii* em até 13,96 ± 5,26%, enquanto *Phaeodactylum tricornutum* suprimiu o crescimento de *Sclerotium rolfsii* e *Rhizoctonia solani* em até 18,35 ± 3,45% (p <0,05). Além disso, *Phaeodactylum tricornutum* e *Scenedesmus obliquus* inibiram o crescimento de *Botrytis cinerea* em até 11,47 ± 2,06% (p <0,05). Assim, esses resultados sugerem que microalgas com atividade fungicida podem contribuir para uma agricultura mais sustentável ao inibir o crescimento de fitopatógenos fúngicos.

No Capítulo III, encontra-se descrita a utilização de microalgas no processo de compostagem. Mais especificamente, este estudo investigou a suplementação de uma mistura de resíduos orgânicos com biomassa de microalgas secas de *Nannochloropsis* sp., *Phaeodactylum tricornutum, Scenedesmus obliquus* e *Chlorella vulgaris*. Até onde sabemos, este é o primeiro relatório que analisou o enriquecimento de materiais de compostagem frescos com pó de microalga seca. Uma vez que as microalgas produzem vários aminoácidos e fitohormonas

que promovem o crescimento das plantas, seria de esperar que elas poderiam melhorar ainda mais as características dos compostos estimulantes das plantas, como a liberação de nutrientes que promovem o crescimento. As taxas de decomposição dependem das atividades metabólicas das populações microbianas que dependem, por sua vez, da disponibilidade de vários micro- e macronutrientes. Portanto, a co-compostagem de biomassa de microalgas rica em nutrientes poderá moldar comunidades microbianas e melhorar a qualidade do composto final com base na riqueza em nutrientes, como fósforo, azoto e potássio. Devido ao seu potencial para transformar e reciclar resíduos de diferentes origens em matéria orgânica, a compostagem terá um papel fundamental no caminho para uma sociedade mais sustentável. Em termos gerais, não foram observadas grandes variações nos parâmetros de pH, condutividade elétrica, matéria orgânica, matéria mineral, temperatura, volume e fitotoxicidade entre todas as pilhas de compostagem modificadas com microalgas, quando comparadas com o composto controlo (fase final). Portanto, o composto fortificado com microalgas poderá ser considerado uma alternativa sustentável promissora para aumentar ainda mais a produtividade das culturas no setor agrícola global, mas que requer ainda verificação experimental em ensaios de campo ou estufa.

Palavras chave: microalgas, agricultura sustentável, bioestimulantes, biopesticidas, fungos patogénicos das plantas

Abstract

Continuous overuse of synthetic fertilizers and pesticides (agrochemicals) has led to excessive anthropogenic nutrient pollution and caused a vast number of environmental degradations. The global expansion of "dead zones" in the world's oceans, where oxygen-depleted water bodies threaten marine life, is just one of many warning signs indicating that counteractive measures are urgently needed. Moreover, long-term exposure to agrochemicals can cause major human health issues. Microalgae-based biostimulants and biopesticides represent a promising alternative to reduce those negative effects and achieve a higher sustainable value in modern agriculture. Microalgal biomass contains numerous plant growth-promoting amino acids and phytohormones that increase crop productivity by stimulating root and shoot growth. Compost can be seen as effective carrier for these bioactive compounds and may be applied as enriching soil amendment. Moreover, microalgae were found to inhibit the growth of several pathogens due to their antimicrobial properties. Hence, they can be seen as sustainable alternative for synthetic fertilizers and pesticides in the agricultural and horticultural sector. In this study we focused on the application of aqueous microalgal extracts as fungicides against the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea* and *Alternaria alternata*. Those fungi are dominant causal agents for common diseases in agriculture and considered as major threat for global food security. Even though microalgae could play a major role in sustainable agriculture development, available literature related to microalgal crop protection and biostimulation is still scarce.

Chapter II describes the promising antifungal application of aqueous extracts from *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Scenedesmus obliquus* and *Spirulina* sp. *in vitro*. Growth suppression was observed against the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani* and *Botrytis cinerea*. In Chapter III, no major parameter variations in pH, electrical conductivity, organic matter, mineral matter, temperature, volume and phytotoxicity were observed among all microalgae-amended composting piles, when compared with the control compost (final phase). Future studies will evaluate the biostimulant properties of these composts *in vivo*.

Keywords: microalgae, sustainable agriculture, biostimulants, biopesticide, fungal plant pathogens

Table of Contents

Index of figures

Chapter 1: State of the art

Chapter 2: Antifungal properties of aqueous microalgal extracts

Chapter 3: Evaluation of microalgae-amended organic compost

Index of tables

Chapter 1: State of the art

Chapter 3: Evaluation of microalgae-amended organic compost

I.) Chapter 1: State of the art

I.I: Mitigation potential of aqueous microalgal extracts on phytopathogenic fungi in vitro

1.) Microalgae - general description

Microalgae constitute a broad group of ubiquitous photosynthetic microorganisms comprising eukaryotic, microscopic algae *sensu stricto*, and Gram-negative prokaryotic cyanobacteria (García et al., 2017). The filamentous cyanobacteria are further separated in heterocystous forms with specialized cells for N fixation, and non-heterocystous forms (Renuka et al., 2018). About 50,000 microalgal species, 4,748 prokaryotic and 46,296 eukaryotic microalgae, have been described in the taxonomic and nomenclatural database of AlgaeBase (Guiry & Guiry, 2020). Algae in general can be seen as a term of convenience comprising photosynthetic organisms or cells that possess (photosynthetic or nonphotosynthetic) plastids (Williams & Keeling, 2003). Keeling (2004) studied the phylogenetic relationships among eukaryotic organisms and found out that the global phylogenetic tree of eukaryotes apparently consists of five "supergroups" (Unikonts, Rhizaria, Excavates, Chromalveolates and Plantae). Algae, excluding cyanobacteria, are present in four of these five supergroups. In 2019, Keeling & Burki published a revised version of the eukaryotic tree of life with primary changes due to the addition of more taxa and the shuffling of lineages (coalescence). The most species-rich microalgal classes are Chlorophyceae (green algae), Cyanophyceae (blue- green algae), Chrysophyceae (golden-brown algae) and Bacillariophyceae (diatoms; García et al., 2017). Microalgae are cosmopolitan in their biogeographical distributions and can be found throughout the biosphere in aquatic, terrestrial and subaerial environments (Metting, 1996). They inhabit the water column, the sediment as well as biofilms in freshwater and marine systems (Dalsgaard, 2003; Mantzorou et al., 2019). Furthermore, brackish environments (e.g., lagoons) and wastewaters, such as municipal sewage or industrial effluents, can serve as potential habitats (Sousa et al., 2014). This is based on the wide range of temperature, pH and salinity values microalgae can withstand (Khan et al., 2018). Moreover, microalgae play an important role in hydrothermal vent communities (Tarasov et al., 2005) and survive in other extreme habitats like deserts, polar crusts or arctic ecosystems (Pushkareva et al. 2016). They appear as unicellular, pluricellular or colonial organisms and, depending on the species, live as individual cells or in symbiosis with other organisms (Carlos et al., 2000). Their growth relies

on light energy, water, CO_2 and mainly nitrogen and phosphorus as essential nutrients (Bhola et al., 2014). As primary producers at the base of marine and freshwater trophic chains, microalgae act as central player in the Earth's carbon cycle, sequestering 40% of the global emitted CO_2 (Scott et al. 2010). Together with bacteria, microalgae build the foundation of aquatic food webs and provide sustenance for primary consumers (Sathasivam et al., 2019). Besides dinoflagellates and coccolithophores, cyanobacteria, diatoms and green algae represent three out of five groups of oceanic phytoplanktonic organisms (Lindsey and Scott, 2010), and therefore are responsible for roughly half of the biosphere's net primary production (Behrenfeld et al., 2006).

Throughout the process of photosynthesis, CO_2 is fixed into sugars using water and light as a source for energy and electrons. Organic carbon (CO_2) can then be biochemically converted into proteins, carbohydrates, lipids and nucleic acids. Because of their submersion in aqueous environments, nutrients, water and CO_2 are easier to access compared to land-based plants, which are less effective in converting solar energy into biomass (Gouveia, 2011; Raja et al., 2014). Although most microalgal species grow photoautotrophically, some species can shift from photoauto- to heterotrophy, while others can grow mixotrophically (Raja et al., 2014).

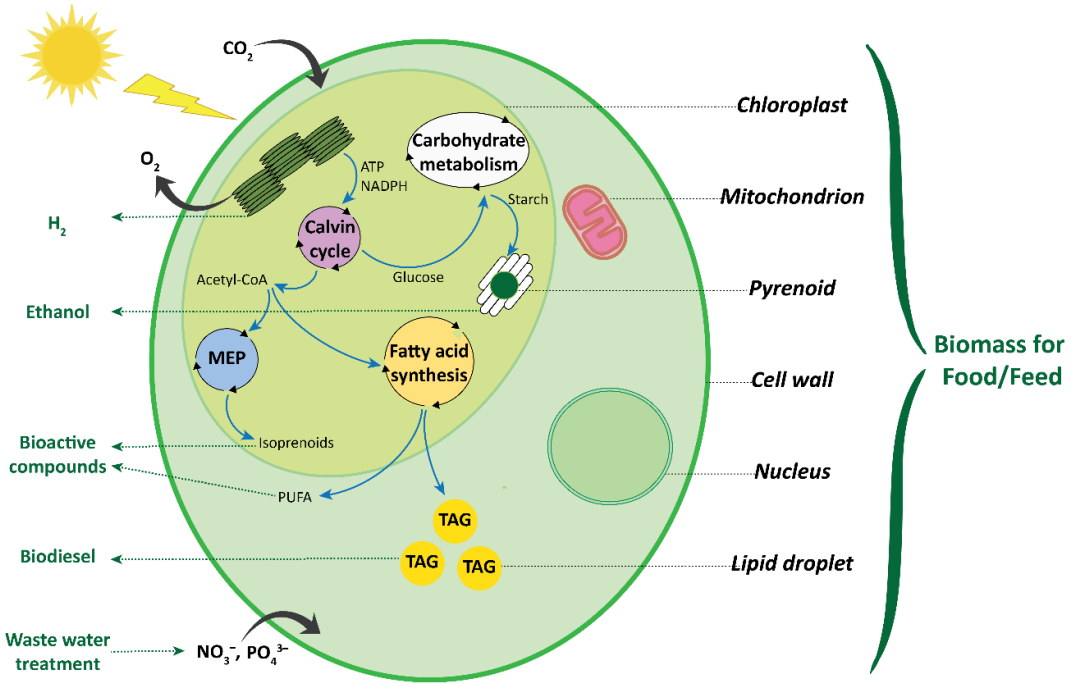

Figure 1.1 - Cell structure and cellular compartments of eukaryotic microalgae. Main metabolic pathways and key biotechnological applications (Raposo, 2017)

Biotechnological industries related to food and feed additives, pharmaceuticals, nutraceuticals, biofuels, bioplastics and bioremediation services see immense potential in microalgal production (Alam et al., 2020). Furthermore, microalgae have been gaining significance in modern, sustainable agriculture due to their multi-functional applications such as feedstocks for biofertilizer production or as potential biocontrol agents (Renuka et al., 2018). They can be seen as a promising alternative for some chemical fertilizers and pesticides, which are finite resources and have negative impacts on the sustainability of agroecosystems (Mantzorou et al., 2019). Because of the broad biochemical diversity of the compounds synthesized by different metabolic pathways among strains (Gimpel et al., 2015), microalgae are promising bioresources for the mass production of proteins, sugars, lipids or polymers (European Commission, 2012). Moreover, they can be applied for the coproduction of high-value biomolecules such as carotenoids, polyunsaturated fatty acids, β-glucans and phycobiliproteins (Enzing et al., 2014).

Nutrient limitations or the exposure to detrimental physical factors such as high light, high temperature or high salinity can promote the overproduction of high-value target metabolites as, for example, *n*-3 polyunsaturated fatty acids and carotenoids (Minhas et al., 2016). On the other hand, manipulation of stress factors can cause oxidative damage to cells, affect microalgal growth and further decrease biomass yields (Albrecht et al., 2016). Two-stage cultivation strategies, supplementation of growth-promoting agents or the development of highly adapted strains are possible solutions to overcome the negative effects of stress-based strategies for inducing the accumulation of target metabolites (Sun et al., 2018).

In addition, microalgal biofilms, which seem to have advantages compared to conventional cultivation systems regarding biomass loss, biomass recovery and water management have become an interesting trend within the microalgal biotechnology field (Polizzi et al., 2017; Toninelli et al., 2016). Biofilm cultivation can result in a higher number of cells per unit volume compared to suspension systems and therefore increase biomass yields (Ozkan et al., 2012). Nevertheless, different cultivation techniques suffer from different bottlenecks, such as high power and water consumptions resulting in high operational costs (OPEX), high capital expenditure (CAPEX) for the purchase and installation of equipment (e.g., photobioreactors and pumps) and problems with biomass production and harvesting (Mantzorou et al., 2019).

The primary products being commercialized on the microalgal market include *β*-carotene, phycobiliproteins, *n*-3 and *n*-6 polyunsaturated fatty acids, sterols, vitamins and biologically active molecules with anticancer, antiviral, antimicrobial and antibacterial properties. (Alam et al., 2020). The global microalgal market was valued at USD 54.64 million in 2018 and estimated to reach USD 76.37 million by 2025 (MarketWatch - stock market news, 2020). In 2019, the European algal sector had an annual turnover of 1.5 billion for direct activities and 240 million for indirect activities, including research (European Blue Economy Report, 2019). Even tough microalgae reveal great potential regarding the realization of a circular, green economy (Wolkers et al., 2011), biomass production needs to become economically viable (Mantzorou et al., 2018). Because of the cost-benefit relationships, industrial microalgal production is often restricted to high-value applications mainly related to aquaculture and human consumption (Borowitzka et al., 2017). The production of biomass in industrial-scale facilities is still in its infancy and there is great interest in finding new ways to reduce costs and boost productivity (Mantzorou et al., 2019). Wastewater, food and feed waste as a source of nutrients is a promising, sustainable way to reduce production costs (Acién et al., 2017). Despite their applications and advantages, only 20 kt of microalgae are annually produced worldwide at a production cost of $20,000/t (Benemann, 2013). Two-thirds of the global microalgae biomass is produced in China, the biggest player regarding microalgae production worldwide (Chen et al., 2015). Algae-related regulations on quality control, specific safety standards, certification requirements and nutrient evaluation are currently not harmonized for European and Asian products; China, for example, allows higher nitrate levels than Europe for the production of *Chlorella* sp. (European Commission, 2018).

2.) Phytopathogenic fungi - general description

Among eukaryotes, fungi are one of the most diverse group of organisms with a conservatively estimated number of 1.5 million species (Hawkswirth, 1991). Molecular phylogenetic comparisons have revealed that the fungal diversity could comprise up to 3.8 million species (Hawksworth & Lücking, 2017) and traditionally applied phenotypic characters not necessarily indicate common origins (Crous et al., 2015). Intraspecific morphological, physiological and metabolic variation complicates the accurate application of nomenclatural rules (Raja et al., 2017). Hence, only around 120,000 species have been

identified, described and accepted by taxonomists as operational taxonomic units so far (Hawksworth et al., 2017).

Kubicek et al. (2014) estimated that around 10% of all described fungal species can cause diseases in plants. Hence, fungi represent one of the most diverse group of phytopathogenic organisms which can cause ecologically and economically relevant threats (Doehlemann et al., 2008). Moreover, plant fungal pathogens are the dominant causal agents for disease in agricultural and horticultural facilities (Agrios et al., 2009). Overall, fungi can be classified as worldwide threat to the food security due to a constantly increasing number of infectious diseases (Hyde et al., 2018). The phyla Ascomycota and Basidiomycota contain the majority of phytopathogenic fungal species (Henson et al., 1999). The two largest groups of plant pathogens, Puccinomycetes (rusts) and Ustilaginomycotina (smuts), belong to the Basidiomycetes (Morrow & Fraser, 2009).

Throughout evolution, phytopathogenic fungal species have developed a vast number of strategies to colonize plants and acquire essential nutrients from their hosts (Rodriguez-Moreno et al., 2017). Fungal pathogens can be divided into biotrophs (obtain nutrients only from living host cells), necrotrophs (obtain nutrients from dead tissues) and hemibiotrophs (switching from biotroph to necrotroph and vice-versa) (Doehlemann et al., 2018). Biotrophic pathogens actively prevent the programmed cell death (PCD) of host cells, an intracellular suicide program to control pathogen invasion (Mukthar et al., 2016). Conversely, necrotrophs produce toxins which actively trigger PCD, cause necrosis or even kill the organism (Mengiste, 2012). The infection process of hemibiotrophs start with a biotrophic followed by a necrotrophic phase in which the pathogen kills host cells for a successful completion of its lifecycle (Mukhtar et al., 2016). Fungal phytopathogens follow a specific cycle of infection (Figure 2): fungal spores, which are dispersed through multiple vectors, attach to host plants and start a process called host recognition (Meng et al., 2009).

