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William Michelon

Microalgae biomass from swine wastewater phycoremediation: metabolic profile and its potential applications

Florianópolis
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Orientador: Prof. Hugo Moreira Soares, Dr.
Coorientador: Alexandre Matthiensen, Dr.

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William Michelon

Microalgae biomass from swine wastewater phycoremediation: metabolic profile and its potential applications

O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

Prof. Cristiano José de Andrade, Dr.
Universidade Federal de Santa Catarina

Prof. Leonardo Rörig, Dr.
Universidade Federal de Santa Catarina

Prof.(a) Estela de Oliveira Nunes, Dr.(a)
Embrapa Suínos e Aves – CNPSA

Prof.(a) Luísa Gouveia, Dr.(a)
Laboratório Nacional de Energia e Geologia - LNEG

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Prof. Hugo Moreira Soares, Dr.
Orientador

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Dedicado este trabalho aos apaixonados por microalgas.

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When you are born in a world you don’t fit in, it’s because you were born to help create a new one (CALIGIURI, Ross)

RESUMO EXPANDIDO

Introdução: A produção e a demanda global por proteína animal aumentaram exponencialmente nas últimas décadas, sendo que atualmente o Brasil é o quarto maior exportador de carne suína. Dessa forma, o sistema de produção animal tem sido estimulado e intensificado para aumentar a produtividade. Porém, uma das desvantagens dos sistemas produtivos intensivos são os elevados volumes de efluentes gerados na unidade de produção, que podem conter matéria orgânica, nutrientes, metais pesados, patógenos (bactérias e vírus) e resíduos de medicamentos veterinários, que se não tratados adequadamente, pode afetar a saúde pública e ambiental. Adicionalmente, os efluentes da indústria de suinocultura são tratados convencionalmente por digestão anaeróbica, para reduzir as cargas de carbono, seguida por um sistema de tratamento terciário para remover nitrogênio e fosfato. Desta maneira, os sistemas de tratamento terciário à base de microalgas têm recebido considerável atenção, principalmente por sua eficiência na biorremediação e pelo potencial de geração de valiosa matéria-prima. **Objetivo:** O presente estudo foi realizado sob o conceito de biorrefinaria e teve como objetivo investigar a presença e recuperação de metabólitos produzidos por conversão biológica em culturas de microalgas sob diferentes condições de cultivo, bem como a remoção de drogas de uso veterinário. **Material e métodos:** O inóculo de microalgas foi obtido de uma lagoa anaeróbia usada para remover nutrientes do digestato de águas residuárias de suínos originado de um biodigestor anaeróbio (EMBRAPA, Concórdia, SC, Brasil). O inóculo foi composto por um consórcio dominado por *Chlorella* spp. Os experimentos foram realizados em escala piloto utilizando biorreatores de 500 L, colocados dentro de uma casa de vegetação, expostos à luz solar direta. O meio de crescimento consistiu em 6 ou 50% (v v^{-1}) de efluente digerido bruto diluído no volume total de água dos reatores. Biomassa foi separada por processo de coagulação/floculação ou centrifugação. Após a separação a biomassa foi submetida a diferentes ensaios e/ou caracterização, com exceção do tópico 6: (1) Produção de açúcares totais a partir da hidrólise ácida e posterior fermentação usando diferentes leveduras (*Saccharomyces cerevisiae e Saccharomyces cerevisiae chardonnay)*. (2) Produção de aminoácidos, ácidos graxos e peptídeos. (3) Produção de extratos com diferentes solventes com atividades antimicrobianas contra bactérias multirresistentes e patogênicas (*Staphylococcus hyicus*, *Enterococcus faecalis* e *Streptococcus suis*). (4) Produção de extratos obtidos com diferentes solventes com atividade virucida. (5) Produção de bio-extratos associada a um fungo com atividade bio-herbicida. (6) A degradação de tetraciclina, oxitetraciclina, clortetraciclina e doxiciclina em águas residuais de suínos tratadas com o processo ficorremediação. **Resultados:** **(1)** Entre as diferentes concentrações de ácido sulfúrico testadas (isto é, 47, 94, 188, 281 e 563 mM), um teor de açúcar significativamente maior foi obtido com 188 mM (0,496 $g_{açúcar}$ $g_{microalgaeDW}^{-1}$). A concentração de açúcar presente nas microalgas não diferiu significativamente entre as biomassas colhidas por centrifugação ou coagulação-floculação. *S. cerevisiae chardonnay* mostrou um consumo significativamente mais rápido de açúcar durante a fase de crescimento exponencial. As cepas de levedura foram capazes de consumir a maior parte do açúcar adicionado ≈ 8 g L^{-1} em 24 h. **(2)** O conteúdo de aminoácidos apresentou concentrações relativamente altas (em % da proteína total) de aminoácidos essenciais como leucina (4,1), lisina (2,5), fenilalanina (2,6) e treonina (2,4). O perfil de ácidos graxos foi composto por 5,3% de poli-insaturados (como C18: 2 e C18: 3) e ≈ 10% de insaturados (principalmente C16:1 e C18:1). Foram identificados cerca de 25 peptídeos bioativos relacionados às diferentes propriedades, tais como: antioxidantes, anti-inflamatórias e anticarcinogênicas. **(3)** A caracterização dos extratos por UHPLC revelou a presença de 23 fitoquímicos com propriedades antimicrobianas reconhecidas. A atividade bacteriostática foi observada em ensaios de plaqueamento pela formação de zonas de inibição variando de 7 a 18 mm de

diâmetro. Apenas os extratos de diclorometano foram inibitórios para todas as três bactérias modelo. A concentração inibitória mínima avaliada para extratos de diclorometano foi 0,5 mg mL^{-1} para *Staphylococcus hyicus* e *Enterococcus faecalis* e 0,2 mg mL^{-1} para *Streptococcus suis*. Não foram observados efeitos bactericidas com extratos de solventes a 2 ou 5 mg L^{-1}. **(4)** O processo de ficorremediação removeu 100% de amônia-N e fosfato-P, com taxas (k_1) de 0,218±0,013 e 0,501±0,038 (d^{-1}), respectivamente. Todos os extratos de microalgas reduziram em 100% a capacidade infecciosa do HSV-1. Os extratos de microalgas obtidos com diclorometano e metanol apresentaram atividades de inibição na menor concentração (3,125 µg mL^{-1}). Ensaios virucidas contra HAdV-5 usando extrato de microalgas de hexano e metanol inibiram a capacidade infecciosa do vírus em 70% em todas as concentrações testadas a 37 ºC. Na concentração de 12,5 µg mL^{-1}, o extrato de microalgas com diclorometano reduziu de 50-80% da capacidade infecciosa do HAdV-5, também a 37 ºC. **(5)** A peroxidase foi a enzima que mais produziu a partir da biomassa fresca e liofilizada, e também quando associada ao *Trichoderma koningiopsis*, com uma atividade máxima de 16.200 U mL^{-1}. Os bio-extratos produzidos apresentaram danos fitotóxicos de até 80% nos primeiros 7 dias e 100% após 15 dias de tratamento na espécie *Conyza bonariensis*. **(6)** A cinética de degradação das tetraciclinas foi ajustada ao modelo de cinética de pseudo-primeira ordem, resultando em: 0,36>0,27>0,19>0,18 (d^{-1}) para tetraciclina, doxiciclina, oxitetraciclina e clortetraciclina, respectivamente. A concentração máxima de biomassa de microalgas (342,4 ± 20,3 mg L^{-1}) foi obtida após 11 dias de cultivo, quando a tetraciclina foi completamente removida. A concentração de clortetraciclina diminuiu rapidamente gerando iso-clortetraciclina e 4-epi-iso-clortetraciclina. A biomassa de microalgas colhida após a remoção dos antibióticos apresentou um conteúdo rico em carboidratos de 52,7 ± 8,1, 50,1 ± 3,3, 51,4 ± 5,4 e 57,4 ± 10,4 (%) quando cultivadas com tetraciclina, oxitetraciclina, clortetraciclina e doxiciclina, respectivamente. **Considerações: (1)** Esses resultados apoiam a noção de que ficorremediação usada como sistema de tratamento terciário para remoção de nutrientes de águas residuárias pode fornecer matéria-prima valiosa para microalgas ricas em açúcares fermentáveis. **(2)** O conceito de biorrefinaria contribui para o avanço da nossa compreensão dos arranjos tecnológicos que combinam o tratamento de águas residuais com a produção de biomassa rica em metabólitos com uma ampla gama de aplicações biotecnológicas. **(3)** Os resultados contribuem para o conhecimento atual sobre o potencial de aplicabilidade de extratos de microalgas como bacteriostáticos contra *S. suis*, *S. hyicus* e *E. faecalis*, conhecido por desempenhar um papel importante em doenças animais e na indústria de alimentos. **(4)** A biomassa de microalgas pode ser cultivada com sucesso com águas residuárias de suínos, e posteriormente colhida para a exploração de produtos farmacêuticos; nesse sentido, tornando-se uma opção pela valorização destes resíduos. Adicionalmente, a busca e o desenvolvimento de novos agentes virucidas ser estimulante, por se tratar de um mercado em expansão e com reais possibilidades de aplicação, principalmente considerando o atual cenário de disseminação de doenças virais. **(5)** A integração de bioprocessos como a ficorremediação de águas residuárias da suinocultura associada ao cultivo de *T. koningiopsis* e produção de enzimas são uma promissora fonte de matéria-prima para a produção de subprodutos como bio-herbicidas. **(6)** A biomassa de microalgas colhida após a remoção das tetraciclinas, apresentou um conteúdo rico em carboidratos, indicando que o tratamento de água residuárias de suínos contendo resíduos de antibióticos, pode ser uma alternativa ambientalmente correta para fornecer matéria-prima para produção de bioetanol e biometano.