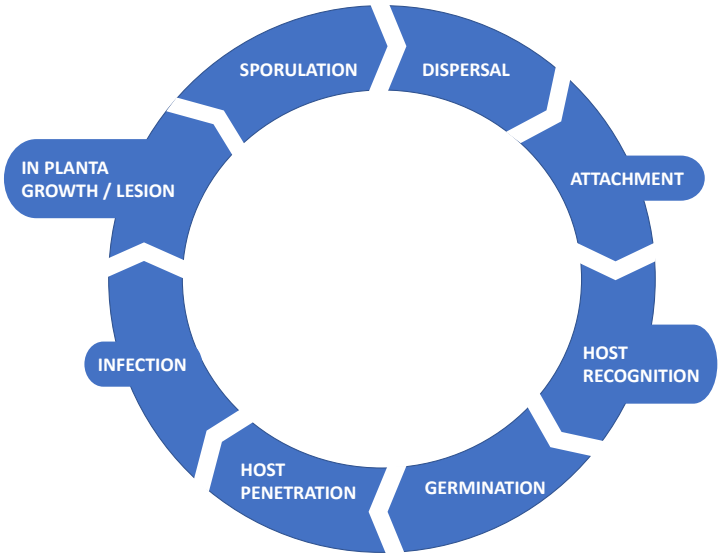

Figure 1.2 - Infection and disease cycle of fungal pathogens (adapted from Meng et al., 2009)

After the first contact with a host plant, fungal pathogens trigger multiple signal pathways comprising ion fluxes, reactive oxygen species (ROS), protein phosphorylation and other signalling transduction processes (Shen et al., 2017). After host recognition, the infection cycle continues with spore germination, defined as formation of a filamentous germ tube (Figure 3; Vega et al., 2012).

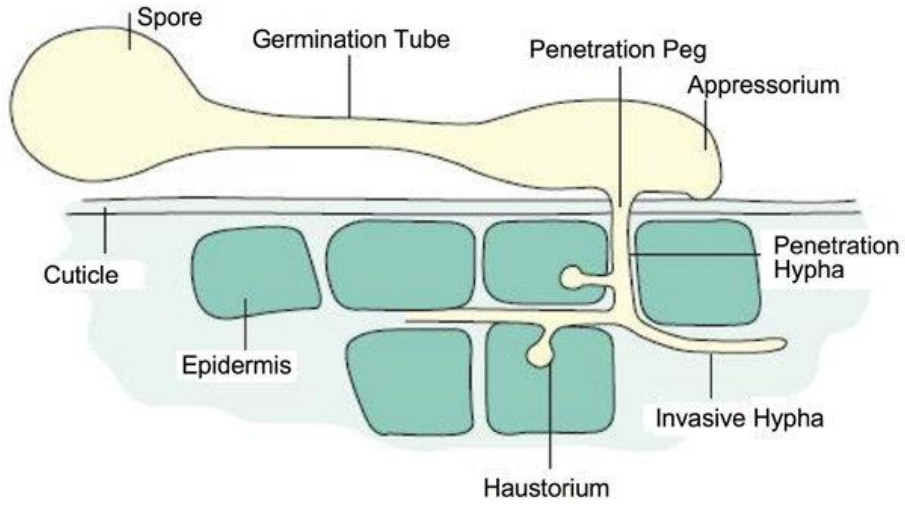

Figure 1.3 - Invasion of a phytopathogenic fungus into a host plant; infection process (Meng et al., 2009)

Subsequently, many pathogenic fungi differentiate dome-shaped appressoria, specialized infection cells which show a complex morphology and physiology (Kleemann et al., 2012).

Appressoria are hyphal organs which generate turgor pressure to rupture cell walls by physical force, followed by a penetration of the epidermis (Ryder et al., 2015). This can be accompanied by a release of cell wall degrading enzymes (CWDE's) (Ryder and Talbot, 2015) and a secretion of effector cells from appressorial penetration pores (Kleemann et al., 2012). CWDE's comprise carbohydrate-active enzymes (CAZymes) such as lyases, glycoside hydrolases or carbohydrate esterases (Rodriguez-Moreno et al., 2017). Those enzymes catalyze the depolymerization of polysaccharide cell wall components in plant tissues prior to the invasion (Kubicek et al., 2014). Some pathogens penetrate the plant cuticle directly through the filamentous germ tube and without differentiating appressoria (Hajek et al., 2002). After successful penetration, invasive hyphae break through the plant tissue, and form specialized cell structures (e.g., haustorium) to acquire nutrients for growth (Meng et al., 2009).

Various defense-associated systems evolved among plants to protect themselves against invasions of fungal pathogens (Shen et al., 2018). Important aspects of those systems are the reinforcement of the cell wall, the induction of pathogenesis-related protein biosynthesis and the accumulation of antifungal secondary metabolites (Aoun, 2017). Cell wall reinforcement is accomplished by deposition of callose or lignin, production of antimicrobial compounds and changes in the biosynthesis of various phytohormones (Macho & Zipfel, 2014). Moreover, pathogen perception enhances the accumulation of reactive oxygen species (ROS), the activation of mitogen-activated protein kinase cascades and ion fluxes (Kubicek et al., 2014). Conversely, pathogenic fungi developed specialized tactics to suppress defense responses of host plants (Kubicek et al., 2016). Those tactics mainly rely on secreted effector molecules which modulate host physiology and suppress plant defense responses (Selin et al., 2016). Fungal effectors appear typically as cysteine-rich proteins, small RNAs (sRNAs) or secondary metabolites (Stergiopoulos & Wit, 2009; Wang et al., 2015). sRNAs regulate biotic and abiotic stress responses in plants as a part of a fast-responding environmental adaptation (Kruszka et al., 2012). They can either be transferred into plant cells or operate in the transition zone between the host and fungal hyphae (Presti et al., 2015).

Fungal pathogens modify or evolve novel effectors to win this genetic arms race (Selin et al., 2016). This dynamic development of resistances results in a continuous co-evolution of host and pathogen, molding the genomes of both "partners" (Presti et al., 2015).

3.) Pesticidal and biocontrol potential of microalgae (sustainable agriculture)

By definition, pesticides are chemical or biological agents which prevent, destroy or control plant pests and diseases (Matthews, 2008). They are classified by their target organisms and include a broad range of fungicides, herbicides, insecticides, nematicides, molluscicides, piscicides, avicides, rodenticides, bactericides, etc. (Tadeo, 2008). The global market of pesticides is controlled by a powerful industry which produces approximately 2 million tons of pesticides annually worldwide and was expected to rise up to 3.5 million tons by 2020 (Sharma et al., 2019). Scientific journals mainly published literature about the negative effects of pesticides, while beneficial attributes are largely ignored (Cooper & Dobson, 2007). The major beneficial effects of pesticides in agriculture are the control of pests and diseases to further improve crop yields and crop quality (Zhang et al., 2018). Liu et al. (2002) found out that almost one-third of the global agricultural production is based on the utilization of pesticides. For example, U.S. exports of soybean, wheat and cotton could decline by 27% without using pesticides. Furthermore, 80% of all fruit and vegetable crops of the United States rely on the application of fungicides (Zhang et al., 2018). Pimentel (2009) estimated that crops can yield up to four dollars for every dollar that is spent in pesticides. The use of chemical pesticides in agriculture significantly accounts for an increase in food production of more than double during the last century (Carvalho et al., 2017). If the human population continues to grow as fast as expected (70 million per year; up to 9.2 billion by 2050), the demand for food will increase up to 70% by midcentury (Popp et al., 2012). Tilman et al. (2011) forecasts an 100-110% increase of the global crop demand between 2005 and 2050 according to the FAOSTAT database (Food and Agriculture Organization of the United Nations; http://faostat.fao.org/). The rapid rise in global food demand will require a more intensive use of agrochemicals in order to further increase crop yields (Sharma et al., 2019). However, excessive use of agrochemicals causes environmental contaminations of terrestrial and aquatic ecosystems (van der Werf, 1996). Therefore, agriculture faces the dilemma of achieving higher crop yields while reducing the environmental impact in future decades (Carvalho et al., 2017). Searching for novel biopesticides has been encouraged in recent years to reduce the utilization of agrochemicals (Costa et al., 2019). Biopesticides derive from biological sources and are classified into microbial, biochemical and plant-incorporated-protectants (PIPs) (Olson et al., 2015). Compared to conventional synthetic pesticides, biopesticides are environmentally friendly and have nontoxic modes of action

(Senthil-Nathan, 2015). Numerous microalgae, particularly cyanobacteria, are considered as promising alternative for chemical pesticides due to their pesticidal, biocontrol and insecticidal properties, mainly due to the accumulation of bioactive metabolites (Renuka et al., 2018). Some of them are shown in Table 1.1.

Table 1.1 - Examples of microalgae which exhibit pesticidal activity through metabolizing multiple bioactive compounds (Costa et al., 2019)

Microalga	Activity	Metabolites	References
Cyanobacteria			
***Anabaena* sp.**	Larvicidal	Anatoxin-a	Méjean et al. (2014); Berry et al. (2008)
Anabaena laxa	Antimicrobial	Laxaphycin B, Laxaphycin C	Hernandez-Carlos et al. (2011); Frankmölle et al. (1992)
Arthrospira platensis	Antifungal	Phenolic compounds	Hussein et al. (2009)
***Calothrix* sp.**	Insecticide	Eremophilone	Höckelmann et al. (2009)
***Fischerella* sp.**	Antimicrobial	Ambiguine isonitrile	Raveh et al. (2007)
Fischerella ambigua	Antimicrobial, molluscicidal	Ambigol A, Ambigol B	Falch et al. (1993)
***Lyngbya* spp.**	Larvicidal	Pahayokolides	Raveh et al. (2007)
Microcystis aeruginosa	Herbicidal	Microcystin	Berry et al. (2008)
***Nostoc* sp.**	Herbicidal	Cryptophycin	Biondi et al. (2004)
Eukaryotic microalgae			
Chlorella vulgaris	Antifungal	Phenolic compounds	Hussein et al. (2009)
Haematococcus pluvialis	Antimicrobial	Propanoic acid, butanoic acid	Rodríguez-Meizoso et al. (2010)

Fungicides account for 17.5% of the total pesticide production worldwide (Sharma et al., 2019). They are defined as biocidal chemical compounds or biological organisms and mainly used by the agricultural industry to control parasitic fungi (MacLean et al., 2018). Moreover, they are applied to treat fungal infections in human and veterinarian medicine (Heusinkveld et al., 2013). Synthetic fungicides might have, however, a significant impact on the environment if residues persist in the soil or enter the water cycle through agricultural runoff (Gavrilescu et al., 2005). Extensive use of copper-based fungicides, for example, results in an accumulation of copper in the soil, harming its long-term fertility (Komárek et al., 2010). Because of the harmful side effects, the risk of generating resistant strains and high costs of synthetic fungicides, scientists worldwide are researching on novel biocides (Khan et al., 2018). Sustainable agriculture requires the urgent establishment of an equilibrium between the control of fungal pathogens and the protection of the environment (Wightwick et al., 2010).

Microalgae are a promising alternative to replace chemical fungicides due to their diversity in bioactive, antifungal compounds synthesized in multiple metabolic pathways (Skulberg, 2000). Many bioactive compounds have antagonistic properties against a wide range of pathogenic fungi through various strategies such as inhibiting protein synthesis and disrupting cytoplasmic membranes (Costa et al., 2019; Swain et al., 2017). Marine microalgae show a greater potential as a source of novel antifungal agents than freshwater species (Cannell et al., 1988). Mudimo et al. (2014) published that the capability to metabolize antifungal compounds probably evolved convergently. Table 2 shows multiple studies which investigated the antifungal capacities of microalgae.

Table 1.2 - Antifungal capacities of numerous microalgae

Microalga	Target organism	References
Green & red microalgae		
Chlorella ellipsoidea	*Candida kefyr*, *Aspergillus fumigatus*, *Aspergillus niger*	Ghasemi et al., (2007)
Chlorella pyrenoidosa	*Aspergillus niger*, *Aspergillus flavus*, *Penicillium herquei*, *Fusarium moniliforme*, *Alternaria brassicae*, *Helminthosporium* sp., *Saccharomyces cerevisiae*, *Candida albicans*	Abedin & Taha, (2008)

Chlorella vulgaris	*Candida kefyr, Aspergillus fumigatus, Aspergillus niger, Cercospora beticola*	Ghasemi et al., (2007); Mostafa et al., (2009)
Chlorococcum humicola	*Candida albicans, Aspergillus niger, Aspergillus flavus*	Bhagavathy et al., (2011)
Coelastrella sp.	*Candida albicans*	Najdenski et al., (2013)
Dunaliella salina	*Aspergillus niger*, *Candida albicans*	Mendiola et al., (2008); Mudimu et al., (2014)
Haematococcus pluvialis	*Candida albicans*	Santonyo et al., (2009)
Heterochlorella luteoviridis	*Candida albicans*	Mudimu et al., (2014)
Nannochloropsis sp.	*Fusarium graminearum*	Scaglioni et al., (2019)
Porphyridium aerugineum	*Candida albicans*	Najdenski et al., (2013)
Porphyridium cruentum	*Candida albicans*	Najdenski et al., (2013)
Porphyridium purpureum	*Candida albicans*	Mudimu et al., (2014)
Rhodella reticulata	*Candida albicans*	Najdenski et al., (2013)
Scenedesmus incrassatulus	*Candida albicans*	Najdenski et al., (2013)
Scenedesmus quadricauda	*Aspergillus niger, Aspergillus flavus, Candida albicans, Penicillium herquei, Fusarium moniliforme, Alternaria brassicae, Helminthosporium sp., Saccharomyces cerevisiae,*	Abedin & Taha, (2008)

Diatoms		
Asterionella glaciallis	*Candida pseudotropicalis, Trichophyton rubrum, Fusarium fuhum, Fusarium oxysporum, Colletotrichum acutatum*	Viso et al., (1987)
Arthrospira platensis	*Candida albicans, Cercospora beticola*	Ozdemir et al., (2004); Hussien et al., (2009)
Chaetoceros diadema	*Candida pseudotropicalis, Trichophyton rubrum, Fusarium fuhum, Fusarium oxysporum, Colletotrichum acutatum*	Viso et al., (1987)
Chaetoceros lauderi	*Candida pseudotropicalis, Trichophyton rubrum, Fusarium fuhum, Fusarium oxysporum, Colletotrichum acutatum*	Viso et al., (1987)
Chaetoceros muelleri	*Candida albicans*	Mendiola et al., (2007)
Haslea karadagensis	*Corollospora maritima, Lulworthia sp., Dendryphiella salina*	Gastineau et al., (2012)
Nitzschia sigma	*Aspergillus niger, Candida neoformans*	Walter & Mahesh, (2000)
Thalassiothrix frauenfeldii	*Candida albicans, Candida glabrata, Candida krusei, Candida tropicalis, Cryptococcus neoformans, Aspergillus niger*	Walter & Mahesh, (2000)
Cyanobacteria		
Anabaena flos-aquae	*Cercospora beticola*	Hussien et al., (2009)
Anabaena oryzae	*Alternaria porri*	Abdel-Hafez et al., (2015)
Anabaena solitaria	*Alternaria alternata, Botrytis cinera, Colletotrichum gloeosporioides, Fusarium oxysporum*	Kim et al., (2006)
Anabaena sp.	*Candida albicans, Aspergillus flavius*	Shishido et al., (2015)
Arthrospira	*Candida albicans*	Najdenski et al.,

fusiformis		(2013)
Arthrospira sp.	*Alternaria porri*	Abdel-Hafez et al., (2015)
Calothrix brevissima	*Alternaria alternata, Botrytis cinera, Colletotrichum gloeosporioides, Fusarium oxysporum, Phytophthora capsici, Pythium ultimum*	Kim et al., (2006)
Chroococcus disperses	*Aspergillus niger, Aspergillus fumigatus, Candida albicans, Candida kefyr*	Ghasemi et al., (2007)
Fischerella ambigua	*Candida krusei*	Ghasemi et al., (2004)
Fischerella sp.	*Candida albicans, Aspergillus flavius*	Shishido et al., (2015)
Gloeocapsa sp.	*Candida albicans*	Najdenski et al., (2013)
Nodularia sp.	*Alternaria alternata, Botrytis cinera, Colleotrichum gloeosporioides, Fusarium oxysporum*	Kim et al., (2006)
Nostoc calcicola	*Candida albicans, Candida krusei*	Vestola et al., (2014)
Nostoc commune	*Fusarium oxysporum f. sp. lycopersici*	Kim & Kim., (2008)
Nostoc humifusum	*Cercospora beticola*	Hussien et al., (2009)
Nostoc minutum	*Alternaria porri*	Abdel-Hafez et al., (2015)
Nostoc muscorum	*Cercospora beticola*	Hussien et al., (2009)
Oscillatoria angustissima	*Alternaria alternata, Botrytis cinera, Colletotrichum gloeosporioides, Fusarium oxysporum*	Kim et al., (2006)
Oscillatoria sp.	*Alternaria porri*	Abdel-Hafez et al., (2015)

Oscillatoria tenuis	*Alternaria alternata, Botrytis cinerea, Phytophthora capsici, Pythium ultimum*	Kim et al., (2006)
Phormidium fragile	*Cercospora beticola*	Hussien et al., (2009)
Scytonema hofmanii C.	*Candida albicans, Aspergillus flavius*	Shishido et al., (2015)
Scytonema sp.	*Candida albicans, Aspergillus flavius*	Shishido et al., (2015)
Spirulina sp.	*Fusarium graminearum*	Scaglioni et al., (2019)
Wollea saccata	*Cercospora beticola*	Hussien et al., (2009)

I.II: Effect of microalgae amendment on the composting process of organic residues

1.) Composting - general description

1.1 Fundamentals

Composting is considered to be an aerobic process of degrading fresh organic material into a stabilized humus-rich product, facilitated through heterotrophic microorganisms (Cooperband et al., 2002). The word compost derives from the Latin word *compositum* ("mixture") and can be applied to the final product which is stable and free of plant seeds and pathogens (Haug et al., 2018). Besides water vapor, CO_2 and heat, composting generates biologically stabilized material through biological oxidative transformation, a similar process to that which naturally occurs in soil (Bertoldi et al., 1983; Stentiford and Bertoldi, 2010), since usually there is no thermophilic phase in nature (Schowalter, 2016). During several stages of composting, exothermic processes of different microbial groups generate internal biological heat (Pietro et al., 2004). If the temperature of the compost mass rises, oxygen consumption of microorganisms increases while moisture decreases (Cooperband et al., 2002). Hence, proper aeration of the compost mass (e.g., manual turning or mechanical ventilation) is essential to maintain a sufficient level of oxygen and water. Gases such as CH_4, NH_3 or N_2O may be released throughout the whole composting process and can cause air pollution (Peigné & Girardin, 2004). Besides oxygen, carbon, nitrogen and water are equally essential as building blocks for metabolic pathways of composting organisms (Bertoldi et al., 1983).