Palavras-chave: Açúcar residual. Antimicrobiano. Aminoácidos. Peptídeos. Virucida. Bio-herbicida.

RESUMO

A remoção de poluentes de águas residuais baseado em microalgas tem ganhado considerável atenção, devido à sua eficiência no tratamento e potencial geração de biomassa como matéria-prima para diversas aplicações biotecnológicas. Assim, a extração de produtos de origem metabólica da biomassa de microalgas e de alto valor agregado, é uma alternativa promissora. O presente estudo foi realizado sob o conceito de biorrefinaria e teve como objetivo investigar a presença e recuperação de metabólitos produzidos por conversão biológica em culturas de microalgas sob diferentes condições de cultivo, bem como a remoção de drogas de uso veterinário. Esta tese foi ordenada em seis capítulos: (1) Açúcar residual da biomassa de microalgas a partir da ficorremediação de águas residuais da suinocultura. A hidrólise foi realizada em diferentes [H_2SO_4], um teor significativo de açúcar de 0,496 $g_{açúcar}\ g_{microalgaDW}^{-1}$ foi obtido com 188 mM. A concentração de açúcar presente nas microalgas não diferiu significativamente entre as biomassas colhidas por centrifugação ou coagulação-floculação. (2) Produção de aminoácidos, ácidos graxos e peptídeos em biomassa de microalgas colhidas durante a ficorremediação de águas residuais de suínos. A biomassa apresentou alto teor de aminoácidos essenciais, tais como: leucina, lisina, metionina, fenilalanina, treonina e triptofano. Condições induzidas por estresse, como limitação de nutrientes, contribuíram para a produção de ácidos graxos. Foram encontrados 25 peptídeos bioativos relacionados a propriedades antioxidantes, anti-inflamatórias e anticarcinogênicas. (3) Microalgas produzidas durante ficorremediação de águas residuais de suínos contêm compostos bacteriostáticos eficazes contra bactérias resistentes a antibióticos. A atividade antibacteriana foi testada contra bactérias resistentes, que são patogênicas aos suínos: *Streptococcus suis*, *Enterococcus faecalis* e *Staphylococcus hyicus*. O extrato de diclorometano demonstrou ação bacteriostática [0.2-0.5 mg mL^{-1}] para todas as bactérias testadas. Vinte e três compostos diferentes foram detectados nos extratos de microalgas. (4) Atividade virucida de extratos de microalgas coletados durante ficorremediação de águas residuais de suínos. Foram usados vírus modelo, com envelope (*Herpes simplex* tipo 1) e sem envelope (*Adenovírus humano* tipo 5). Todos os extratos de microalgas reduziram em 100% a capacidade infecciosa do HSV-1. Já os extratos de microalgas obtidos de diclorometano e metanol apresentaram atividades de inibição na menor concentração testada. (5) Produção de bio-herbicidas a partir de biomassa de *Chlorella* spp. e *Trichoderma koningiopsis*. A produção de atividade enzimática e bio-herbicida, com e sem associação fúngica, foi testada contra plantas daninhas. A enzima peroxidase foi a mais produzida, com uma atividade máxima de 16.200 U mL^{-1}. Os bio-extratos produzidos apresentaram danos fitotóxicos de até 80% nos primeiros 7 dias e 100% após 15 dias de tratamento. (6) Remoção de antibióticos de uso veterinário em água residuais de suínos pelo processo baseado em microalgas. Tetraciclina, oxitetraciclina, clortetraciclina e doxiciclina foram os fármacos utilizados para avaliação da capacidade de remoção pelo processo de ficorremediação. A tetraciclina foi completamente removida após 11 dias de ficorremediação. A degradação da clortetraciclina gerou metabolitos como iso-clortetraciclina e 4-epi-iso-clortetraciclina. Em resumo, a biomassa de microalgas produzida durante a ficorremediação de águas residuárias de suínos mostrou-se uma fonte promissora de matéria-prima alternativa para produção e extração de produtos de alto valor agregado, além de eficiente na remoção de antibióticos de uso veterinário.

Palavras-chave: Açúcar residual. Antimicrobiano. Aminoácidos. Peptídeos. Virucida. Bio-herbicida.

ABSTRACT

The removal of pollutants from wastewater using microalgae-based processes has gained considerable attention, due to its efficiency in the treatment and potential generation of biomass as feedstock for several biotechnological applications. Thus, extraction of metabolic source products of biomass of microalgae with high added value, is a promising alternative. The present study was carried out under the concept of biorefinery and aimed to investigate the presence and recovery of metabolites produced by biological conversion in microalgae cultures under different cultivation conditions, as well as the removal of veterinary drugs. Therefore, the thesis was ordered into six chapters: (1) Residual sugar from microalgae biomass harvested from phycoremediation of swine wastewater digestate. The hydrolysis was carried out in different [H_2SO_4], a significant sugar content of 0.496 $g_{sugar}\ g_{microalgaeDW}^{-1}$ was obtained with 188 mM. The concentration of sugar present in the microalgae did not differ significantly between the biomasses harvested by either centrifugation or coagulation-flocculation. (2) Amino acids, fatty acids and peptides in microalgae biomass produced from phycoremediation of swine wastewaters. Biomass showed a high content of essential amino acids such as mainly leucine, lysine, methionine, phenylalanine, threonine and tryptophan. Stress-induced conditions, as nutrients limitation, contributed to the production of fatty acids. Twenty-five bioactive peptides related to antioxidant, anti-inflammatory and anticarcinogenic properties were found. (3) Microalgae produced during phycoremediation of swine wastewater contains effective bacteriostatic compounds against antibiotic-resistant bacteria. The antibacterial activity was performed against pathogenic resistant bacteria that cause disease in swine: *Streptococcus suis*, *Enterococcus faecalis* and *Staphylococcus hyicus*. Dichloromethane extract was bacteriostatic (0.2-0.5 mg mL^{-1}) for all tested bacteria. Twenty-three different compounds were detected in the microalgae extracts. (4) Virucidal activity of microalgae extracts harvested during phycoremediation of swine wastewater. Antiviral models, enveloped (*Herpes simplex* type 1) and non-enveloped (*Human Adenovirus* type 5) were used. All microalgae extracts reduced 100% of the infectious capacity of HSV-1. Yet, the microalgae extracts with dichloromethane and methanol showed inhibition activities at the lowest concentration of 3.125 μg mL^{-1}. (5) Production of bio-herbicides from biomass of *Chlorella* spp. and *Trichoderma koningiopsis*. The production of enzymatic and bio-herbicidal activity, with and without fungal association, against weeds were tested. Peroxidase enzyme was the most produced, with an activity maximum of 16,200 U mL^{-1}. The bio-extracts produced showed phytotoxic damage of up to 80% in the first 7 days and 100% after 15 days of treatment. (6) Removal of veterinary antibiotics from swine wastewater using microalgae-based process. Tetracycline, oxytetracycline, chlortetracycline and doxycycline were the drugs used to evaluate the removal capacity by the phycoremediation process. Tetracycline was completely removed after 11 days of phycoremediation. The degradation of chlortetracycline generated metabolites such as iso-chlortetracycline and 4-epi-iso-chlortetracycline. In summary, the microalgal biomass produced during the phycoremediation of swine wastewater proved a promising source of alternative feedstock for the production and extraction of high value-added products, as well as being efficient in removing veterinary antibiotics.

Keywords: Residual sugar. Antimicrobial. Amino acids. Peptides. Virucide. Bio-herbicide.

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LIST OF ABBREVIATIONS AND ACRONYMS

A549 - Adenocarcinomic Human Alveolar Basal Epithelial

AA - Amino Acid

ABPA - Associação Brasileira de Proteína Animal

AOAC - Association of Official Analytical Chemists

AOCS - American Oil Chemists Society

APHA - American Public Health Association

BCAA - Brazilian Compendium of Animal Nutrition

BOD - Biological Oxygen Demand

CT - Chlortetracycline

DC - Doxycycline

DHA - Docosahexaenoic Acid

DNS - Dinitrosalicylic Acid

DW - Dry Weight

EPA - Eicosapentaenoic Acid

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FA - Fatty Acid

FAO - Food and Agriculture Organization of the United Nations

HAdV-5 - *Human adenovirus virus* type 5

HSV-1 - *Herpes simplex virus* type 1

IBGE - Instituto Brasileiro de Geografia e Estatística

MIC - Minimum Inhibitory Concentration

MUFA - Monounsaturated Fatty Acid

NR - Nitrification-denitrification Reactor

OT - Oxytetracycline

PBR - Photobioreactor

PPFD - Photosynthetic Photon Flux Density

PUFA - Polyunsaturated Fatty Acid

SBCPD - Brazilian Society of Weed Science

SFA - Saturated Fatty Acid

TC - Tetracycline

TOC - Total Organic Carbon

UASB - Upflow Anaerobioc Sludge Blanket

UFAs - Unsaturated Fatty Acid

UHPLC - Ultra High-Pressure Liquid Chromatography

USEPA - United States Environmental Protection Agency

VERO - Kidney epithelial derived from African green monkey

CONTENTS

INTRODUCTION

The global production and demand for animal protein have increased exponentially in recent decades. Brazil is the fourth larger exporter of swine meat, with 4.4 million tons in 2020 (ABPA, 2021). In this manner, the animal production system has been stimulated to increase productivity. However, one of the disadvantages of intensive farming systems is the high volumes of wastewater generated for the unity of production, which can contain organic matter, nutrients, heavy metals, pathogens (bacteria and virus) and veterinary drugs, which if not properly treated, can affect public and environmental health (VIANCELLI et al., 2013).

In this context, the agro-industrial sector needs of low-cost and easy-to-operate technologies to promote the removal of these organic compounds present in wastewater. The use of microalgae as a tertiary or polishing treatment (phycoremediation) is attractive, due to its bioconversion capacity in biomass with potential biotechnological application. The integration of processes through the recovery and reuse of surplus energy, mass and water from agro-industrial processes, has been considered one of the most important approaches to the economic viability of processes and products (FREITAS et al., 2021). Recently, phycoremediation was associated to the biorefinery concept, since the microalgae biomass produced can be a source of valuable feedstock or raw material to produce bioenergy, nutraceuticals and fertilizers (UBANDO et al. 2020). Additionally, the microalgae could be applied on the removal of residual veterinary drugs from swine wastewater. These residues came from the excretion of active form of the antibiotics administered during the swine production chain. The problem generated by these residues is the increase of resistant bacteria strains.

For instance, a wide range of high-value molecules synthesized by microalgae, such as essential fatty acids, especially the long-chain polyunsaturated (omega-3 and 6), and essential amino acids (threonine, methionine, lysine, cysteine and tryptophan) are compounds of special interest for nutraceutical and animal industry. Additionally, the microalgae biomass can contain compounds with nutraceutical and medical properties including antioxidant, anticancer anti-inflammatory and antimicrobials (BASHEER, 2020).

Other novelty related to microalgae is the use of biomass to produce bio-herbicide compounds. This is of particular interest, once that the agriculture dependence on synthetic herbicides has been questioned for many years, since the long-term use of chemical herbicides favors the occurrence of phenological adaptations and selection of resistant genotypes.

OBJECTIVES

General objective

To investigate the biological properties of microalgae, obtained from phycoremediation of swine wastewater, in the perspective of biorefinery concept.

Specific objectives

- To evaluate whether the harvesting process of microalgae biomass (centrifugation, coagulation/flocculation) changes the final concentration of sugars;

- To evaluate whether the qualitative and/or quantitative profile of peptides, amino acids and fatty acids are altered under different nutritional conditions;

- To investigate whether microalgae biomass extracts obtained from different solvents bactericidal/bacteriostatic activity against multidrug-resistant pathogenic bacteria;

- To investigate whether microalgae biomass extracts obtained from different solvents present virucidal activity;

- To evaluate whether the microalgal biomass associated with a fungus has bio-herbicidal activity;

- To investigate whether the phycoremediation process in swine wastewater is efficient for the removal of residual veterinary antibiotics.

CONTENT OF THE THESIS

This thesis was divided into 6 chapters:

Chapter 1: Residual sugar from microalgae biomass harvested from phycoremediation of swine wastewater digestate.

Chapter 2: Amino acids, fatty acids and peptides in microalgae biomass produced from phycoremediation of swine wastewaters.

Chapter 3: Microalgae produced during phycoremediation of swine wastewater contains effective bacteriostatic compounds against antibiotic-resistant bacteria.

Chapter 4: Virucidal activity of microalgae extracts harvested during phycoremediation of swine wastewater.

Chapter 5: Production of bio-herbicides from biomass of *Chlorella* spp. and *Trichoderma koningiopsis*

Chapter 6: Removal of veterinary antibiotics in swine wastewater using microalgae-based process.

- CHAPTER 1 -

1 RESIDUAL SUGAR FROM MICROALGAE BIOMASS HARVESTED FROM PHYCOREMEDIATION OF SWINE WASTEWATER DIGESTATE [1]

Abstract: The present study assessed the carbohydrate and sugar production from *Chlorella* spp. biomass harvested from a field scale reactor simulating phycoremediation of swine wastewater. The microalgae biomass was mainly composed by (%): carbohydrates (41±0.4), proteins (50±0.4), and lipids (1.3±0.5). The residual sugar present in the biomass was extracted via acid hydrolysis. Among different concentrations of sulfuric acid tested (i.e., 47, 94, 188, 281 and 563 mM) significantly higher sugar content was obtained with 188 mM (0.496 g_{sugar} $g_{microalgaeDW}^{-1}$). The concentration of sugar present in the microalgae did not differ significantly between the biomasses harvested by either centrifugation or coagulation-flocculation. Two commercially available strains of yeast (i.e., *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae chardonnay*) were tested for their capability to ferment sugar from lyophilized microalgae biomass. *S. cerevisiae chardonnay* showed a significantly faster consumption of sugar during the exponential growth phase. Both strains of yeast were capable to consume most of the sugar added ≅ 8 g L^{-1} within 24 h. Overall, the results suggest that carbohydrate-rich microalgae biomass obtained from the phycoremediation of swine wastewaters can play an important role in green design for industries seeking alternatives sources of feedstock rich in sugar.

1.1 INTRODUCTION

Concerns about the uncertain availability of fossil fuels in the near future have motivated the scientific community to search for alternative sources of renewable energy. For instance, ethanol derived from crops, such as corn and sugar cane, became a commodity with a multibillion-dollar industry that still threatens to push up the price of these plants for food (HO et al., 2013a; BRASIL et al., 2015). There are several environmental implications associated with current production of energy crops, such as atmospheric emissions associated with the use

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of fire in sugar-cane fields; the excessive use of water for irrigation; contamination of groundwater and soil by pesticides; territorial expansion; soil erosion; among others (ABBASI; ABBASI, 2010). In this regard, lignocellulosic materials have been considered, although production of sufficient biomass requires logging and deforestation (CHENG; TIMILSINA, 2011). Production of ethanol from lignocellulosic biomass that does not compete with the food industry are still struggling to take off due to high costs (KHOO 2015; CHENG; TIMILSINA, 2011).