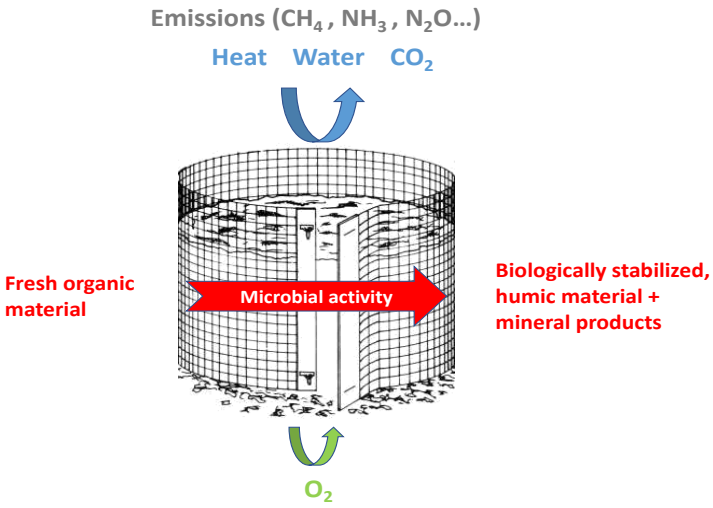

Figure 1.4 - Schematic representation of the fundamental composting process (adapted from Rynk, 1992)

Finstein et al. (1986) described the composting process with the following chemical equation: Fresh organic matter + O_2 → Humus-like substances + CO_2 + H_2O + Energy (heat) + mineral products

1.2 Composting material

The composting procedure begins with the proper selection of raw organic materials in order to obtain a carbon/nitrogen ratio between 25 and 30 of the initial mixture (Füleky & Bendek, 2010). This ratio ensures an appropriate carbon/nitrogen balance for microbial metabolic activities, necessary to decompose and transform raw materials into precursors of humus (Sánchez et al., 2017). A raw material that is rich in nitrogen is commonly considered as "green material", whereas carbonaceous material is considered as "brown material" (Schwarz & Bonhotal, 2011). Compost can be obtained from almost all organic plant and animal materials as long as the initial mixture contains the right amounts of nitrogen and carbon (Baldwin & Greenfield, 2009), although other elements are important, namely phosphorus (Jakubus, 2016). The initial mix of composting material should have a moisture content in the range of 55-65% (Rynk, 2000), depending on particle size. Wojcieszak *et al.* (2015) reported 30-50% as optimal dry matter values along the composting process. Bertoldi et al. (1983) stated that in principle all kinds of raw organic material with a pH range from three to 11 can be composted. Examples of composting materials are fruit and vegetable waste, animal manure and bedding (e.g., straw), human waste and sewage sludge (Epstein, 1996; Table 3). Because frequently used feedstocks are commonly poor in nitrogen, nitrogenous materials, mainly manure or chemical nitrogen sources have to be supplemented (Sánchez et al., 2017). Prior treatments usually include particle size adjustment (Epstein, 1996). If raw materials are too coarse, particle size reduction is required to increase microbiological activity by increasing the surface/volume ratio, while maintaining sufficient internal aeration (Zhang & Sun, 2014). Conversely, fine materials might need to be mixed with a bulking agent to increase natural aeration, preventing a rapid decrease of oxygen in the medium (Eftoda & McCartney, 2004).

Composting is a promising approach to recycle various types of biowaste (Füleky & Benedek, 2010). Agricultural waste and agro-industrial residues are the main resource for raw materials and therefore, composting contributes to the concept of sustainable agriculture and to an ecofriendly waste management behavior (Gajalakshmi & Abbasi, 2008).

Bundela et al. (2010) suggests that using compost in agricultural applications is the most cost-effective option to recycle municipal solid waste (MSW).

Table 1.3 - Commonly used composting material and characteristics (adapted from Cooperband, 2002)

Composting material	Moisture content %	C:N ratio	Bulk density (lb/yd^3)
High in Carbon			
Bark (paper mill waste)		100-130	
Brush, wood chips		100-500	
Cardboard	8	500	250
Corn silage	65-68	40	-
Corn stalks	12	60-70	32
Fall leaves		30-80	100-300
Hay	8-10	15-30	
Mixed paper		150-200	
Newspaper	3-8	400-800	200-250
Sawdust	20-60	200-700	350-450
Straw	5-20	40-150	50-400
High in Nitrogen			
Coffee grounds		20	
Cull potatoes	70-80	18	1500
Dairy manure	80	5-25	1400
Grass clippings		15-25	
Hog manure	65-80	10-20	
Poultry manure	20-40	5-15	1500
Sewage sludge		9-25	
Vegetable wastes		10-20	

1.2.1 Moisture content of composting materials

An optimum moisture content is important for all microorganisms in the composting mass to assimilate dissolved nutrients and to maintain mobility (Pellejero et al., 2015). Size and physical state of the particles in the composting mass are the main factors determining the optimal moisture content (Bertoldi et al., 1983; Table 4). Low moisture contents cause early dehydration of the composting mass which further prevents microorganisms to keep various metabolic pathways active (Sharma et al., 1997). This causes a temperature decrease and affects biological processes, resulting in a physically stable but biologically unstable product (Bertoldi et al., 1983). Conversely, high moisture content, on the opposite, can reduce oxygenation by clogging pores that are essential for air circulation (Cooperband, 2002). In practice, coarse materials can be successfully composted with a higher moisture content than fine materials.

Table 1.4 - Optimum moisture contents of various composting materials (adapted from Guo et al., 2012)

Optimum moisture content	Composting material
69%	Poultry manure with wheat straw
< 80%	Swine manure and corncob
60-70%	Sewage sludge
50-60%	Pig manure with sawdust
65-70%	Solid fraction of poultry manure with straw

1.3 Composting process (overview)

The key parameters that influence the length of the composting process are the nature of the composting material (e.g., C/N ratio, chemical composition, moisture, particle size), oxygen availability and the applied composting technology (Sánchez et al., 2017). A high C/N ratio of the initial mix of composting materials generally delays the process of composting (Guo et al., 2012), while a low C/N ratio increases nitrogen emissions (Hwang et al., 2020). If the oxygen supply is not sufficient, anaerobic decomposition can occur, slowing down the composting process (Cooperband, 2002) and increasing bad odors due to the accumulation of several chemical substances, which are eventually phytotoxic (Garrett et al., 2012). Moreover, the growth of microbial communities that do not support the composting process condition can be promoted (Sánchez et al, 2017). Sharma et al. (1997) observed that aeration and moisture content (MC) are closely interrelated: if moisture is too high, the pores which are essential for air to circulate, can be clogged with clumped material and air flow may be interrupted. In contrast, low moisture values prevent microbial activity, enhancing the importance of a proper particle size distribution at the beginning and during the composting.

The composting process can commonly be separated into four phases, characterized by the temperature reached in the hottest zone of the composting mass, as a consequence of the metabolic activity of different microbial communities (Figure 5; Cooperband, 2002). During the first stage of composting, mesophilic microorganisms (15-40 °C), such as various soil bacteria, fungi and actinomycetes, break down organic material through the hydrolysis of sugars, amino acids and lipids (Bertoldi et al., 1983). Exothermic metabolic activity of mesophilic microbes is able to enhance the temperature of the composting mass up to a maximum of 85°C (Sánchez et al., 2017).

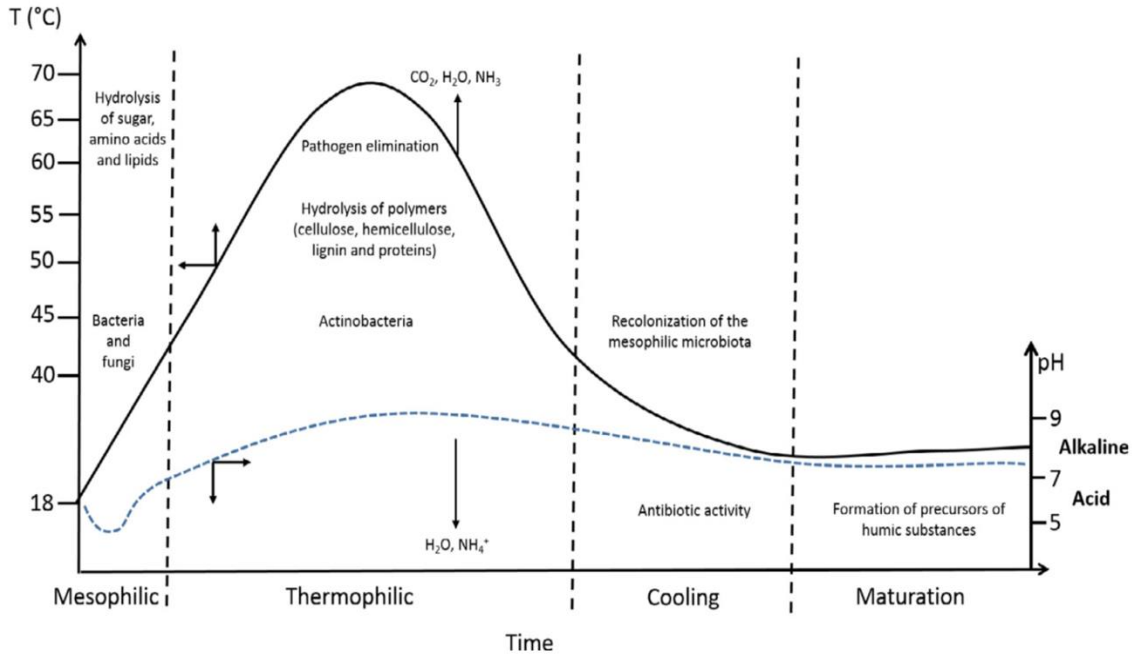

Figure 1.5 - Schematic representation of the composting process. Time profile of the average pile temperature and pH profile (Sánchez et al., 2014).

Because of the rise in temperature, thermophilic microorganisms replace mesophilic microorganisms and initiate the second phase of composting, defined as the "thermophilic phase" (Insam & de Bertoldi, 2007). In this phase, thermophiles (mainly actinobacteria) degrade complex molecules like lignin, cellulose, hemicellulose and proteins through enzymatic activities (Bernal et al., 2009). Moreover, high temperatures prevent germination of seeds, eliminates or degrades phytotoxic and pathogenic agents (Cooperband, 2002). As soon as energy sources are depleted, the composting mass cools down ("cooling phase") and reaches temperatures between 15 °C and 35 °C, causing a second colonization by mesophilic microbiota (Sánchez et al., 2017). During the maturation phase of the compost, humus-like substances are formed (Cooperband, 2002).

1.3.1 Oxygenation / aeration

Along the whole composting process, the availability of oxygen is of primary importance for various metabolic pathways of microorganisms (Cooperband, 2002). In order to maintain the availability of oxygen and to avoid anoxic zones, the composting mass needs to be aerated (Bertoldi et al., 1983). Therefore, periodic aeration such as turning, or continuous aeration such as mechanical ventilation is essential (Cooperband, 2002). Haug (2018) indicates three basic purposes of aeration: stoichiometric demand (oxygen demand for organic

decomposition), drying demand (air picks up moisture and support the drying process) and heat removal demand (removal of heat generated by exothermic metabolic activities of microbes). Diaz et al. (2002) found out that successful composting is mainly influenced by the optimal aeration rate (AR). The key parameters that influence optimal AR are the nature of the composting material (e.g., texture and particle size) and the ventilation method (Guo et al., 2012). Insufficient aeration leads to anaerobic decomposition and slows down the composting process (Cooperband, 2002). Excessive aeration on the other hand causes excessive losses of heat, water and ammonia, which delays the composting process as well (Guo et al., 2012). Moreover, the AR influences the nitrification process, ammonification and ammonia emission and is therefore considered to be a main parameter influencing nitrogen dynamics (de Guardia et al., 2008). Epstein (1996) proposed three principal aeration methods: convective air flow (natural), physical turning and mechanical aeration, sometimes in combination.

1.4 Evaluating compost

Compost evaluation can be achieved by analyzing many different parameters which may vary according to the end use application. The following sections focus on some compost characteristics that are of primary importance to apply the finished product as plant fertilizers in soil, or as plant growing media in soilless culture.

1.4.1 Phytotoxicity

Germination tests are bioassays that quantify the inhibitory effects of phytotoxins such as the delay of seed germination or the inhibition of plant growth (Wrap, 2002). Phytotoxins are compounds produced by pathogenic fungi or bacteria that can cause disease symptoms in plants (Strobel, 1982). The maturity of the compost, regarded as its potential of use, is often defined as the total amount of degradation of phytotoxic substances and can be estimated by determining the germination rate index (GI) (Gao et al., 2010; Zucconi et al., 1981 a, b). Some GI's combine relative root elongation (L%) and relative seed germination (G%), mainly from garden-cress seeds (*Lepidium sativum*), germinating on aqueous compost extracts (Ranal & Santana, 2006). Phytotoxicity tests are important to confirm that the compost is suitable for agricultural purposes and to avoid negative effects of immature compost on the agricultural ecosystem (Warman, 1999). One of the most utilized germination

tests is that reported by Zucconi et al. (1981a, b), which can also be considered as compost maturity test, when it reaches values above 60% (Selim et al., 2011).

1.4.2 Organic matter or volatile solids content

Precise determination of organic matter content or volatile solids is necessary to analyze the decomposition dynamics of organic matter over a period of time (Hogsteen et al., 2015). The initial organic matter content of typical feedstocks differs greatly and decreases throughout the different stages of composting (Alsanius et al., 2016; Fig. 1.5). Organic matter can be determined by the loss of ignition (LOI), a methodology in which a compost sample is ignited to high temperatures in a muffle furnace (Matthiessen et al., 2005). The weight lost during combustion, referred to as ash content, is used to estimate organic matter content by reciprocation (Hsu et al., 1999). In literature, the ignition temperature ranges from 375º to 1025 ºC (Donkin, 1991) and the heating time varies unevenly (Matthiessen et al., 2005), but 560ºC is a typical value. There is no universal standard protocol and multiple factors, such as ignition temperature, furnace type and heating time may influence the precision of measurements (Hogsteen et al., 2015).

1.4.3 Physical and chemical analysis

Physical and chemical properties of the composts are important quality measurements for producers and users (Epstein, 1996). Common approaches to access compost quality and maturity are analysis of the compost's chemical composition including total organic carbon, volatile solids, cation exchange capacity, total nitrogen, inorganic nitrogen, phosphorus, potassium, calcium, magnesium and micronutrients (Sullivan & Miller, 2001). Another way to determine compost quality, relevant when the compost is used as plant growing media component, are the analysis of physical parameters, such as gravimetric moisture content, bulk density, gravimetric water holding capacity, particle size and other mechanical properties such as porosity (Agnew & Leonard, 2003).

1.4.3.1 Salinity

Electrical conductivity (EC) is a measure that describes the salinity level of composting masses and indicates the total amount of soluble ions, which may cause phytotoxic or phytoinhibitory effects (Zaha et al., 2013). EC is influenced by various soil fertility parameters such as pH, available phosphorus and potassium, exchangeable calcium and

magnesium, availability of micronutrients and cation exchange capacity (CEC) (Carmo, 2016). Optimal EC values for using compost as biofertilizer lie between 2.0 and 3.5 dS m^{-1} (Zaha et al., 2013). Li et al. (2008) found out that soil electrical conductivity is highly correlated to crop yield potential of cotton plants and thus, EC could be considered as an additional soil fertility index (Carmo et al., 2016).

1.4.3.2 pH

Although, in principle, all kinds of organic materials with a pH range from three to 11 can be composted, optimal pH values for successful composting lie between 5.5 and 8.0 (Bertoldi et al., 1983). As composting proceeds, the organic material undergoes different phases of pH levels, depending on the chemical composition of the composting mass and the actual composition of microbial communities (Sundberg et al., 2013). Changes in pH are predictable as the pH values of the composting mass follow a characteristic curve (Figure5; Sánchez et al., 2017). During the initial phase of composting, a drop in pH, mainly enhanced through the accumulation of organic acids, can be observed (Reinhardt, 2002). Thereafter, pH increases due to the consumption of organic acids and the production of ammonium (Beck-Friis et al., 2003).

1.4.4 Quantification of microbial populations

The community structure of microbial functional groups accurately represents biological processes and can, for example, be used to determine N availability by means of the abundance of N-fixing microorganisms (Abril et al., 2011). Therefore, molecular analysis of bacterial and fungal populations is a common scientific strategy to evaluate compost quality in different stages of the process (Kutu et al., 2019). The chemical composition of raw materials, its physical characteristics and nutrient composition define microbial communities in the compost (Tiquia et al., 2005; Villar et al., 2016). Ishii and Takii (2003) stated that the concentration of dissolved organic substances is the main factor affecting the community structure. Moreover, due to their high sensitivity to compost parameters and management, microbial dynamics can be applied as a tool to measure the compost quality (Abril et al., 2011). Although most studies focus on the population dynamics of early composting stages (Ryckeboer et al., 2003), microbial community structures of finished products represent its level of fertility (Fracchia et al., 2006).

2.) Algal compost

2.1 Algae as compost enricher for sustainable agriculture

Microalgae have recently attracted considerable attention in agriculture, namely in horticulture, due to their immense potential as a multifunctional bioresource with many application possibilities (Renuka et al., 2018). This section focusses on the application of microalgae as plant biostimulants, one of the Product Function Categories (PFCs) of EU fertilizing products, as defined in the Annex I of the Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019. According to this regulation, *a plant biostimulant shall be an EU fertilizing product the function of which is 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.*

2.1.1 Application of microalgae as biofertilizers, plant growth promoting agents and soil conditioners

Based on their ability to promote plant growth and improve soil quality / fertility in several ways, microalgae can be considered as a promising sustainable and renewable alternative to synthetic fertilizers (Dineshkumar et al., 2018; Kawalekar, 2013). Until now, biofertilizers are defined as biological substances that must contain living microorganisms which promote plant growth when applied to soil, seed or plant surfaces (Calvo et al., 2014). The forthcoming regulation (EU) 2019/1009 on EU fertilizing products, which will be applied on 16 July 2022, will modify the rules for the European market of fertilizing products. Hereafter, plant biostimulants are considered as biofertilizers and may contain microorganisms or not. Microalgae can also be considered as plant biostimulants due to their ability of stimulating biological and chemical processes in plants and/or plant growth-promoting microbes, which enhances nutrient use efficiency, crop performance and stress resilience (Calvo et al., 2014; Chiaiese et al., 2018). Several ways how microalgae promote plant growth are discussed in the following sections:

a) Biomineralization and soil enrichment

Soil amendment with algal biomass enhances the bioavailability of nutrients based on the capability of microalgae to modulate the rhizosphere microbiome (Hamed et al., 2018; Ronga

et al., 2019). The mobilization ability of microalgae (geochemical nutrient cycling and microbial transformations) increases the overall activity of soil microbes and supports the growth and interactions of beneficial microorganisms (Dalsgaard, 2003; Prasanna et al., 2013). For example, plant growth promoting rhizobacteria (PGPRs) can improve plant growth through the production of growth-promoting hormones as well as through the suppression of pathogens and pests (Vessey, 2002). Microalgae can promote the solubilization and mineralization of nutrients by microbes, stimulating nutrient fluxes from the soil to the plant (Sanchéz et al., 2017). This facilitation of precipitating mineral compounds by the aid of living organisms is known as biomineralization (Skinner & Jahren, 2003). Microalgae can significantly contribute to the mineralization and solubilization processes through the production of organic acids (Sanchéz et al., 2017). Moreover, microalgae represent important sources of organic matter, based on their capability to capture and utilize atmospheric carbon dioxide in order to produce biomass (photosynthesis) (Han et al., 2014). Many cyanobacteria and green algae can excrete carbon (e.g., exopolysaccharides) into the surrounding environment, promoting the growth of soil microbes (Najdenski et al., 2013). Exopolysaccharides (EP) are defined as high molecular-weight polymers that are composed of organic or inorganic compounds, which can support plant growth and draught tolerance (Ashraf et al., 2004). Naseem et al. (2014) reported that EPS improved plant biomass, root and shoot length, leaf area and moisture contents of maize. EPS are secreted by various microorganisms and count as primary sources of organic carbon in soils (Raliya et al., 2014).

b) Nitrogen fixation

Soil enrichment with N (Nitrogen)-fixing cyanobacteria is commonly known as "Algalization" (Cresswell et al., 1989). They can differentiate specialized nitrogen-fixing cells called heterocysts, which carry out nitrogen fixation under aerobic conditions during nitrogen starvation (Kumar et al., 2010). Heterocystous cyanobacteria can significantly increase soil N-content based on their capability of fixing atmospheric N_2 and therefore providing plant nitrogen nutrition (Cresswell et al., 1989). Moreover, heterocystous cyanobacteria have been shown to colonize phyllospheres (leaf surfaces), where N-fixation rates depend on light exposure and microclimatic conditions (Fürnkranz et al., 2008).

c) Phytohormones

Many species from both groups, cyanobacteria and green algae, can produce and excrete hormones (e.g., auxins and cytokinins) that promote plant growth (Ronga et al., 2019). Hussain & Hasnain (2011) studied the phytostimulation and biofertilizaton potential of cyanobacteria under axenic and field conditions. The authors showed that cytokinin and IAA levels positively correlate with seed weight, spike length, stem length and root length of plants. Moreover, the authors reported that synthetic and natural phytohormones can increase crop yields and productivity.

d) Elicitation of plant defense mechanisms

Results of various studies have shown that interactions between plants and microalgae promote plant immunity and improve biotic and abiotic stress resistance (Alam et al., 2020). Cyanobacteria are reported to modulate and elicit plant defense mechanisms by increasing the activity of plant defense enzymes such as chitinase, peroxidase, catalase, polyphenol oxidase and others (Renuka et al., 2018).

e) Colonization of plant tissues

Microalgae are reported to use various strategies to colonize different parts of vascular and non-vascular plants, as well as the rhizosphere microbiome (Alam et al., 2020). This can promote plant growth, seed germination, disease control, productivity and soil fertility (Renuka et al., 2018). Babu et al. (2015) studied the colonization of six cyanobacteria strains on roots of wheat plants using biochemical and molecular tools. The authors found out that the nitrogen-fixing potential significantly correlates with the inoculation of cyanobacteria what could lead to increased crop yields and savings in nitrogen.

2.2. Peculiarities of algae as compost enrichers

Since algae are known to be rich in micro- and macronutrients, amino acids, vitamins, phytohormones and many more plant growth promoting substances, they can be used to enrich composting masses and further stimulate the overall plant productivity (Renuka et al., 2018). Moreover, the application of algal biomass as fertilizer or soil conditioner diminishes agricultural pollution such as eutrophication, soil infertility and biodiversity loss (Calvo et al., 2014). Compost can be an effective carrier for the use of algal biomass as biofertilizer, plant growth promoting agent or soil conditioner (Renuka et al., 2017). It is reported that algae, mainly macroalgae, were successfully used as cost-effective composting feedstock, leading to

a mature and highly stable compost (Han et al., 2014; Michalak & Chojnacka, 2013; Michalak et al., 2016; Michalak et al., 2017). Throughout the composting process, algal biomass is biologically decomposed and stabilized (Michalak et al., 2017). If the final product is sufficiently stable, it can be used as sustainable fertilizer and soil conditioner (Renuka et al., 2017). Most studies focus on the composting of algal biomass collected from eutrophication events such as green tides (Han et al., 2014), which can endanger the environment (e.g., fish populations) and cause serious problems to the human population (e.g., interruption of domestic and industrial water supply, blocking of port channels, etc.) (Han et al., 2014; Seymour, 1980;). Keesing et al. (2009) reported that green tides in the Yellow Sea off China are linked to upwelling events and the eutrophication of water environments. The authors found out that many green macroalgal blooms are correlated with high nitrogen levels in the water body, mainly caused by excessive agricultural runoff. Composting algae can lead to a better waste management of algal biomass, harvested from algal blooms or green tide events (Han et al., 2014).

As mentioned previously, a proper carbon/nitrogen ratio is of great importance for successful composting (Guo et al., 2012). It has been reported that algae show a relatively low carbon/nitrogen ratio, ranging from 8-11 (Cuomo et al., 1995; Han et al., 2014; Maze et al., 1993). In order to achieve a suitable C/N ratio for composting (around 25- 30), the algal biomass has to be mixed with composting material with a high C/N ratio (Han et al., 2014). Moreover, the generally high moisture content of algal biomass is a critical parameter for successful composting (Michalak & Chojnacka, 2013). If the moisture of the composting mass is too high, aeration will be restricted, and microbial activity can be inhibited (Han et al., 2014). Furthermore, high salinity levels, especially in marine algal species, and the accumulation of heavy metals in these marine organisms can cause problems regarding compost quality (Michalak & Chojnacka, 2013).

Literature about co-composting of microalgae is still scarce and most studies focus on the composting of fresh macroalgal biomass. Prasanna et al. (2015) investigated the potential of cyanobacteria-enriched compost and showed that the addition of *Anabaena* sp. and *Calothrix* sp. promoted seed germination and crop yields of cotton plants, when applied as substrate. Farrag et al. (2017) showed that the application of compost tea and cyanobacteria filtrate by foliar spraying promoted vegetative growth and fruit yield of *Cucumis melo L*. Wang & Husain (2015) reported that *Anabaena* sp. additions improve the chemical qualities of

compost due to its biodegradation ability. The authors showed that *Anabaena* sp. can enrich the compost with nitrogen and biodegrade the majority of organic pollutants. El-Gamal (2011) studied the addition of living cyanobacteria to compost and found out that microbial activities were enhanced, leading to an acceleration of the composting process. Dukare et al. (2011) evaluated cyanobacteria enriched compost and compost tea preparations as biocontrol agents in tomato. The authors showed that cyanobacteria suppress diseases caused by *Fusarium oxysporum*, *Pythium debaryanum*, *Pythium aphanidermatum* and *Rhizoctonia solani*. Moreover, the study revealed that cyanobacteria amended compost promotes seed germination, seedlings length and biomass production.

I.III: Objectives of the thesis

The main objective of the present study is to determine the biostimulant and biofungicide properties of five microalgal species: *Scenedesmus sp.*, *Chlorella vulgaris*, *Nannochloropsis sp.*, *Arthrospira (Spirulina) sp.* and *Phaeodactylum tricornutum*. To achieve the aforementioned goal, the specific objectives of this dissertation are as follows: (1) evaluate the control of plant diseases with aqueous microalgal extracts under laboratory conditions; and (2) evaluate and characterise microalgae-enriched composting processes.

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II.) Chapter 2: Antifungal properties of aqueous microalgal extracts

Antifungal properties of aqueous microalgal extracts

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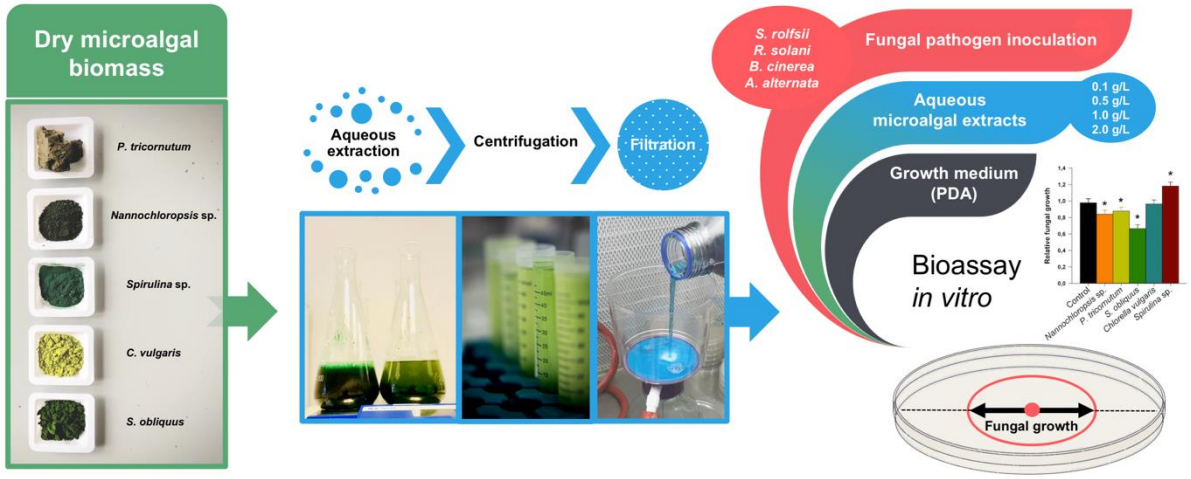

Figure 2.1: Graphical abstract

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1.) Abstract

Microalgal species are a promising source for non-environmentally harmful antifungal agents that can potentially reduce the usage of synthetic fungicides and limit the ecological impact of the agricultural sector. Since most studies focus on antifungal properties of prokaryotic cyanobacteria, the present study aims to fill the gap of knowledge concerning the use of eukaryotic microalgae as antifungal agents. To avoid complex extraction methods and purification steps, which increase costs and restrict large-scale applications of algae-based fungicides, a simple water-based extraction was used. Hence, properties of aqueous extracts from eukaryotic (*Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Scenedesmus obliquus* and *Chlorella vulgaris*) and prokaryotic (*Spirulina* sp.) microalgae were investigated *in vitro* regarding their antagonistic activity against the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea* and *Alternaria alternata*. Statistical analysis revealed that *Scenedesmus obliquus* showed the highest antifungal activity of all microalgal strains against *Sclerotium rolfsii*, with growth inhibitions of up to 32.01 ± 4.82 %. *Nannochloropsis* sp. mitigated *Sclerotium rolfsii* by up to 13.96 ± 5.26 %, while *Phaeodactylum tricornutum* suppressed the mycelial growth of *Sclerotium rolfsii* and *Rhizoctonia solani* by up to 18.35 ± 3.45 % ($p<0.05$). Moreover, *Phaeodactylum tricornutum* and *Scenedesmus obliquus* inhibited *Botrytis cinerea* growth by up to 11.47 ± 2.06 % ($p<0.05$). Taken together, these results suggest that microalgae with fungicidal activity might contribute for a more sustainable agriculture by inhibiting the growth of fungal phytopathogens.

Keywords: natural fungicides, microalgae, phytopathogenic fungi, sustainable agriculture

2.) Introduction

Phytopathogenic fungi are the dominant causal agents for diseases in agriculture [1] and can be classified as a worldwide relevant threat to food security [2]. The rapid rise in global food demand and the need for controlling fungal pathogens requires an intensive use of synthetic pesticides [3], which negatively impacts the sustainability of agroecosystems and affects human health [4]. The World Health Organization [5] identified agrochemical pollution as a major environmental and health issue, particularly in developing countries that rely on the agricultural sector economically. Synthetic fungicides affect the diversity, abundance, reproduction, ecological interactions and performance of aquatic and terrestrial non-target species [6]. Moreover, long-term exposure to synthetic fungicides can increase the risk of

cancer development and may cause reproductive disorders [7,8]. Hence, sustainable agriculture faces the dilemma of reducing these negative impacts while achieving increasingly higher crop yields [9].