The use of microalgae for biofuel production has been discussed extensively. Compared to conventional crops, the growth rates and yield of microalgae are significantly superior. Other advantages include less demand for consumable resources (e.g., water and soil) (MATA et al., 2010; KHAN et al., 2018) and the increased potential for CO_2 mitigation (MU et al., 2014; ULLAH et al., 2014). Microalgae biomass is composed of large amounts of carbohydrates (polysaccharides) in cell walls and across the intracellular matrix that can be converted into fermentable sugars (HARUN et al., 2011). Residual sugar concentrations (wt wt $^{-1}$) of 80% [*Spirulina platensis* (MARKOU et al., 2013) and *Synechococcus* sp. (MÖLLERS et al., 2014)], 45-70% [*Chlorella vulgaris* KMMCC-9; (KIM et al., 2014)], and 37.9-44.3% [*Scenedesmus* sp. CCNM 1077; (PANCHA et al., 2016)] were reported.

It is worth noting, however, that the economic feasibility of microalgae production in an industry-relevant setting is largely influenced by the availability and costs of water and/ or nutrients. In an attempt to reduce costs, the use of wastewater has been considered (POPP et al., 2014; SERVICE, 2011). Many industries are contemplating the use of algae-based phycoremediation treatment to remove nutrients from wastewater effluents while simultaneously producing valuable microalgae biomass (BRASIL et al., 2015). The microalgae produced in this process can have different biochemical composition depending on nutrient concentration present in the wastewater used as growth medium (LEE et al., 2015). For instance, nutrient-rich wastewaters such as those generated from confined swine production may constitute an alternative growth medium to produce microalgal biomass rich in carbohydrates (MICHELON et al., 2015; ÖZÇIMEN and İNAN, 2015). Variations in carbohydrate content from microalgae can also occur depending on the harvesting method, i.e., mechanical centrifugation or chemical coagulation/flocculation (LEE et al. 1998; BORGES et al. 2011; COWARD et al. 2014). This effect, however, is not always observed (NDIKUBWIMANA et al., 2016), thus suggesting the need to be assessed on a case-by-case basis.

Also, considering that microalgae biochemical composition can vary depending on the harvesting method used, ancillary investigation was performed to determine whether centrifugation or coagulation-flocculation (the two most conventional harvesting methods) could affect the total amount or residual sugar present in the biomass.

1.2 MATERIAL AND METHODS

Figure 1.1 shows a schematic diagram of the material and methods. Steps are described in the sections below.

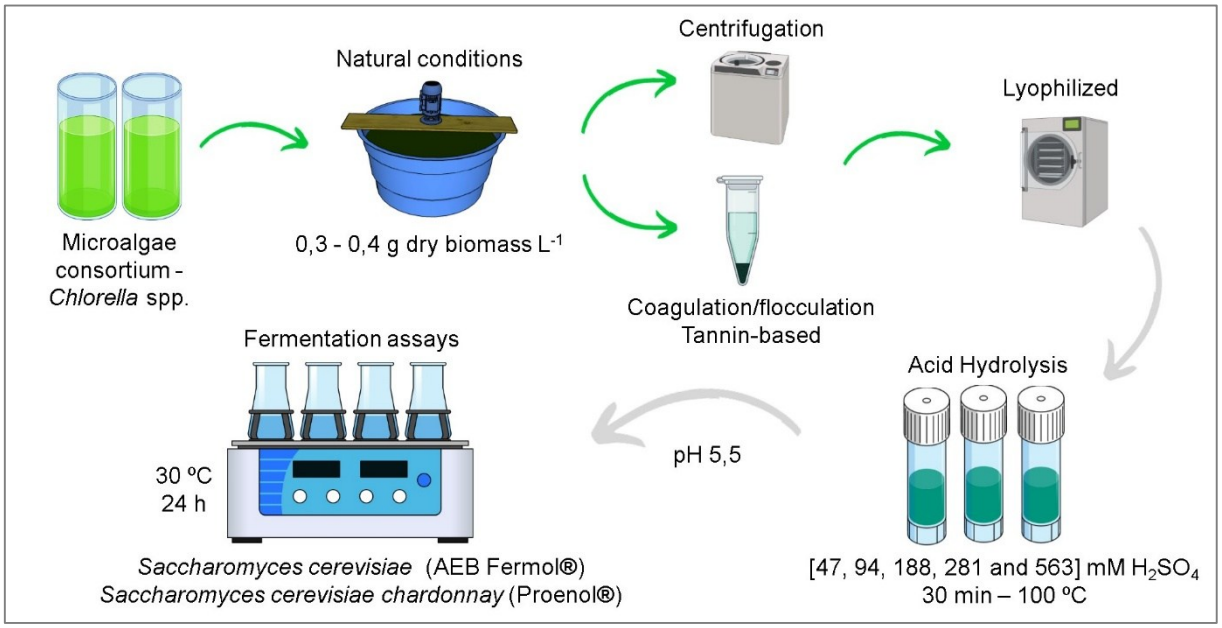

Figure 1.1. Schematic diagram of methodology

1.2.1 Experimental set up

Microalgae inoculum was obtained from a field scale lagoon used to remove nutrients from swine wastewater digestate originated from an anaerobic biodigester (Brazilian Agricultural Research Corporation, EMBRAPA, Concórdia, SC, Brazil). The inoculum was composed by a consortium dominated by *Chlorella* spp. as previously identified (MICHELON, et al., 2015) (Supplementary material I). Experiments were performed in pilot scale using 500-L reactors (121.2 cm internal Ø; 58.4 cm height) placed inside a greenhouse, exposed to direct sun light (photosynthetic photon flux density average and standard deviation of 321.5±411.4 µmol m^{-2} s^{-1}) and under ambient average temperature of 31.7±16.3 °C. These measurements (n=3) were taken in the morning (8 am), mid-day (12 am) and afternoon (4 pm). Reactors were inoculated with 30% of inoculum (volume-based) containing 70 mg dry weight microalgae L^{-1}. The growth medium was continuously mixed in the reactor using a submersible aquarium pump (flow rate of 1,200 L h^{-1}). The growth medium consisted of 6% v v^{-1} of raw digestate

effluent diluted in the reactors total volume of water. Dilution of digestate was necessary to enhance light penetration and microalgae growth. The chemical composition of the growth medium used in the reactor at the beginning of the experiments (i.e., at time zero) was (average mg L^{-1}±standard deviation): total organic carbon (100±5.2), biological oxygen demand (BOD_5 90.8±0.9), alkalinity as $CaCO_3$ (190±10), total nitrogen (50.3±0.9), ammonia-N (40.1±0.7) and phosphate-P (10.5±4.6). pH was 7.9±0.6.

In this work, we focused on the microalgae only. The efficiency of phycoremediation as treatment approach to remove nutrients from swine wastewater digestate was discussed elsewhere (MICHELON et al., 2015; PRANDINI et al., 2015; MEZZARI et al., 2013).

1.2.2 Harvesting

After 5 days of cultivation, the biomass in the reactor reached 0.3–0.4 g dry weight microalgae L^{-1}. At this point, biomass was harvested by either centrifugation (3,000 ×g, at 25 ˚C for 30 min; EVODOS, T10, Netherlands) or chemical coagulation-flocculation. A tannin-based cationic polyphenolic organic polymer produced through ammonium chloride and formaldehyde reaction was used as coagulant. The use of tannin was chosen due to its biodegradability (BEUCKELS et al., 2013; VANDAMME et al., 2013) as opposed to other types of coagulants (e.g., aluminum) that may jeopardize water quality (ROSSELAND et al., 1990). The tannin used was extracted from Acacia tree (*A.mearnsii*) bark and is available commercially in liquid form with 30% w v^{-1} of tannic acid solution (flavan- 3,4-diol) and weight distribution of 830-1940Da (CAS # 85029-52-3; Veta OrganicTM, Brazilian Wattle Extracts, Canoas, Brazil). This tannin was chemically modified through the Mannich reaction to improve cationic strength properties by the addition of an ammonium quaternary functional group. Coagulation was performed directly in the reservoirs by adding 0.01% v v^{-1} of tannin. The coagulation/ flocculation method proved to recover >95% of microalgae biomass at neutral pH (MEZZARI et al., 2014).

The concentration of sugars present in microalgae can decrease significantly within days at room temperature or when stored in the refrigerator (4 °C) (ADAMSON, 2015). For this reason, the harvested microalgae biomass was immediately frozen (-40 °C) and lyophilized (Model 030-JJ LJI Scientific) on the same day.