Recently, scientists have suggested replacing toxic agrochemicals by microalgal extracts as an environmentally friendly alternative [10,11]. Biologically active compounds of these algal extracts should effectively suppress the growth of pathogenic fungi which cause damage to the worldwide production of crops [12,13]. The target fungal species *Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea* and *Alternaria alternata* were selected regarding their economic impact on global crop or yield losses [14,15,16,17]. Each of these phytopathogenic fungi has different characteristics [18,19,20]. For example, *S. rolfsii* penetrates plant tissues prior to colonization through the production of cell-wall degrading enzymes or oxalic acid as a toxic agent [18]. Likewise, the phytopathogenic fungus *Rhizoctonia solani* is classified as a soil-borne basidiomycete that causes a wide range of commercially significant diseases, primarily through seed germination inhibition ("post emergence damping off") of various important food crops [15]. The ascomycete *Botrytis cinerea* (grey mould), a causal agent for more than 500 plant diseases, causes annual global losses of up to $100 billion [16]. *B. cinerea* is difficult to control due to various specific infection strategies and a broad host range [21]. The pathogen *Alternaria alternata* causes fungal blight in over 400 plant species and may produce host-specific phytotoxins, depending on its *forma specialis* [17,22].

Microalgal extracts contain bioactive metabolites with antifungal properties [11,12,23,24,25,26]. For example, inhibition of phytopathogenic fungi was demonstrated by Shishido *et al*. [27] upon studying the antifungal activity of various microalgal strains belonging to the orders Stigonematales and Nostocales against *Aspergillus flavus* and *Candida albicans.* The authors showed evidence that nine of those strains inhibited growth of *A. flavus*, while ten strains acted antagonistically against the growth of *C. albicans*. However, further studies are required to investigate the efficiency of aqueous microalgal extracts with the perspective of using them as alternative fungicides on a large scale [28]. Research about eukaryotic microalgae as antifungal agents is still scarce and most studies investigate prokaryotic cyanobacteria [27,29]. Moreover, while most research focuses on the application of fungicidal microalgae in human pathogens, the efficiency against plant pathogens is virtually unknown [29]. Aiming to fill this gap, the present study focuses on the antifungal

properties of *Nannochloropsis* sp., *P. tricornutum*, *Scenedesmus obliquus*, *Chlorella vulgaris* and *Spirulina* sp. against *S. rolfsii*, *R. solani*, *B. cinerea* and *A. alternata*.

3.) Material and methods

Pathogenic isolates and culturing conditions

All experiments were carried out at the Campus of Gambelas, University of Algarve, Portugal (37°02′35.45″N, 7°58′20.64″W).

Isolates of four fungal target species (*Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea* and *Alternaria alternata*) were obtained from the mycological collection of the Mediterranean Institute for Agriculture, Environment and Development (University of Algarve). All fungi were grown on potato dextrose agar media (PDA; Biokar, France) 7 days at 25 ± 2 °C to obtain mycelial discs as inoculum.

Microalgal biomass cultivation

Dry biomass of *Nannochloropsis* sp., *Phaeodactylum tricornutum* and *Spirulina* sp. was obtained from NECTON S.A. (Faro, Portugal), while *Scenedesmus obliquus* and *Chlorella vulgaris* were obtained from Allmicroalgae Natural Products (Leiria, Portugal). Cultures were grown in tubular photobioreactors and the harvested biomass was either freeze- or spray-dried, two procedures that are known to cause cell disruption and leakage of cell contents [30].

Preparation of aqueous microalgal extract

A stock solution was prepared in a conical flask with distilled water (Milli-Q Synthesis System, Millipore) and 10 g L^{-1} dry powder of each microalgal strain, followed by an overnight extraction (dark conditions) with an IKA RO 10 magnetic orbital laboratory shaker (IKA-Werke GmbH & Co. KG, Germany) at 350 rpm. The extract was then transferred into conical centrifuge tubes and centrifuged twice (Heraeus Megafuge 16R, Thermo Fisher Scientific, Netherlands) at 906 RCF for 5 minutes. The supernatant was then poured into filter paper funnels using multifold, qualitative filter paper (⌀ = 150mm, nominal pore size= 15~20 μm) (Pratdumas, France). Subsequently, the filtrate was passed through filter papers with a pore size of 5.0, 0.7 and 0.45 μm, using a Buchi V-700 (Richmond Scientific, United

Kingdom) vacuum pump. After pre-filtration, a sterile 0.2 μm bottle-top filter (Nalgene Rapid-Flow, Thermo Fisher Scientific, Netherlands) was used under a laminar flow hood as final filtration processing step. The sterile filtrates were then transferred into 50 mL conical centrifuge tubes aliquots and stored in the freezer for further usage.

Bioassay preparation

The PDA medium containing 39 g L^{-1} (15 g L^{-1} agar, 20 g L^{-1} dextrose and 4 g L^{-1} potato extract) was sterilized by autoclaving, as well as conical flasks with distilled water (Mediterranean Institute for Agriculture, Environment and Development, University of Algarve), pipette tips and L-shaped cell spreaders. After autoclaving, the PDA media was stored hermetically sealed in an incubation chamber at 50 ºC to prevent hardening. A sterile thermometer was used to control the temperature of several liquid media in order to prevent microalgal biomass disruption.

Bioassay procedure

For the *in vitro* bioassays with *S. rolfsii* and *R. solani*, Petri dishes with a diameter of 90 mm were used. *B. cinerea* and *A. alternata* were inoculated as mycelial disc into Petri dishes with a diameter of 60 mm. A volume of 12.5 mL (Petri dishes with a diameter of 90 mm) or 8.5 mL (Petri dishes with a diameter of 90 mm) of PDA media was poured into sterile Petri dishes under a laminar flow hood. The interior of the flow chamber was previously sterilized with an integrated UV-C germicidal lamp for 15 minutes. Antimicrobial activities were evaluated *in vitro* by using diffusion methods adapted from Ambika and Sujatha [31] and Machado *et al.* [32]. The stock solution was added to previously autoclaved conical flasks with distilled water to obtain the final aqueous algal extracts with the concentrations 0.1, 0.5, 1.0 and 2.0 g L^{-1}. Distilled water was used as a negative control group. When the PDA media reached a semi solid state, 750 μL (Petri dishes with a diameter of 90 mm) or 350 μL (Petri dishes with a diameter of 60 mm) of aqueous algal extract was pipetted into the PDA-coated Petri dishes and uniformly distributed with a sterile L-shaped cell spreader. Each bioassay was performed in triplicate (n=3). After an overnight incubation at 25 ± 2 °C, the fungal pathogen was inoculated as active mycelial disc in the geometric centre of each Petri dish under a laminar flow hood. A perpendicular straight line was drawn on the bottom of each Petri dish and stretchable adhesive tape was used for closing and sealing. After inoculation,

the culture plates were stored in an incubation chamber at 25 ± 2 °C for three days and radial fungal growth (cm) was measured every 24 hours.

Statistical analysis

Analyses of covariances (ANCOVA) were performed to detect differences in the relative fungal growth of *S. rolfsii*, *R. solani*, *B. cinerea* and *A. alternata* on day 3 after inoculation among all concentrations of aqueous extracts (0.1, 0.5, 1.0 and 2.0 g L^{-1}) of the microalgal strains *Nannochloropsis* sp., *P. tricornutum*, *S. obliquus*, *C. vulgaris* and *Spirulina* sp. Data for relative fungal growth were previously normalized to the control group (for each fungal strain and algal strain) and then illustrated as adjusted means ± 95 % confidence interval obtained from Tukey's post hoc test (ANCOVA). Analysis of variances (ANOVA) were performed to test the effect of different extract concentrations at each day (independent, qualitative variables) on fungal growth (dependent, quantitative variable). Data points at each day are shown as mean ± SD (n=3). Dunnett's two sided post-hoc test (ANOVA) was performed to indicate significant differences of the relative fungal growth between microalgal extracts and the control group. A significance level (α) of 0.05 was used for all performed tests. Standard deviations are represented with error bars.

4.) Results

4.1 *Sclerotium rolfsii*

An overall ANCOVA model (R^2=0.709) fitted to the mycelial growth of the pathogenic fungus *Sclerotium rolfsii* on day 3 of the experiment revealed major effects of the tested strain (F= 100.58, p<0.05) and minor effects of the used extract concentration (F=7.31, p>0.05). The adjusted means from the Tukey's post-hoc test indicated that aqueous extracts of *Nannochloropsis* sp., *Phaeodactylum tricornutum* and *Scenedesmus obliquus* suppressed the growth of *S. rolfsii* by up to 13.96 ± 5.26, 10.25 ± 5.36 and 32.01 ± 4.82 %, respectively (p<0.05; Fig. 1a). On the other hand, *Chlorella vulgaris* showed no significant suppression of *S. rolfsii* growth (p>0.05) while *Spirulina* sp. promoted the pathogen growth by up to 20.79 ± 6.25 % (p<0.05, Fig. 1a).

Analysis of variances (ANOVA) fitted to extract concentrations at each time point (day 1, 2 and 3) revealed that all aqueous extracts of *Nannochloropsis* sp. (0.1, 0.5, 1.0 and 2.0 g L^{-1}) significantly inhibited the pathogen growth on day 3, resulting in an average fungal radius of 3.19 ± 0.17 cm (Fig. 1a) compared to that of the control group (3.82 ± 0.24 cm). Likewise, *P.*

tricornutum suppressed the pathogen growth at concentrations of 0.1 and 2.0 g L^{-1} with an average fungal growth of 3.45 ± 0.34 cm compared to the growth of the control group (4.12 ± 0.14 cm; Fig. 1c), while extract concentrations of 0.5 and 1.0 g L^{-1} showed no effect. Strikingly, *S. obliquus* showed the highest inhibition of *S. rolfsii* growth in all tested concentrations (2.70 ± 0.46 cm; day 3) compared to that of the control group (4.20 ± 0.00 cm; day 3; Fig. 1d). Bioassays with 0.1 g L^{-1} *Spirulina* sp. extract showed an average fungal growth of 1.92 ± 0.31 cm, compared to 1.64 ± 0.16 cm in the control treatment (day 3, $p<0.05$, Fig. 1f). *C. vulgaris* extracts showed no significant effect on fungal growth (Fig. 1e).

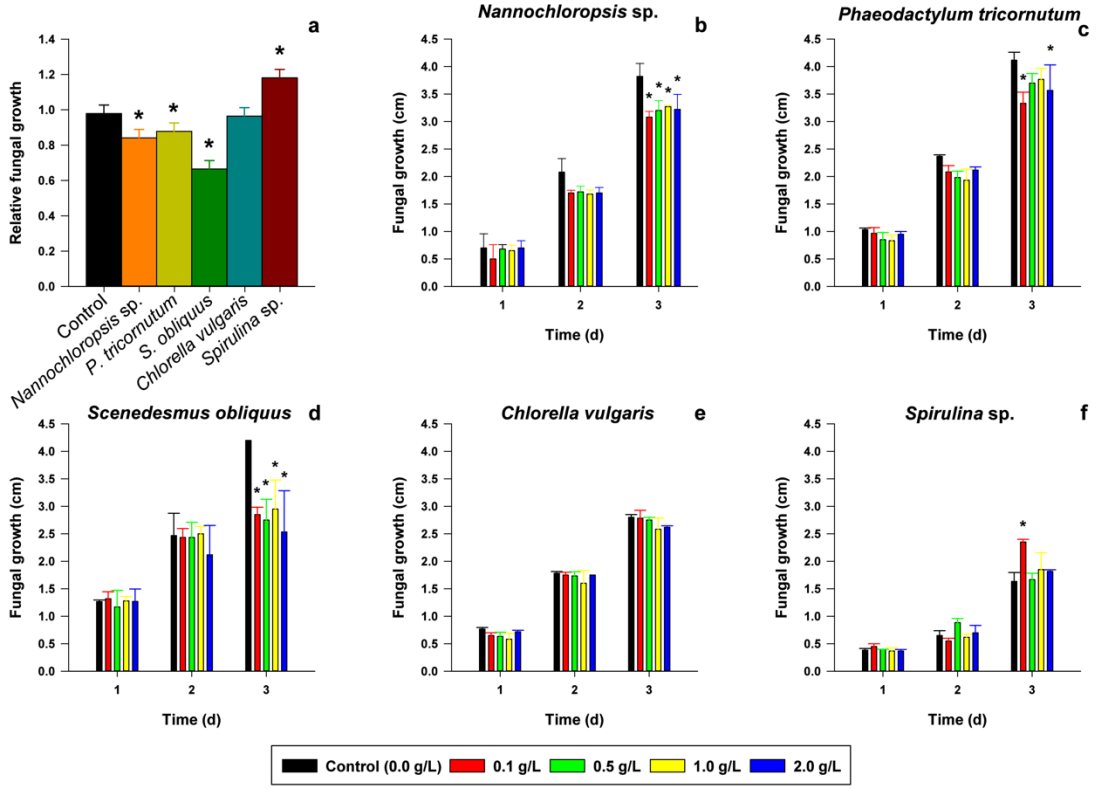

Figure 2.2: Sclerotium rolfsii exposed to microalgal extracts. Relative differences among algal strains on fungal growth at day 3 (a) are given as adjusted means ± 95 % confidence interval obtained from Tukey's post hoc test (ANCOVA). Panels b-f illustrate the absolute average radial growth of *S. rolfsii* exposed to aqueous extracts of *Nannochloropsis* sp. (b), *Phaeodactylum tricornutum* (c), *Scenedesmus obliquus* (d), *Chlorella vulgaris* (e) and *Spirulina* sp. (f) at different days (1, 2 and 3) and concentrations (0.1, 0.5, 1.0 and 2.0 g L^{-1}. Dunnett's two sided post-hoc test (ANOVA) indicated significant differences (*) compared to the control group (b-f). Error bars represent standard deviations. Data points at each day (b-f) are shown as mean ± SD (n=3).

4.2 *Rhizoctonia solani*

An overall ANCOVA model (R^2=0.398) considering extract concentrations as co-variate showed significant differences in mycelial growth of the pathogenic fungus *R. solani* on day 3 of the experiment. Type III sum of squares analysis showed a significant effect of the microalgal strains tested (F=28.38), while the effects of the concentrations used were insignificant (F=2.17). Tukey`s post-hoc test revealed significant growth suppression of *R. solani* by up to 5.07 ± 3.69 % by *Nannochloropsis* sp. extracts, while aqueous extracts of *P. tricornutum* suppressed the average radial growth by up to 18.35 ± 3.45 % (p<0.05, Fig. 2a). *S. obliquus* and *C. vulgaris* extracts showed no antifungal effect (p>0.05), whereas *Spirulina* sp. extracts suppressed the pathogen growth by up to 5.56 ± 3.68 % on day 3 (p<0.05, Fig. 2a).

The statistical analysis of *P. tricornutum* extract concentrations at each time point revealed a growth suppression of the pathogen up to 3.14 ± 0.41 cm (0.1, 0.5 and 1.0 g L^{-1}) compared to 4.08 ± 0.06 cm in the control group (Fig. 2c). Moreover, *R. solani* mycelia grew until reaching 3.75 ± 0.05 cm in aqueous *Spirulina* sp. extracts at the concentration of 1.0 g L^{-1}, compared to 4.10 ± 0.05 cm in the control group (p<0.05). Bioassays at the concentrations of 0.1, 0.5 and 2.0 g L^{-1} resulted in insignificant suppression of the pathogenic fungus growth (Fig. 2f). *Nannochloropsis* sp., *S. obliquus* and *C. vulgaris* extracts of all concentrations at each time point showed no significant effects on fungal growth (Fig. 2b, d and e).