1.2.3 Determination of carbohydrate, lipid, protein and ash content

The cellular lipid content was determined by ether extraction (Ankom XT15) (AOCS, 2013). Protein content was measured by the combustion method (Leco FP-528) (AOAC, 1990). Ash content was determined according to the Brazilian Compendium of Animal Nutrition, method 36 (BCAA, 2009). Carbohydrate was determined by subtracting total cell dry weight from the measured lipid, protein and ash concentrations (BI; HE, 2013).

1.2.4 Recovery of residual sugar

Acid hydrolysis was used to recover sugar from microalgae biomass. A fixed amount of collected biomass (15 g L^{-1} re-suspended in distilled H_2O) was used as substrate for reaction assays. Different concentrations of sulfuric acid were tested (i.e., 47, 94, 188, 281 and 563 mM) to determine the most effective concentration. Hydrolysis assays were conducted in Erlenmeyer flasks. Reaction took place at 100 ˚C for 30 min (Waiser Lab. Products NC EST - 011). Samples were cooled at room temperature and then centrifuged at 3,200 ×g at 20 ˚C for 8 min (Excelsa® II model 206 BL). The supernatant containing the residual sugars was collected and the pH adjusted to 5.5 using 1M NaOH. The residual sugar concentration was analyzed using the DNS (dinitrosalicylic acid) method with glucose as standard for calibration curves (MILLER, 1959). After mixing 0.75 mL of glucose with 0.5 mL of DNS reagent, samples were heated at 100 ˚C for 5 min. Samples were cooled at room temperature and then 3 mL of water was added. Sugar concentrations were determined spectrophotometrically (Varian, Inc. Cary® 50 UV-Vis) at 540 nm.

1.2.5 Fermentation assays

Prior to fermentation tests, the supernatant containing the residual sugar was sterilized in autoclave at 121˚C for 15 min (Phoenix® Av-75/2). Fermentation assays were performed in triplicates using two different strains of yeasts: *Saccharomyces cerevisiae* (AEB Fermol®) and *Saccharomyces cerevisiae chardonnay* (Proenol®). Pre-inoculum was prepared in sterile Erlenmeyer flasks by adding 20 g L^{-1} of yeast into sterile deionized water containing 0.2 g L^{-1} nutrient medium YPD broth medium (Himedia®). After approximately 1 h of incubation, yeast suspensions were washed three times in phosphate buffer and transferred to 500 mL (3% w v^{-1}) Erlenmeyer flasks containing 200 mL of the sterile hydrolyzed sugar solution. Incubation took place at 30 ˚C for 24 h. Samples were taken over time for determination of sugar concentration as described above.

1.2.6 Statistical analysis

Experiments were performed in triplicate (n=3). The results were presented as mean ± standard error. Data were tested for normality and homoscedasticity and the statistical differences between group means were determined by one-way ANOVA. Tukey's honestly significant difference post hoc test was conducted after the determination of the homogeneity of variances ($p \geq 0.05$). Statistical analyses were performed using SASTM (2012). The level of significance considered for all the analyses was 5% ($p \leq 0.05$).

1.3 RESULTS AND DISCUSSION

1.3.1 Biochemical composition of microalgae

Microalgae can accumulate considerable amounts of lipids and carbohydrates under different nutrient-deficient conditions, making them one of the most versatile and sustainable sources for biofuel production (FAN et al., 2014). Most microalgae have carbohydrates content ranging between 5-25% of the total cell biomass, depending on the species (BILLER; ROSS, 2011; BRUTON et al., 2009; PRAJAPATI et al., 2014). The amount of carbohydrate content in microalgae biomass can be increased once cells are deprived from nutrients and/ or exposed to additional sources of atmospheric CO_2 (CHEN et al., 2013, HO et al., 2013b). Our previous studies corroborate these findings (MICHELON et al. 2015). Table 1.1 compares the carbohydrate content from different species of microalgae grown under controlled conditions using synthetic growth medium amended or not with CO_2. Most of the experimental conditions tested may not realistically represent the environmental dynamics (e.g., variations in light and temperature) expected at field scale. In this work, the microalgae produced from a pilot scale experiment exposed to field conditions were rich in proteins (50.3%) and carbohydrates (41%) but low in lipids (1.3%). Thus, the cultivation of microalgae biomass as proposed here may not be attractive for industries seeking oil and its derivates such as Omega-3; Omega-6, etc.

Table 1.1. Biochemical composition of biomass changes according to species and growth conditions.

Microalgae species	Growth medium	Growth conditions	Dry cell weight ($g\ L^{-1}$)	Content (%)			Yield of Hydrolysis (%)	Reference
				Carbohydrates	Proteins	Lipids		
Chlorella variabilis	synthetic	2% CO_2 (CO_2−air)	0.43	37.8	19.8	24.7	-	Cheng, et al., 2013
Chlorella vulgaris FSP-E	synthetic	2% CO_2 (CO_2−air)	7	13.3 - 54.4 [a]	20.1 - 58.8	11 - 15	90.4	Ho, et al., 2013a
Chlorella vulgaris KMMCC-9	synthetic	Bubbling air	-	22.4	-	-	45 - 70	Kim, et al., 2014
Scenedesmus sp. CCNM 1077	synthetic	-	-	45.2	31.2	-	37.9 - 43.4	Pancha, et al., 2016
Scenedesmus dimorphus	synthetic	2% CO_2	4 - 5	45 - 50	10 - 32.5	7.5 - 35	-	Wang, et al., 2013
Spirulina platensis	synthetic	Bubbling air	2 - 2.2	58 [b]	24	4	80	Markou, et al., 2013
Synechococcus sp.	synthetic	1% CO_2 (CO_2−air)	0.9 - 3.7	40 - 59	-	-	80	Möllers, et al., 2014
Chlorella spp.	non-sterile swine digestate	Open to atmosphere	0.3 - 0.4	41	50.3	1.3	49.6	**This study**

[a] Cultivated under nitrogen limited conditions

[b] Cultivated under phosphorus limited conditions

1.3.2 Acid hydrolysis

Acid pre-treatment is a method used regularly to disrupt microalgae cell walls before proceeding to enzymatic hydrolysis. The disruption facilitates the release of entrapped carbohydrates present in the cell wall. To determine the most adequate concentration of acid for hydrolysis pretreatment, different concentrations of sulfuric acid ranging from 47 to 563 mM were tested (Figure 1.2). More diluted acid concentrations are always preferred for hydrolysis because the process becomes less harsh and costly. The use of low concentrations of acids for hydrolysis can still be more effective than other hydrolysis methods (e.g. enzymatic hydrolysis) (HO, et al., 2013c). The optimum concentration of sulfuric acid, that led to a significant ($p<0.01$) increased sugar recovery (0.496 g_{sugar} $g_{microalgaeDW}^{-1}$; Table 1.1), was 188 mM (Figure 1.2). The lowest and highest concentrations of acid tested, i.e. 47 and 563 mM, were not as effective to recover sugar with only 0.13 and 0.22 g_{sugar} $g_{microalgaeDW}^{-1}$, respectively. The sugar yield obtained here was comparable to other specific microalgae strains grown under controlled laboratory conditions using synthetic medium amended with CO_2 (29.5 to 98.2%) (COWARD et al., 2014; WAN et al., 2015; XU et al., 2013; LEE et al., 2015; MÖLLERS et al., 2014) (Table 1.1).

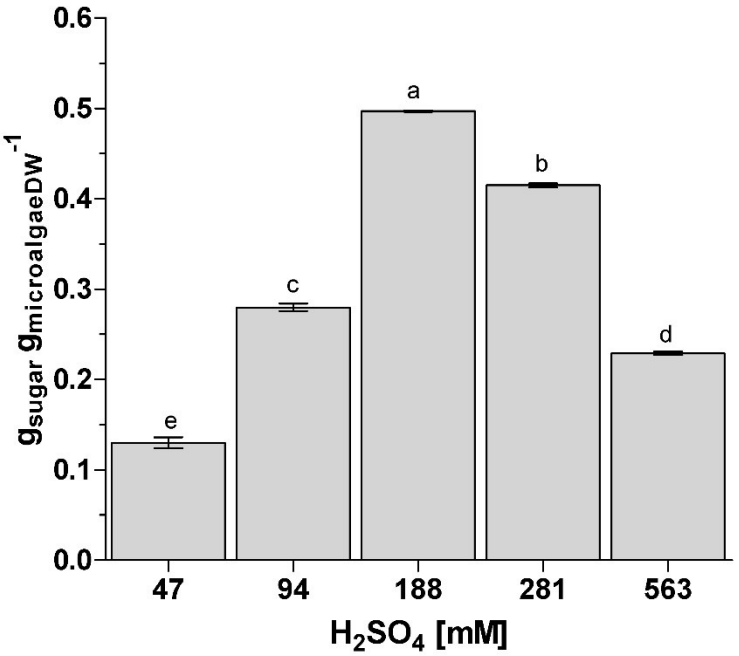

Figure 1.2. Effects of different concentrations of sulfuric acid on acid hydrolysis and sugar recovery from microalgae biomass. Bars depict standard error. Different letters denote significant differences ($p\leq0.05$) according to Tukey test.

1.3.3 Effect of coagulation-flocculation or centrifugation on sugar content

There are various mechanical and chemical methods available for harvesting microalgae, such as centrifugation, flocculation, filtration and screening, gravity sedimentation, and flotation (COWARD et al., 2014). Among these approaches, coagulation and flocculation with organic polymers are the most appropriate and cost effective option for large scale operations (WAN et al., 2015; XU et al., 2013; MEZZARI et al., 2014). There are conflicting lines of evidence showing that different harvesting processes result in microalgae biochemical composition changes. (BORGES et al. 2011, COWARD et al., 2014, MICHELON et al., 2015; NDIKUBWIMANA et al., 2016). Thus, it is important to determine on a case-by-case basis if the harvesting method of choice can ultimately affect microalgae composition and residual sugar concentration. To address this question, the microalgae residual sugar yield harvested from centrifugation was compared to microalgae collected via coagulation-flocculation. No significant ($p \leq 0.05$) differences in sugar concentration were observed independently of the method of harvesting used (Figure 1.3).

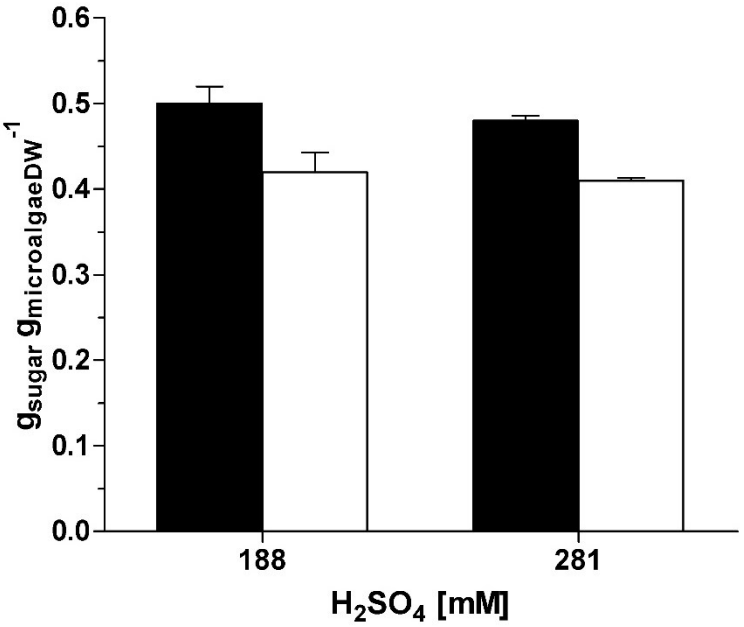

Figure 1.3. The concentration of residual sugar recovered from biomass did not change significantly as result of harvesting methods i.e., coagulation-flocculation (white) versus centrifugation (black). The *p* value obtained from one-way ANOVA was 0.89 and 0.26 for the 188 and 281 [mM] sulfuric acid, respectively. Bars depict standard error.

1.3.4 Sugar consumption

One limitation of bioethanol production from microalgae carbohydrates is that not all residual sugars are suitable for yeast fermentation (LEE et al., 2015). In this regard, less

complex sugars, such as glucose or fructose, are usually preferred (MARKOU et al., 2013). Two different commercially available strains of *Saccharomyces* were used in the fermentation assays to investigate which yeast could lead to higher sugar consumption. These yeast strains were used because of their broad metabolic capabilities and capacity to adapt in response to changes in the environment conditions, ultimately increasing bioethanol yield (SHARMA et al., 2016; MOHD AZHAR et al., 2017). Suspended cells of *S. cerevisiae* (Fermol Aromatic Group - AEB®) and *S. cerevisiae* (Fermol Chardonnay Group - AEB®) consumed 39.8% and 70.6%, respectively of the initial glucose concentration after 12 h of experiment. *S. cerevisiae chardonnay* was capable to remove 91.6% of the initial glucose present in the medium after 24 h of incubation (Table 1.2). The rate of sugar consumption was significantly higher ($p<0.0001$) for *S. cerevisiae chardonnay* during the exponential growth phase (between 50 min and 6 h of incubation) (Table 1.2).

Table 1.2. Consumption of sugar over time by the two strains of yeast tested in this work.

Incubation time	**Sugar consumption (g L^{-1})**		*p value*
	S. cerevisiae	*S. cerevisiae chardonnay*	
10min	8.617±0.080 ^{a}	8.614±0.003 ^{a}	0.9720
15min	8.680±0.039 ^{a}	8.543±0.038 ^{a}	0.0166
30min	8.549±0.041 ^{a}	8.556±0.004 ^{a}	0.8643
50min	8.549±0.041 ^{a}	8.350±0.008 ^{b}	<0.0001
4h	6.887±0.426 ^{b}	4.763±0.012 ^{c}	<0.0001
6h	4.997±0.008 ^{c}	2.441±0.008 ^{d}	<0.0001
20h	3.020±0.855 ^{cd}	0.691±0.031 ^{d}	0.0104
24h	2.299±0.779 ^{d}	0.452±0.008 ^{d}	0.0240
p value	<0.0001	<0.0001	

Data shown as means±std error. Different letters denote significant differences ($p\leq0.05$) according to Tukey test.

Table 1.3 shows the theoretical ethanol yield expected from microalgae biomass in comparison to other conventional feedstocks. Data from microalgae were estimated based on microalgae yield coefficient (0.3 g L^{-1} obtained every 5 days of cultivation) and the concentration of sugar recovered from biomass.

Table 1.3. Theoretical production of sugar from conventional agricultural feedstock sources.

Feedstock	Biomass Yield (ton ha^{-1} yr^{-1})	Residual sugar (ton ha^{-1} yr^{-1})	Reference
Corn	10.73	-	USDA, 2015
Sugar-cane	150	18	FAO, 2014
***Chlorella* spp.**	**87.6[a]**	**43.4[b]**	**This study**

[a] Estimated using the equation = [10^4 m^2/ha × 0.4 m (*h*; raceway depth) × microalgae yield (0.3 g-algae/L/5d) × 10^3 L/m^3 × 1 ton/ 10^6 g × 365 d/ yr].

[b] Estimated using the equation = (87.6 ton/ha/yr × 0.496 wt-reducing sugar/wt-biomass).

Microalgae biomass yield was 8-fold higher than corn and 2-fold lower than sugarcane. However, the higher concentration of carbohydrate present in microalgae biomass outweighs its lower yield in comparison to sugar cane. Hence, the potential for sugar production from microalgae (43.4 ton ha^{-1} yr^{-1}) is comparatively superior to sugarcane (18 ton ha^{-1} yr^{-1}). In this regard, microalgae biomass can play an important role in the development of sustainable biorefineries that are less dependent on arable land and water for irrigation. The existing concerns about the use of arable land for production of biofuels instead of food are diminished because microalgae can be produced in areas unsuitable for agricultural practices (e.g., desert, sand, etc.) (MUSSGNUG et al. 2010). The water footprint to produce microalgae can range between 200 to 1,000 m^3 ton^{-1} (assuming typical yields of 1–5 g fresh weight L^{-1}) which is considerably higher than the estimated global average water footprint of sugar cane, i.e., 209 m^3 ton^{-1} (GERBENS-LEENES; HOEKSTRA, 2012). However, the water used for microalgae growth can be reused postharvest (MEZZARI et al., 2014), thus significantly minimizing the amount of water needed. Another advantage of the use of microalgae is the short harvesting cycle (1-20 days) compared to sugarcane that is harvested once or twice a year. The frequent harvesting provides uninterrupted supply of raw material to meet the constant demands imposed by industries. Once in the industry, the operational costs associated with biomass pretreatment are expected to be lower with the use of microalgae because cells lack hemicellulose and lignin facilitating saccharification (CARRIERI et al., 2010; BABADZHANOV et al., 2004). Despite these advantages, however, the industry of microalgae for biofuels is still struggling to take off, mostly due to high operating operation costs with harvesting and dewatering, as well as low cell productivity (KHAN, et al., 2018). Consequently, the production costs of microalgae are still much higher (673 to 700 US$ ton $biomass^{-1}$) (KANG et al., 2015; HOFFMAN, 2016) than sugarcane (20–26 US$ ton $biomass^{-1}$) (CARDOSO et al., 2019). Yet, the high cost to produce microalgae can be offset by extracting and marketing residual byproducts for the nutraceutical and pharmaceutical industries. Although the production of microalgae biomass can unfold a

promising feedstock alternative to ethanol production, it still needs to be further investigated by encompassing more comprehensive techno-economic analysis.