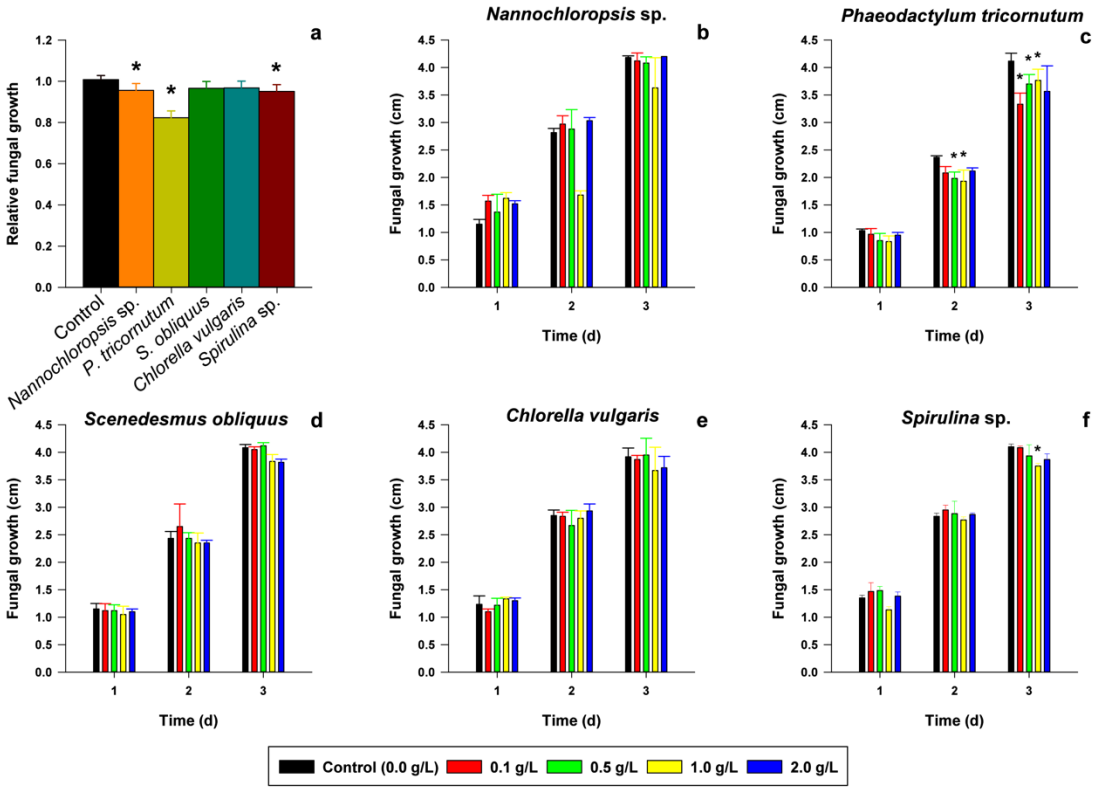

Figure 2.3: Rhizoctonia solani exposed to microalgal extracts. Relative differences among algal strains on fungal growth at day 3 (a) are given as adjusted means ± 95 % confidence interval obtained from Tukey's post hoc test (ANCOVA). Panels b-f illustrate the absolute average radial growth of *R. solani* exposed to aqueous extracts of *Nannochloropsis* sp. (b), *Phaeodactylum tricornutum* (c), *Scenedesmus obliquus* (d), *Chlorella vulgaris* (e) and *Spirulina* sp. (f) at different days (1, 2 and 3) and concentrations (0.1, 0.5, 1.0 and 2.0 g L^{-1}). Dunnett's two sided post-hoc test (ANOVA) indicated significant differences (*) compared to the control group (b-f). Error bars represent standard deviations. Data points at each day (b-f) are shown as mean ± SD (*n*=3).

4.3 *Botrytis cinerea*

The ANCOVA model (R^2=0.610) revealed major effects of the tested strain (F=66.90; p<0.05) and the used concentration (F=34.62; p<0.05). *P. tricornutum* and *S. obliquus* significantly suppressed *B. cinerea* growth up to 11.47 ± 2.06 % Fig. 3a). *Nannochloropsis* sp., *C. vulgaris* and *Spirulina* sp. extracts showed no antifungal activity against *B. cinerea* on day 3 (p>0.05; Fig. 3a).

Statistical analysis (ANOVA) of extract concentrations at each time point revealed that aqueous extracts of *P. tricornutum* at the concentrations of 0.1, 0.5 and 1.0 g L^{-1} significantly inhibited the pathogen growth. This growth inhibition resulted from an average mycelial

growth of 2.31 ± 0.09 cm (0.1, 0.5 and 1.0 g L^{-1}) compared to 2.55 ± 0.05 cm in the control group (Fig. 3c). The growth of *B. cinerea* in bioassays with aqueous *S. obliquus* extracts at the concentrations of 0.1 and 0.5 g L^{-1} (2.18 ± 0.12 cm) significantly differed from the control group (2.55 ± 0.05; Fig. 3d). *Nannochloropsis* sp. and *Spirulina* sp. extracts of all concentrations at each timepoint showed no significant difference to the control group (Fig. 3b and f).

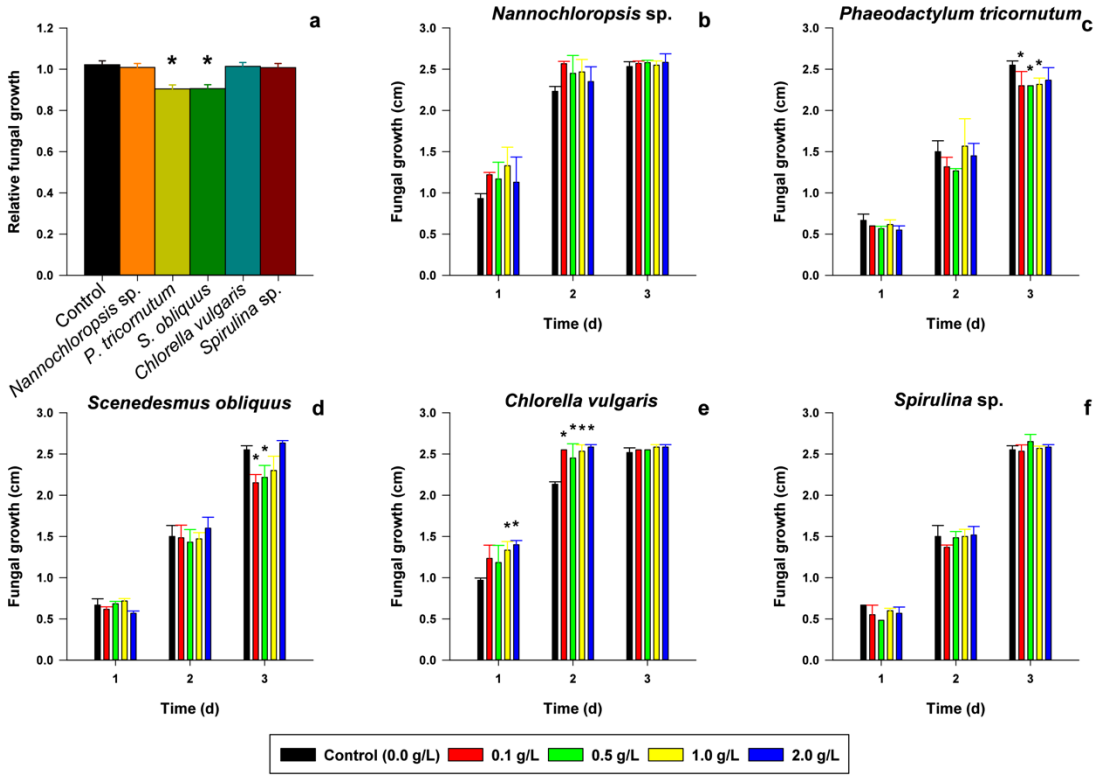

Figure 2.4: Botrytis cinerea exposed to microalgal extracts. Relative differences among algal strains on fungal growth at day 3 (a) are given as adjusted means ± 95 % confidence interval obtained from Tukey's post hoc test (ANCOVA). Panels b-f illustrate the absolute average radial growth of *B. Cinerea* exposed to aqueous extracts of *Nannochloropsis* sp. (b), *Phaeodactylum tricornutum* (c), *Scenedesmus obliquus* (d), *Chlorella vulgaris* (e) and *Spirulina* sp. (f) at different days (1, 2 and 3) and concentrations (0.1, 0.5, 1.0 and 2.0 g L^{-1}). Dunnett's two sided post-hoc test (ANOVA) indicated significant differences (*) compared to the control group (b-f). Error bars represent standard deviations. Data points at each day (b-f) are shown as mean ± SD (*n*=3).

4.4 Alternaria alternata

An overall ANCOVA model (R^2=0.202) fitted to the mycelial growth of the pathogenic fungus *A. alternata* on day 3 of the experiment revealed minor effects of the tested microalgal strains (F= 9.53, p<0.05) and the used extract concentration (F=5.29, p>0.05). *A. alternata* mycelia grew faster in bioassays with aqueous extracts of *Nannochloropsis* sp. (16.05 ± 12.78 %, p<0.05), while no significant antifungal activities of the remaining microalgal extracts were found (*Nannochloropsis* sp., *P. tricornutum*, *S. obliquus*, *C. vulgaris* and *Spirulina* sp.; Fig.4a). Analysis of variances (ANOVA) showed that aqueous *Nannochloropsis* sp. extracts of all tested concentrations (0.1, 0.5, 1.0 and 2.0 g L^{-1}) significantly promoted the pathogen growth, resulting in an average fungal radial growth of 1.63 ± 0.05 cm, compared to 1.18 ± 0.25 cm in the control group (day 3, Fig. 4b).

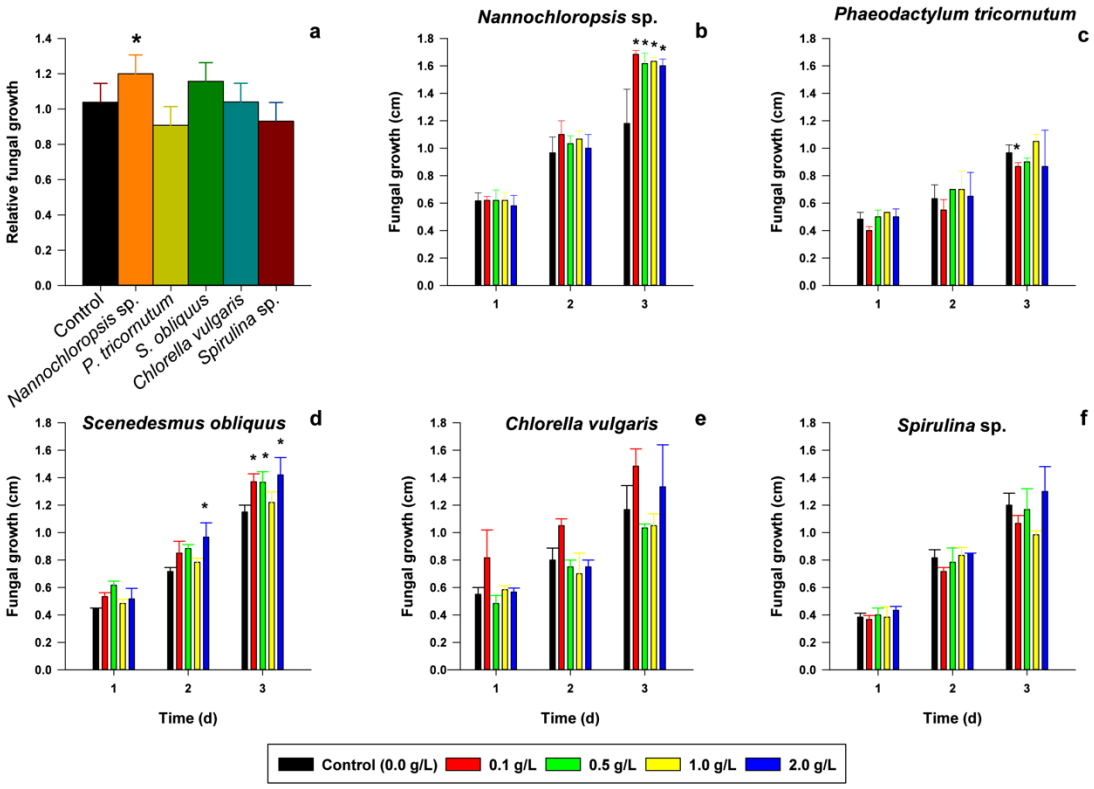

Figure 2.5: Alternaria alternata exposed to microalgal extracts. Relative differences among algal strains on fungal growth at day 3 (a) are given as adjusted means ± 95 % confidence interval obtained from Tukey's post hoc test (ANCOVA). Figures b-f illustrate the absolute average radial growth of *A. alternata* exposed to aqueous extracts of *Nannochloropsis* sp. (b), *Phaeodactylum tricornutum* (c), *Scenedesmus obliquus* (d), *Chlorella vulgaris* (e) and *Spirulina* sp. (f) at different days (1, 2 and 3) and concentrations (0.1, 0.5, 1.0 and 2.0 g L^{-1}). Dunnett's two sided post-hoc test (ANOVA) indicated significant differences (*) compared to the control group (b-f). Error bars represent standard deviations. Data points at each day (b-f) are shown as mean ± SD (n=3).

5.) Discussion

Our results indicated specific suppression of the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani* and *Botrytis cinerea*. *Phaeodactylum tricornutum* was the only microalgal species showing antifungal effects against all target fungi except *Alternaria alternata*. The most significant antagonistic effects were obtained against *S. rolfsii*. Our statistical analysis revealed this pathogen growth was inhibited by up to 32.01 ± 4.82 % upon incubation with *Scenedesmus obliquus* extracts (day 3), followed by those of *Nannochloropsis* sp. (13.96 ± 5.26 %) and *P. tricornutum* (10.25 ± 5.36 %). However, antagonistic impacts were highly dependent on the combination of microalga and target fungus, which might indicate specificity in the interaction observed between them. Strong target specificity is a significant concern in the application of biocontrol agents and maximizes their effectiveness against the fungus whose growth needs to be inhibited [33]. Notably, there is a trend in the literature that various eco-friendly methods of natural pathogen control are considered as "biocontrol agents" without fitting the definition [34]. According to Eilenberg *et al*. [35], biological control is defined as the use of living organisms that suppress the impact of specific pests and diseases. Since the present study focused on the application of dried microalgal powder, we define microalgae with inhibitory effects against phytopathogenic fungi as "algal fungicides".

To the best of the authors' knowledge, aqueous extracts of *Nannochloropsis* sp., *P. tricornutum* and *S. obliquus* have not previously been reported to impact the growth of the phytopathogenic fungi tested. Scaglioni *et al*. [28] showed that natural free phenolic extracts of *Nannochloropsis* sp. and *Spirulina* sp. mitigated contamination by fungal phytopathogens of the *Fusarium* complex. This may be related with the antifungal properties of various bioactive compounds with a phenolic acid profile such as chlorogenic acids [28,36]. Moreover, recent studies suggested that antimicrobial peptides should also be considered as possible agents for microbial growth inhibition [37,38]. Another possibility would be the occurrence of carotenoid pigments such as astaxanthin, *β*-carotene, canthaxanthin, neoxanthin, violaxanthin or zeaxanthin, which are considered to be powerful antioxidant pigments with antimicrobial properties [39,40]. Furthermore, eicosapentaenoic acid, a polyunsaturated fatty acid biosynthesized by the eukaryotic microalga *P. tricornutum*, showed antibacterial properties against various Gram-negative and Gram-positive bacteria [41,42]. However, since our study researched the effect of aqueous microalgal extracts, the successful growth inhibition of *S. rolfsii* and *R. solani* (by up to 32%) is unlikely to be related

with either carotenoids or fatty acids, which are extracted mainly by solvents with lower polarity. Instead, water soluble compounds such as flavonoids have been reported to exhibit diverse biological activities including antibacterial, antifungal and antiviral effects [43]. Moreover, Ulanowska *et al*. [44] showed that microbial DNA, RNA and protein synthesis was powerfully inhibited by flavonoids. The successful growth inhibitions of *S. rolfsii* (10.25 ± 5.36 %), *R. solani* (18.35 ± 3.45 %) and *B. cinerea* (11.47 ± 2.06 %) by *P. tricornutum* may thus be explained with the antifungal activities of these bioactive compounds. However, further studies are required to evaluate *P. tricornutum* biomass as an algal fungicide.

Several studies found strong evidence for the antimicrobial properties of *Scenedesmus* sp. extracts [45,46,47]. For example, Marrez *et al*. [47] found that aqueous *S. obliquus* extracts inhibited the growth of various mycotoxigenic fungi (*Aspergillus flavus, Aspergillus steynii, Aspergillus westerdijikia* and *Aspergillus carbonarius*). Furthermore, Dantas *et al*. [48] evaluated aqueous *Scenedesmus subspicatus* extracts as growth inhibitors of *Bacillus subtilis*. Our statistical analysis revealed that *S. obliquus* showed the strongest mycelial growth inhibition of *S. rolfsii* by up to 32.01 ± 4.82 %, which may be related with the cytotoxic activity of bioactive *S. obliquus* metabolites towards the pathogen [47,49]. However, usage of aqueous *S. obliquus* extracts as sources of algae-based fungicides requires further study.