1.4 CONCLUSIONS

In this work, field scale experiments simulating phycoremediation of swine wastewater produced microalgae rich in proteins (50.3%) and carbohydrates (41.0%). Among the concentrations of sulfuric acid tested for the recovery of sugar from biomass, the concentration of 188 mM showed best results with 0.497±0.001 g_{sugar} $g_{microalgae\text{-}DW}^{-1}$. The use of mechanical or chemical coagulation-flocculation for harvesting the microalgae biomass had insignificant effect on the biomass residual sugar. Compared to *S. cerevisiae*, *S. cerevisiae chardonnay* showed significantly faster consumption of sugar during the exponential growth phase, consuming 92% of the total sugar added (8 g L^{-1}) within 24 h. These results support the notion that phycoremediation used as tertiary treatment system for removal of nutrients from wastewaters could provide valuable microalgae feedstock rich in fermentable sugars.

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- CHAPTER 2 –

2 AMINO ACIDS, FATTY ACIDS AND PEPTIDES IN MICROALGAE BIOMASS PRODUCED FROM PHYCOREMEDIATION OF SWINE WASTEWATERS [2]

Abstract: Algae-based wastewater tertiary treatment systems have been drawing attention to eco-friendly companies due to high remediation effectiveness and production of valuable raw material. The amino acids, fatty acids and peptides from microalgae harvested from a pilot-scale phycoremediation system treating swine wastewater was determined. The maximum microalgae concentration of 247±3.4 mg L^{-1} was obtained after 11 days when phosphate and ammonium were completely removed. The amino acids content showed relatively high concentrations (as % of total protein) of essential amino acids such as leucine (4.1), lysine (2.5), phenylalanine (2.6) and threonine (2.4). The fatty acids profile was composed of 5.3% polyunsaturated (as C18:2 and C18:3) and ≈10% of unsaturated (mainly C16:1 and C18:1). About 25 bioactive peptides related to antioxidative, anti-inflammatory and anticarcinogenic properties were found. Therefore, microalgae biomass produced during phycoremediation of swine wastewaters seems promising as a source of alternative feedstock for the production and extraction of high value-added products.

2.1 INTRODUCTION

In recent years the global demand for animal protein has increased significantly (YARNOLD et al., 2019). For instance, production of swine meat in Brazil (fourth larger exporter) increased from 3.2 in 2010 to 4 million tons, in 2019 (ABPA, 2019). Thus, the animal production system was enforced to increment productivity, which was mainly achieved by raising the number of confined animals per unit of area. However, one of the main drawbacks of such production systems is the large volumes of wastewater generated, containing high organic matter, nutrients, heavy metals and veterinary drug residues, in which if not properly treated prior to disposal, can harm the environment (e.g. eutrophication) (VERONEZE et al., 2019).

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Phycoremediation is an effective and low-cost wastewater tertiary treatment for the removal of N and P from wastewaters. The process aids CO_2 sequestration and the produced biomass utilized as feedstock or raw material to produce fertilizers, biofuels, food supplements, pharmaceutics and cosmetics (SINGH; DHAR, 2019). For example, a wide range of high-value molecules synthesized by microalgae, such as essential FA (fatty acid), especially the long-chain polyunsaturated fatty acids (PUFAs), γ-linoleic acid (18:3 omega-6), arachidonic acid (20:4 omega-6), EPA (20:5 omega-3) and DHA (22:6 omega-3), are of special interest for nutraceutical, aquafeed and animal industry (JACOB-LOPES et al., 2019; SHARMA et al., 2020). Moreover, the presence of several essential AA (amino acid) in microalgae biomass, such as threonine, methionine, lysine, cysteine and tryptophan, are also appealing as feed supplements (MADEIRA et al., 2017). Additionally, recent study showed that peptides extracted from microalgae have interesting nutraceutics properties including antioxidant, antihypertensive, immunomodulating, antithrombotic and anticancer (COSTA et al., 2020).

The composition of FA (WANG et al., 2012; ZHU et al., 2013a) and AA (CANIZARES-VILLANUEVA et al., 1995; MOHEIMANI et al., 2018) in microalgae biomass growing in piggery wastewaters have already been discussed. Nonetheless, to the best of our knowledge, there is no report on peptides content in microalgae biomass on these systems. It is worth noting that the synthesis and intracellular storage of carbohydrates, proteins and lipids can vary greatly among species and/or the cultivation conditions such as variations in pH, temperature, light intensity, nutrients bioavailability and wastewater chemical composition (KHAN; SHIN; KIM, 2018; MICHELON et al., 2015). In addition, previous studies on FA and AA characterization focused on the use of species-specific microalgae and/or controlled laboratory scale conditions utilizing pretreated or sterile wastewaters (CANIZARES-VILLANUEVA et al., 1995; ZHU et al., 2013a). Thus, scarce information (if any) is available on the characterization of AA, FA, and peptides present in microalgae biomass harvested from field scale pilot experiments, utilizing non-sterile raw wastewater and indigenous consortium of microalgae. It is expected that at more realistic conditions it could clearly elucidate the effects of *in situ* phycoremediation treatment on microalgae composition changes during different stages of cell growth.

Therefore, the aim of this study was to characterize the concentration profiles of AA, FA, and peptides in a consortium of indigenous microalgae biomass (mainly dominated by *Chlorella* spp.) cultivated in a pilot scale reactor, simulating phycoremediation of swine wastewater. Wastewaters from an anaerobic digester and from a nitrification-denitrification

reactor (NR) were used to assess the effects of these two different wastewaters with distinctive physical-chemical characteristics on microalgae biochemical composition.

2.2 MATERIAL AND METHODS

Figure 2.1 shows a schematic diagram of the material and methods. Steps are described in the sections below.