Antimicrobial activities of *Chlorella vulgaris* towards pathogens were extensively investigated and many authors highlighted *C. vulgaris* as potential source for bioactive compounds [13,50,51]. Chlorellin, a fatty acid mixture, was the first reported isolated antimicrobial compound that showed inhibitory effects towards Gram-negative and Gram-positive bacteria [52]. However, to the best of our knowledge, chlorellin was not evaluated as antifungal substance and hence should be addressed in future research. In addition, Vehapi *et al*. [13] reported antifungal activities of *C. vulgaris* extracts against *A. niger* and *Fusarium oxysporum*. Ghasemi *et al*. [53] showed antifungal activities of *C. vulgaris* against *Candida kefyr, Aspergillus fumigatus* and *Aspergillus niger*. Vehapi *et al*. [13] reported strong mycelial growth inhibition of *A. niger* by *C. vulgaris*, which may be related with the presence of bioactive terpenes in this microalgae strain [54]. However, since our study focused on aqueous extraction of microalgal biomass, liposoluble or hydrophobic compounds (e.g., terpenes and fatty acids) are unlikely to be the source of fungal inhibition. Interestingly, in the latter report, unlike the strain tested in the present study, aqueous extracts of *C. vulgaris*

were efficient regarding fungal inhibition. Therefore, it seems as though fungal inhibition may be a strain-specific bioactivity that needs to be further explored.

Most of the reviewed studies screened antifungal properties of prokaryotic cyanobacteria whereas the antifungal properties of many eukaryotic microalgae are still unknown. Cyanobacteria are widely known as potential source for bioactive compounds that might inhibit the growth of phytopathogenic fungi [29]. The production of such compounds may be responsible for the growth inhibition of *R. solani* by *Spirulina* sp. (3.75 ± 0.05 cm, compared to 4.10 ± 0.05 cm in the control group on day 3) in the present study [55,56]. Shishido *et al*. [17] studied antifungal properties of cyanobacteria and detected the antifungal macrolide scytophycin in methanolic extracts of *Anabaena* sp. HAN21/1, *Anabaena cf. cylindrica* PH133, *Nostoc* sp. HAN11/1 and *Scytonema* sp. HAN3/2. Moreover, the authors found evidence for the antifungal potential of the glycolipopeptide hassallidin metabolized by *Anabaena* spp. (BIR JV1 and HAN7/1) and *Nostoc* spp. (6sf Calc and CENA 219). Similarly, most antimicrobial peptides (AMP), such as cyclic hassallidin peptides, are known to exert their antifungal activity on the cell wall of the pathogen [57]. Hassalidins are synthesized by non-ribosomal biosynthetic enzymes (NRPS) [27]. Battah *et al*. [55] identified the antifungal potential of 50 purified antagonistic agents from *Spirulina maxima* and showed a growth inhibition of *Penicillium oxalicum* (91 %), *Fusarium solani* (65 %) and *R. solani* (20 %). Vestola *et al*. [58] found evidence that hassallidin D, produced by cyanobacteria of the genera *Anabaena*, *Aphanizomenon*, *Nostoc* and *Tolypothrix*, exhibits antifungal activity against *Candida* strains. Many other representatives of bioactive compounds were shown to exhibit antifungal activities including fischerellin A, balticidins, hapalindole, carazostatin, phytoalexin, tolytoxin, scytophycin, toyocamycin, tjipanazole, nostocyclamide, nostodione and nostofungicidine [17]. Furthermore, Rajamanickam *et al*. [59] showed that *Spirulina* sp. extracts can be considered as promising source for silver nanoparticles with antifungal potential. Conversely, our statistical analysis revealed that *Spirulina* sp. extracts promoted the growth of the fungal pathogen *S. rolfsii* up to 20.79 ± 6.25 %. *Spirulina* sp. biomass was reported with high contents of potent high purity polysaccharides such as the high-molecular-weight polysaccharide fraction immolina [60]. Notably, carbohydrate polymers constitute the build-up material for fungal cell and rapid fungal growth might be related with potent high purity polysaccharides metabolized from *Spirulina* sp. [61]. Growth promotion was also observed for *A. alternata* growth in aqueous *Nannochloropsis* sp. extracts (1.63 ± 0.05 cm,

compared to 1.18 ± 0.25 cm in the control group on day 3). As pathogen-specificity of algal fungicides is evident, the use of microalgae as sources of fungicidal activity should be implemented with caution in order to exclude growth promotion of pathogens. Therefore, the positive identification of the pathogen is recommended before scaling up the application of algae-based fungicides. Applying microalgae-based fungicides should be restricted to microalgae that do not promote the growth of any pathogen as, for example, *P. tricornutum.* Since *P. tricornutum* is a diatom, future studies may include other closely related microalgae to examine if this property is exclusive to this particular species or can be considered as a trait found in other ochrophytes.

No growth response relationship between extract concentrations and fungal growth were found. Algal extracts at the concentrations 0.1, 0.5 and 1.0 g L^{-1} inhibited fungal growth more effectively (7 growth inhibitions each) than 2.0 g L^{-1} extracts (4 growth inhibitions). Recent studies reported a greater antifungal potential of natural microalgal extracts than synthetic extracts, suggesting that purification prior to their application is not essential [28,62]. Purification steps and the complexity of extraction methods are still a limiting factor in the large-scale application of algal fungicides [63]. Hence, this study investigated the antifungal properties of natural aqueous microalgal extracts *in vitro* at concentrations 0.1, 0.5, 1.0 and 2.0 g L^{-1}.

6.) Conclusion

This study showed the promising antifungal application of aqueous extracts from *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Scenedesmus obliquus* and *Spirulina* sp. *in vitro*. Growth suppression was observed against the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani* and *Botrytis cinerea*. Statistical analysis revealed the highest inhibition values overall for *S. obliquus* extracts which inhibited *S. rolfsii* growth by up to 32.01 ± 4.82 %, followed by *Nannochloropsis* sp. extracts (13.96 ± 5.26 %). Of the five microalgae under study, only *P. tricornutum* extracts inhibited the growth of the three pathogens *S. rolfsii* (10.25 ± 5.36 %), *R. solani* (18.35 ± 3.45 %) and *B. cinerea* (11.47 ± 2.06 %). Antagonistic activities varied widely depending on the combination of microalga and target fungus. This indicates strong target specificity and requires further investigation to ensure successful biological control of fungal pathogens. Since fungal growth promotion was observed for *A. alternata* and *S. rolfsii* in aqueous extracts of *Nannochloropsis* sp. and *Spirulina* sp.

respectively, further studies need to be performed to clarify these exceptions. Taken together, these results suggest that algal fungicides should be considered as a promising eco-friendly alternative to achieve a higher sustainability in modern agriculture by limiting the overuse of agrochemicals. While most research focuses on antagonistic activities of isolated bioactive compounds, our study shows that aqueous extracts of dried microalgal powder can be applied to fight pests without prior compound isolation. Moreover, as microalgal biomass is also considered as source of plant growth promoting compounds (e.g., phytohormones), biostimulation of crops could be achieved as a secondary outcome, which would be prevented if bioactive compounds were extracted and used separately. Future research should address the fungus-alga antagonism with models that assay the interaction between plants, the phytopathogenic fungus and algal extracts *in vivo*. In order to overcome the limitations of the current *in vitro* study, research on such models will be carried out in the near future.

7.) Acknowledgements

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III.) Chapter 3: Evaluation of microalgae-amended organic compost

1.) Abstract

The main goal of this study is to evaluate the influence of supplemented dry microalgal powder on important descriptive composting parameters. In the final phase of the composting process, all microalgae-amended piles showed no major parameter variations in comparison with the control group (pH, electrical conductivity, organic matter, mineral matter, temperature, volume, phytotoxicity). Moreover, all microalgae-amended composts were approved as non-phytotoxic due to germination indexes ranging between 68.0 and 70.4%. Hence, composting of microalgae should be considered as safe methodology to enrich composting masses without negatively influencing important physical and chemical composting parameters of the final product.

For the aforementioned purpose, this study has investigated the supplementation of an organic residue's mixture with dried microalgal biomass of *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Scenedesmus obliquus* and *Chlorella vulgaris*. To the best of our knowledge, this is the first report analyzing the enrichment of fresh composting materials with dry microalgal powder. Since microalgae metabolize numerous plant growth-promoting amino acids and phytohormones, they could further improve plant stimulating characteristics of composts such as the release of growth promoting nutrients. Decomposition rates depend on metabolic activities of microbial populations that rely on the availability of various micro and macronutrients. Hence, co-composting of nutrient-rich microalgal biomass may mold microbial communities and improve final compost quality based on a richness in nutrients such as phosphorus, nitrogen and potassium. Therefore, microalgae fortified compost should be considered as promising sustainable alternative to further increase crop yields among the global agricultural sector. Because of its potential to transform and recycle wastes of different origins into organic material, composting will play a key role on the way to a sustainable society. However, the true potential of microalgae-amended compost still awaits validation by means of field or greenhouse trials.

Keywords: Algal compost, sustainable agriculture, biostimulation, microalgae

2.) Introduction

Microalgal biomass is considered as promising feedstock for multi-functional applications in modern sustainable agriculture (Renuka et al., 2018). Because of their richness in plant stimulating bioactive metabolites, microalgae can lead to higher agronomic yields and may represent a key role in reducing the excessive usage of agrochemicals in future decades (Ronga et al., 2019). Long-term exposure to agrochemicals causes major human health effects and negatively impacts the sustainability of agroecosystems (Kim et al., 2017; Bretveld, 2006; Bassil, 2007). Therefore, alternative and sustainable strategies are of urgent need to achieve increasingly higher crop yields ensuring the coverage of a rising global food demand (Tilman et al., 2011; Popp et al., 2012).

Composting is considered as microorganism-mediated, aerobic process of degrading fresh organic material into a stabilized humus-rich product (Cooperband et al., 2002). Metabolic activities of different microbial populations along the composting process might be enhanced by the addition of microalgae and bioconversions of organic materials accelerated (El-Gamal, 2011; Jiang et al., 2012). Moreover, compost can serve as a microalgae-carrier for plant growth stimulation and plant disease protection (Dukare et al., 2011; Renuka et al., 2018). Various studies evaluated the microalgae as biostimulant and reported soil amendment with either dry or suspended liquid algal biomass as booster for crop productivity (Coppens et al., 2016; Elarroussia et al., 2016; Ronga et al., 2019). Conversely, reports on microalgae-amended compost are scarce and do not contain clear guidelines for large scale applications (Han et al., 2014). Prasanna *et al.* (2015) investigated the potential of cyanobacteria fortified compost and showed that the addition of *Anabaena* sp. and *Calothrix* sp. promoted seed germination and crop yields of cotton plants, when used as substrate. Moreover, Renuka *et al.* (2017) used compost as carrier for selected microalgal strains and observed a significant enhancement of soil micronutrient availability. Since algae are known to be rich in micro- and macronutrients, amino acids, vitamins, phytohormones and many other plant growth promoting substances, they can be used to enrich composting masses and further stimulate the overall plant productivity (Renuka et al., 2018). Based on the ability of microalgae to promote plant growth and improve soil quality, compost amendments should be further investigated as promising alternative strategy to limit the use of environmentally harmful chemical fertilizers (Elarroussi et al., 2016; Han et al., 2014; Ronga et al., 2019). While most published literature focusses on co-composting macroalgal biomass, microalgal compost

amendments are yet to be researched in earnest (Han et al., 2014). Furthermore, reports about co-composting dried algal biomass are still scanty and further research is required to evaluate the influence on physical and chemical composting parameters.

Large scale applications of algal composts as fertilizer or soil conditioner could diminish agricultural pollution such as eutrophication, biodiversity loss and soil infertility (Calvo et al., 2014). Despite these positive indications for agricultural applications, potentially high toxin contents, accumulations of metals and high salinity may have negative consequences for the final use as fertilizer (Han et al., 2014).

3.) Material and methods

The experiments were carried out at the Campus of Gambelas, University of Algarve, Portugal (37°02′35.45″N, 7°58′20.64″W)

Microalgae-amended compost production

Dry microalgal biomass of *Nannochloropsis* sp., *Phaeodactylum tricornutum, Scenedesmus obliquus* and *Chlorella vulgaris* was co-composted with grass cuttings, grape mark residues and non-conform oranges (1.1:1:0.9 v/v). Six cylindric composting bins were manufactured with wire net and plastic sun scream to allow aeration while controlling excessive heat losses in order to maintain a high temperature. The composting piles were filled stepwise with 785 L of mixed raw composting materials and dry microalgal biomass in order to obtain a carbon-to-nitrogen ratio close to 25-30. According to Fong *et al.* (1999), a C/N ratio between 25 and 30 is considered as optimal range for the initial phase of composting. The composting piles were aerated by manual turning, 7 times along the composting process, when temperatures remained steady or decreased (on days: 7,19,33,53,80,108 and 165). We produced one composting pile per microalgae (1.0 g L^{-1}), plus an additional pile with *C. vulgaris* at a concentration 0.1 g L^{-1} (P6), as well as one control pile without microalgae amendment (P3; Table 3.1).

Table 3.5: Composting piles

Pile	Microalgae amendment	Concentration
P1	*Nannochloropsis* sp.	1.0 $g.L^{-1}$
P2	*Phaeodactylum* sp.	1.0 $g.L^{-1}$
P3	Control	-

P4	*Scenedesmus obliquus*	1.0 $g.L^{-1}$
P5	*Chlorella vulgaris*	1.0 $g.L^{-1}$
P6	*Chlorella vulgaris*	0.1 $g.L^{-1}$

Monitoring of various compost-parameters

Various parameters were determined along the whole composting process of 207 days and used as indicators for compost maturity. Aqueous compost extracts (1:2 v/v) were prepared for pH measurements (potentiometer; Crison Micro pH, 2001) and the filtered suspension used to determine electrical conductivity (conductivity meter; Crison 522). Moreover, organic and mineral matter (Ramos et al., 1987), as well as dry matter (Martinez, 1992) were monitored. All previously mentioned analyses were performed on days 1,3,7,19, 33, 53, 80, 108, 165 and 207 of the composting processes. Variations in temperature were monitored with a portable probe that measures the temperature inside the composting pile at 6 different vertical points (TP 62, Umwelt Elektronik GmbH & Co.KG, Germany). Temperature measurements were averaged and stopped when values remained constant. In order to calculate the loss of volume during the composting process, height-measurements of the biomass inside the composting pile were taken at 4 different spots and averaged. Germinations tests with *Lepidium sativum* L. were performed according to Zucconi *et al.*, (1985) on days 80, 108, 165 and 207 to evaluate the phytotoxicity of the composts.

Microalgal biomass

Dry biomass of *Nannochloropsis* sp. and *Phaeodactylum tricornutum* was obtained from NECTON S.A. (Faro, Portugal), while *Scenedesmus obliquus* and *Chlorella vulgaris* was obtained from Allmicroalgae Natural Products (Leiria, Portugal). The detailed biochemical composition is provided in Table 3.1.

Statistical analysis

Apart from the germination test, all analyzed parameters were treated as non-independent observations and considered as technical replicates. Dunnett's two sided post-hoc test (ANOVA) was performed to indicate significant differences in phytotoxicity between microalgae enriched composts and the control group. A significance level (α) of 0.05 was used for all performed tests.

4.) Results & Discussion

a) pH

As composting proceeds, the organic material undergoes different phases of pH levels, depending on the chemical composition of the composting mass and the actual composition of microbial communities (Sundberg et al., 2013). Hence, changes in pH are predictable as the pH values of the composting mass follow a characteristic curve (Sánchez et al., 2017). The release of organic acids through volatilization enhances a characteristic pH decrease during the initial phase of composting (Beck-Friis et al., 2003). Except for *Nannochloropsis* sp. and *P. tricornutum* amended compost mixtures, none of the compost piles showed this predicted initial drop in pH (Fig. 3.1a). This may be related with the low pH values (3.62 - 4.74) of all piles at the beginning of the composting process, most likely enhanced by the high acidity of the raw composting materials, particularly that of the oranges (Fig. 3.1a). De Bertoldi et al. (1983) reported that all kinds of organic materials with a pH range from 3 to 11 can be composted, while optimal pH values for successful composting lie between 5.5 and 8.0. The pH values in all piles tended to rise gradually towards alkaline levels along the composting process (Fig. 3.1a). This characteristic rise in pH is mainly related with ammonium release, enhanced by mineralization of organic nitrogen (Beck-Friis et al., 2003; Finstein & Morris, 1975). The pH curves of *S. obliquus* (P4) and both *C. vulgaris* (P5 and P6) amended compost piles showed a faster increase and steeper slopes, while pH values in the final phase of composting lay within a range of 8.39 and 8.85 for all piles (Fig. 3.1a). According to Karak *et al.* (2013), final pH values of all piles are not in the acceptable range of 6.5 - 7.5 for mature compost. Bunt (1988) reported 5.2 - 6.3 as optimal pH range for plant growing media. Therefore, high pH values of the final compost should be taken into account when applying compost as growing media component for alkaline-sensitive plants (Benito et al., 2006). In summary, microalgae amendment showed no major influences on pH variations along the composting process when compared with the control pile (Fig. 3.1a).

b) Electrical conductivity (EC)

Electrical conductivity indirectly describes the salinity level and indicates the total amount of soluble ions, which may cause phytotoxic or phytoinhibitory effects such as nutrient stress or plant antioxidant enzyme activities when in excess (Zaha et al., 2013; Ding et al., 2018). EC is influenced by various soil fertility parameters such as pH, available phosphorus and potassium, exchangeable calcium and magnesium, availability of micronutrients and cation

exchange capacity (CEC) (Carmo, 2016). Moreover, high levels in salinity can cause lysis of microbial cells and might harm the compost microbiome (Brock et al., 1994). Santamaria-Romero and Ferrera-Cerrato (2001) reported negative effects on the composting process when values exceed 8 dS m^{-1}. On day 1, *P. tricornutum* amended compost showed 4.53 ± 0.32 dS m^{-1}, the highest EC value, while all other piles showed EC values in a range between 2.11 and 2.94 dS m^{-1} (Fig. 3.1b). Both *C. vulgaris* composts showed unexpected peaks on day 3 and 7. The decomposition process of organic materials may enhance an EC-increase through the release of mineral salts such as ammonia and phosphate (Gondek et al., 2020). After the first turn (day 19), all microalgae amended composts showed no major differences in EC compared to the control compost (Fig. 3.1b). EC values in all piles tended to rise until the 4^{th} turn (day 80) followed by a drop at turn 5 (day 108; Fig. 3.1b). In the final phase of composting, all piles showed EC values within a range of 3.40 and 4.30 dS m^{-1}. According to Zaha *et al.*, 2013, only P6 (*C. vulgaris* 0.1 g/L) shows final EC values (3.40 ± 0.20 dS m^{-1}) that lie in the optimal range for the use of compost as biofertilizer (2.0 - 3.5 dS m^{-1}). Since marine algae are considered as highly saline composting feedstock (Han et al., 2014), greater variations between the control group and all microalgae amended composts were expected. Coelho *et al.* (2020) studied similar composting processes without the addition of microalgal biomass and obtained relatively high EC values as well. Therefore, the addition of dried microalgae (0.1% and 1%) appears to have no influence on EC values of the final composts. Sullivan *et al.* (2018) reported that acceptable EC values for mature compost also depend on various application-related factors such as irrigation water management, soil texture and compost application rate. When applying highly saline composting masses as substrate for salt intolerant plants, germination processes and yields will be affected (Liu et al., 2014), hence application doses must be adjusted to the salinity of the product.

c) Dry matter

Compost moisture was determined and monitored by conventional dry matter determination according to Martinez (1992). In the initial phase of composting, dry weight contents between 32.2 and 38.0% were measured in all piles. This is most likely related with the high moisture content of composted oranges. Moreover, changing atmospheric conditions influence dry matter content of composts (Wojcieszak et al., 2015). On day 7, unexpected peaks in the range between 40.8 and 52.1% were observed for all piles except the *Nannochloropsis* sp. amended pile (P3; Fig. 3.1c). This may be related with primary

degradations of oranges and the associated water release. Monitoring moisture during biodegradation might optimize the composting rate since sufficient compost stabilization is only given when the composting materials are neither too dry nor too wet (Hamelers & Richard, 2001). Wojcieszak *et al.* (2015) reported 30-50% as optimal dry matter values along the composting process. According to Day & Shaw (2005), moisture values below 40% can interfere with the composting process, while an excessive moisture content (> 60%) prevents oxygen diffusion and causes asphyxiation, often combined with the emission of odors. In the final days of composting, dry weight of all piles stabilized in a range between 45.3 and 48.7% (Fig. 3.1c). Hence, co-composting of all tested microalgae strains showed no major effect on dry weight variations in comparison with the control pile.

d) Organic matter (volatile solids content)

Initial organic matter content ranged from 87.66 to 90.44% for all produced composts and decreased gradually with slight deviations until the final day of composting (Fig 3.1d). In different phases of the composting process, heterotrophic microbes break down complex organic compounds into stabilized humus-rich compost (Cooperband et al., 2002). Hence microorganism-induced organic matter degradation affects temperature evolution, high organic matter content may prolong the thermophilic phase. Therefore, precise determination of organic matter content is necessary to analyze the decomposition dynamics of organic matter over a period of time (Hogsteen et al., 2015). Final organic matter content ranged from 58.0 to 62.8% (Fig 3.1d), indicating a successful degradation of composting material. In comparison with the control pile, co-composting of all tested microalgae strains showed no major effect on organic matter content variation during the entire composting process. Organic matter was determined by the loss of ignition (LOI), a methodology in which a compost sample is ignited to high temperatures in a muffle furnace (Ramos et al., 1987). The weight lost during combustion, referred as ash content, was used to estimate organic matter content by reciprocation (Hsu et al., 1999). In literature, the ignition temperature ranges from 375º to 1025 ºC (Donkin, 1991) and the heating time varies unevenly (Matthiessen et al., 2005), but 560ºC is a typical value. There is no universal standard protocol and multiple factors, such as ignition temperature, furnace type and heating time may influence the precision of measurements (Hogsteen et al., 2015).

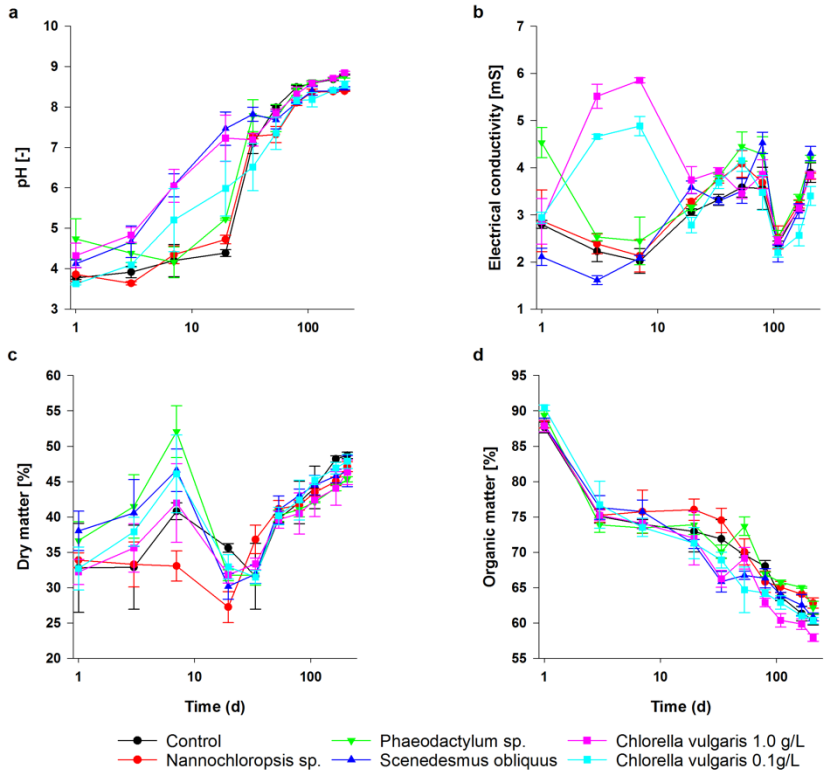

Figure 3.1 - Changes in pH (a), electrical conductivity (b), dry matter (c) and organic matter (d) over time. Data points at each day are shown as mean ± SD (n=3). Error bars represent standard deviations.

e) Temperature

Initial temperature for all composts ranged from 22.67 to 31.33 °C (Fig. 3.2a). During the first stage of composting, mesophilic microorganisms (15-40 °C) break down organic material through the hydrolysis of sugars, amino acids and lipids (de Bertoldi et al., 1983). Exothermic metabolic activity of mesophilic microbes might increase the temperature of the composting mass up to a maximum of 85°C (Sánchez et al., 2017). In order to remove potential pathogens such as *E. coli* and *Salmonella*, the composting mass must have temperatures above 55 °C for at least two weeks (Droffner and Brinton, 1995). Between days 30 and 50, P1(*Nannochloropsis* sp.), P2 (*P. tricornutum*) and P4 peaked (*S. obliquus*) between 58.9 and 64.3 °C. P6 (*C. vulgaris* 0.1 $g.L^{-1}$) showed the highest value for temperature with 69.9 °C on day 51, followed by P5 (*C. vulgaris* 1.0 $g.L^{-1}$) with 63.1 °C on day 52. P1 peaked again on day 62 with 52.5 °C (Fig. 3.2a). While all microalgae-amended composts showed temperatures between 36.6 and 47.6 °C on day 55, P3 (control) showed the lowest temperature, 21.9 °C (Fig. 3.2a). The rise in temperature enhances thermophilic

microorganisms to be replaced by mesophilic microorganisms, which initiates the second phase of composting, defined as the "thermophilic phase" (Insam & de Bertoldi, 2007). This phase is characterized by the presence of thermophiles (mainly actinobacteria) that degrade complex molecules like lignin, cellulose, hemicellulose and proteins through enzymatic activities (Bernal et al., 2009). High temperatures are essential to eliminate the viability of seeds and to degrade phytotoxic agents (Cooperband, 2002). As soon as energy sources are depleted, the composting mass cools down ("cooling phase") and reaches temperatures between 15 °C and 35 °C, causing a second colonization by mesophilic microbiota (Sánchez et al., 2017). During the maturation phase of the compost, humus-like substances are formed (Cooperband, 2002). After 100 days of composting, temperature for all composts stabilized in a range between 16.10 and 18.20 °C (Fig 3a).

f) Volume

The relative reduction of volume along the composting process ranged from 70.0 to 77.2% for all compost piles (Fig. 3.2b). The average initial volume of all piles (785 L) decreased to final values between 179.00 and 235 L and remained constant after day 100 (Fig. 3.2b). Reduction of mass and volume during the composting process can be considered as important key parameter regarding compost operation management and facility design (Breitenbeck & Schellinger, 2013).

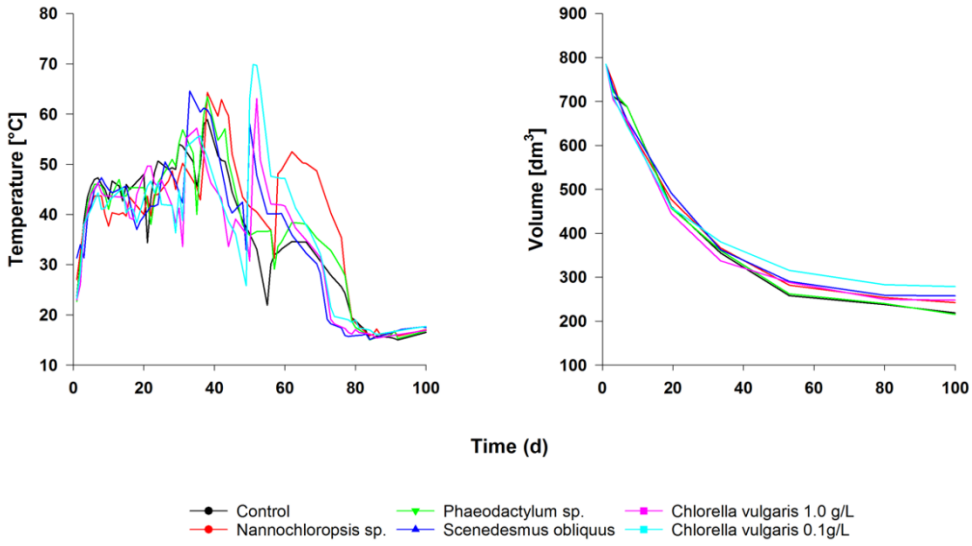

Figure 3.12 - Changes in temperature (a) and volume (b) over time. Figure a represents average temperatures of 6 different vertical points of the composting pile.

g) Phytotoxicity

Germination tests are bioassays quantifying the inhibitory effects of phytotoxins that can cause disease symptoms in plants (Strobel, 1982; Wrap et al., 2002). Zucconi *et al.* (1985) and Zucconi & De Bertoldi (1987) reported composts with germination rate index (GI) values above 60% as non-phytotoxic. The first germination test was performed on day 80 of the composting process and revealed significant difference between the control group (23.6 ± 0.39% GI; P3) and all microalgae-amended composts, except the *S. obliquus* enriched pile (11.6 ± 9.78% GI; P4; Fig 3.3). GI's for *Nannochloropsis* sp. (P1), *P. tricornutum* (P2) and both *C. vulgaris* (P5, P6) fortified composts ranged from 1.1 to 8.9% (Fig. 3.3). All composts tended to rise gradually towards GI values above 60% which indicate non-phytotoxicity (Zucconi et al., 1985; Zucconi & De Bertoldi, 1987; Emino & Warman, 2004; Rey et al., 2008). On day 108, P2 showed 48.6%, a significantly higher GI ($p<0.05$) than that of the control pile (26.6%). On the final day of composting, all microalgae-amended compost piles were approved as non-phytotoxic due to GIs ranging from 68.0 to 70.4% (Zucconi et al., 1985; Emino & Warman, 2004; Rey at al., 2008). However, all composting piles amended with microalgae, showed significantly lower GI values than the control group (84.8 ± 5.47%).

Germination tests are bioassays that quantify the inhibitory effects of phytotoxins such as the delay of seed germination or the inhibition of plant growth (Wrap, 2002). Phytotoxins are compounds produced by pathogenic fungi or bacteria that can cause disease symptoms in plants (Strobel, 1982). The maturity of the compost, regarded as its potential of use, is often defined as the total amount of degradation of phytotoxic substances and can be estimated by determining the germination rate index (GI) (Gao et al., 2010; Zucconi et al., 1981 a, b). Some GI's combine relative root elongation (L%) and relative seed germination (G%), mainly from garden cress seeds (*Lepidium sativum*), irrigated with aqueous compost extracts (Ranal & Santana, 2006). Phytotoxicity tests are important to confirm that the compost is suitable for agricultural purposes and to avoid negative effects of immature compost on the agricultural ecosystem and to confirm that the compost is suitable for agricultural purposes (Warman, 1999). One of the most applied germination tests is that reported by Zucconi *et al.* (1981a, b), which can also be considered as compost maturity test, when it reaches values above 60% (Selim et al., 2011).

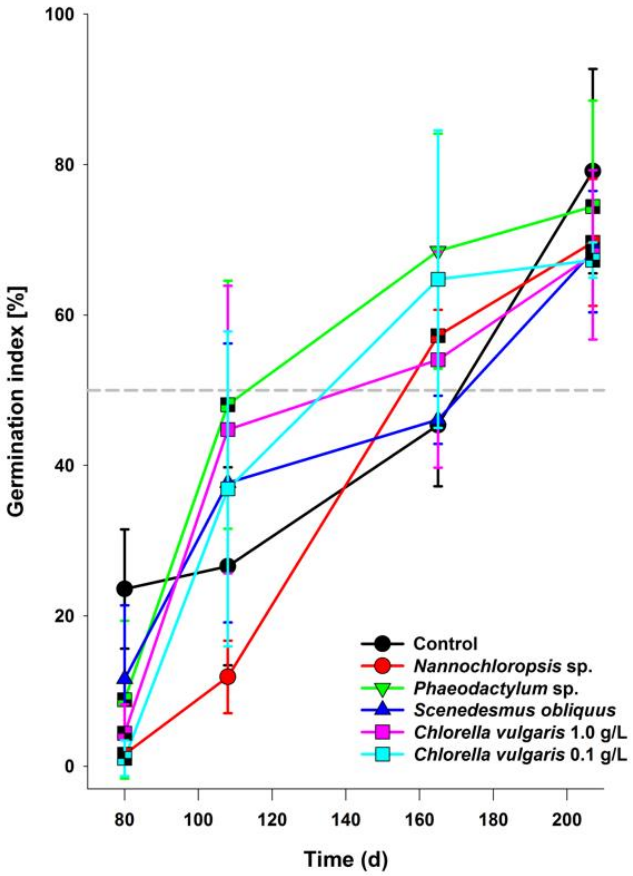

Figure 3.13 - Germination index of all composting piles along the experiment. Dunnett's two sided post-hoc test (ANOVA) indicated significant differences (half colored squares) compared to the control group. Error bars represent standard deviations. Data points at each day are shown as mean ± SD (n=3).

5.) Conclusion

This study investigated in the amendment of organic compost with dry microalgal powder of *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Scenedesmus obliquus* and *Chlorella vulgaris*. Seven chemical and physical parameters were monitored along the composting process (pH, electrical conductivity, organic matter, mineral matter, temperature, volume, phytotoxicity). No major parameter variations were observed among all treated composting piles in comparison with the control compost during the final phase of the process. Measurements for pH and electrical conductivity revealed non-optimum values of the majority of composts for the use as biofertilizer, including the control pile. Therefore, applying the obtained compost as growing media for alkaline-sensitive and salt-sensitive plants must be done with caution. Future studies will address the application of the final composting masses as biofertilizer and further investigate in bio-stimulating properties.

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