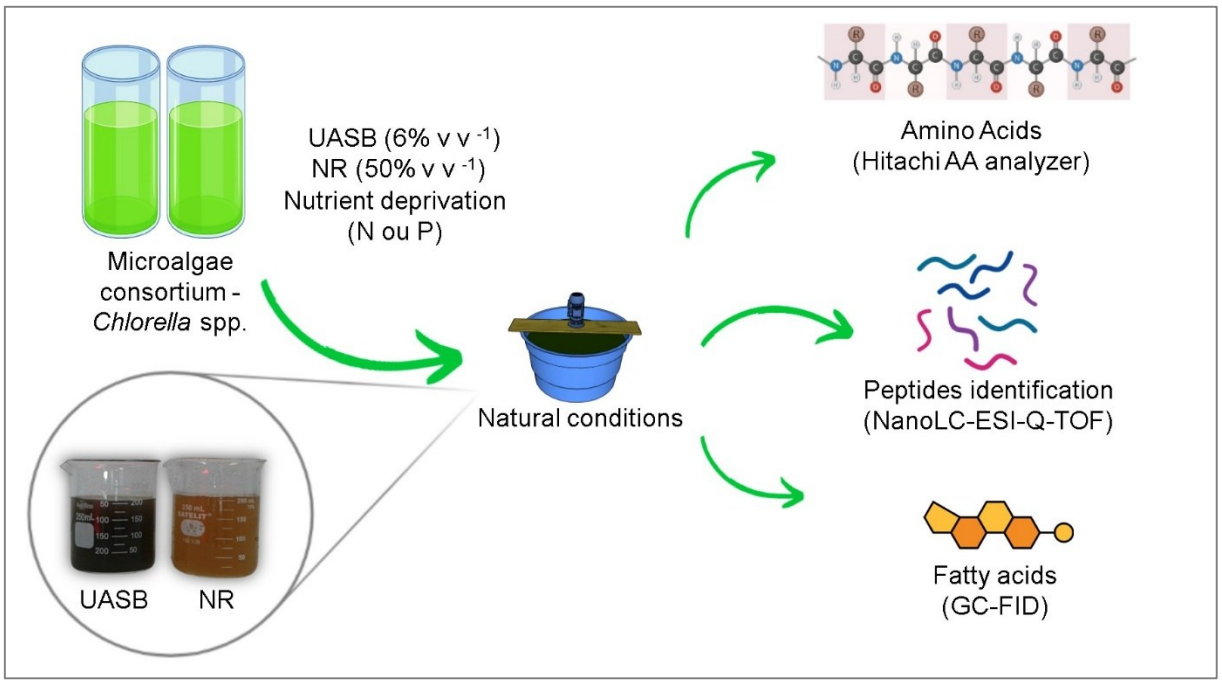


Figure 2.1. Schematic diagram of methodology

2.2.1 Microalgae inoculum

The microalgae consortium used as inoculum in this study was previously obtained from a field scale swine wastewater treatment system composed by an upflow anaerobic sludge blanket reactor (UASB) and a facultative pond as tertiary treatment (Brazilian Agricultural Research Corporation, EMBRAPA, Concordia, Brazil) (MICHELON et al., 2015). The collected microalgae inoculum was then acclimated in 12 L glass photobioreactors (PBRs; 20 cm Ø ID), filled with water containing 5% v v^{-1} of non-sterile digestate from the UASB (Supplementary material I). Dilution of digestate was needed to decrease effluent turbidity, enhancing light penetration required for microalgae growth. PBRs were kept at room temperature (23 ^{o}C) under mixotrophic conditions using 40-W fluorescent lamps [photosynthetic photon flux density (PPFD) of 44.8 µmol m^{-2} s^{-1}] and continuous agitation using recirculation mechanical pumps (Sarlobetter brand).

2.2.2 Pilot scale experiment

Experiments were conducted using three 500 L reactors, located in a greenhouse, exposed to natural sunlight (PPFD of 321.5±411.4 μmol m^{-2} s^{-1}) and under temperature-controlled conditions (25 °C). Reactors were operated in fed-batch mode using effluents from either, a field scale UASB or an air-sparged nitrification-denitrification tank placed downgrading the UASB for the removal of nitrogen compounds. Effluent from the UASB was diluted by adding 30 L (6% v v^{-1}) into 320 L (64% v v^{-1}) chlorine-free tap water. Effluent from the nitrification-denitrification tank was also diluted by mixing 250 L (50% v v^{-1}) into 100 L (50% v v^{-1}) chlorine-free tap water. Reactors were inoculated with 75±0.5 mg_{DW} microalgae L^{-1} (30% v v^{-1}). The reactors were kept under continuous agitation using a mechanic pump (flow rate of 1,200 L h^{-1}).

The chemical composition of the diluted UASB effluent, prior to inoculation (i.e., at time zero) was (mg L^{-1}): total organic carbon (100±5.2), biological oxygen demand (BOD_5 90.8±0.9), alkalinity as $CaCO_3$ (190±10), total nitrogen (50.3±0.9), ammonia-N (45.1±0.7) and, phosphate-P (10.5±4.6). pH was 7.9±0.6. The chemical composition of the nitrification-denitrification effluent prior to inoculation (i.e., at time zero) was (mg L^{-1}): total organic carbon (210±10), biological oxygen demand (BOD_5 100.8±8.9), alkalinity as $CaCO_3$ (500±15.1), total nitrogen (30±0.7), ammonia-N (26.1±0.2) and phosphate-P (11.5±6.2). pH was 7±0.5. After 12 days following reactors inoculation, N and P were completely removed. At this point in time the biomass in stationary growth phase was harvested by centrifugation at 3,000 ×*g* (EVODOS, T10, Netherlands) and immediately frozen (-40 °C) and lyophilized (Model 030-JJ LJI Scientific) for further analyses. Fresh biomass average weight was 4±1 g microalgae L^{-1}.

A third reactor was utilized to assess the effects of nutrients limitation on microalgae composition changes particularly on AA and FA. For this particular experiment, microalgae biomass previously grown in the UASB digestate (MICHELON et al., 2015) was harvested via centrifugation (3,000 ×*g*; EVODOS, T10, Netherlands), and the cell pellet resuspended in the 500-L reactor containing fresh chlorine-free tap water. After 12 days following inoculation, the biomass was harvested via centrifugation (3,000×*g*; EVODOS, T10, Netherlands) for further analyses. It is recognized that nutrients limitations (e.g., N and/ or P) can alter the composition of carbohydrates, proteins and lipids (CHU et al., 2013). Therefore, studies were conducted to investigate the effects of N and/ or P starvation on microalgae AA and FA composition. Thus, one reactor after 12 days of phycoremediation, containing microalgae biomass was harvested via centrifugation and cells were re-suspended in 500 L nutrient-free water and a second reactor

containing N but not P. In the latter, to avoid N depletion during the tests, the concentration of nitrate was continuously monitored and added (50 mg NO_3^- L^{-1}) when needed (MICHELON et al., 2015). After 25 days, the cells were harvested by centrifugation (3,000 ×*g*; EVODOS, T10, Netherlands) and the cell pellet stored for further analysis.

2.2.3Analytical Methods

Phosphate-P was quantified by the ascorbic acid colorimetric method (APHA, 2012). Ammonia (NH_3-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) concentrations were determined by flow injection analysis (FIAlab–2500). Total organic carbon (TOC) was measured using a TOC analyzer (Multi C/N 2100, Analytik Jena). Alkalinity (as mg $CaCO_3$) was determined by automatic titration (Metrohm 848 Titrino Plus). Light intensity was measured with a Luximeter (DX-100, Japan). pH was monitored using pHmeter (pH–mV, Hanna Instruments, Inc.). A satisfactory correlation (r^2=0.98) between dry matter (DW) biomass content as measured by suspended solids (APHA, 2012) and optical density (OD_{570}) (mg_{DW} L^{-1}=543.84×OD_{570}nm – 37.726). Therefore, microalgae growth over time was assessed using a spectrophotometer (Varian, Inc. Cary® 50) analysis at 570 nm.

2.2.4 Quantification of AA and FA in microalgae

The concentration of AA was determined by the AOAC method 994.12 (AOAC, 2000) using a Hitachi (L-8900) AA analyzer. The FA were first extracted with dichloromethane and methanol and then by volatilization using sodium hydroxide and methanol described in AOCS (AOCS, 2013). FA were dissolved in 1 mL hexane and solution was dried with anhydrous sodium sulfate. 2 µL were injected on a GC Varian CP-3800 (Walnut Creek, Palo Alto, CA, USA), equipped with a split/splitless injector (1:100), a capillary column CP Sil 88 (50 m × 0.25 mm i.d. × 0.2 µm film thickness), a flame ionization detector (FID). Oven temperature was set to rise from 80 °C to 150 °C at 5 °C min^{-1}, then from 150 °C to 220 °C at 2 °C min^{-1}, and held at 220 °C for 6 min. Nitrogen was used as carrier gas at 1 mL min^{-1}. FA were identified by comparison of the peak retention times between each sample and the authentic standards (Sigma Aldrich). FA quantification in the sample solutions was done by external calibration using a methyl stearate curve (r^2=0.992). The AA and FA were reported as percentage of the total weight of protein and lipid, respectively.

2.2.5 Identification of hydrolyzed peptides from microalgal biomass

Digested peptides from microalgae biomass were obtained via protein digestion using trypsin enzyme (Sigma-Aldrich) solubilized in 400 µL of 50 mM ammonium bicarbonate (NH_4HCO_3), to a final concentration of 0.05 µg enzyme μL^{-1}. A 50 µL solution (1:50, enzyme: protein) was then prepared and incubated at 37 °C for 24h. Then, 10 µL of 10% (v v^{-1}) trifluoroacetic acid was added into solution and kept for 90 min at 37 °C. Samples were centrifuged for 30 min at 17,400 ×*g*, kept at 6 °C for further analysis.

Digested peptides identification was performed by high performance liquid nanochromatography coupled to the mass spectrometer using a NanoLC-ESI-Q-TOF system (Thermo Scientific UltiMate 3000 nano LC and Bruker Daltonics ESI-Q-TOF Impact II model), containing nanoeletrospray ionization source and TOF quadrupole mass analyzer. The peptides were separated into PepMap nanocolumn (C18, 5 µm particles, pore size 300 Å, 15 cm long, 75 µm internal diameter; Thermo Scientific) using a gradient of 3 to 97% (v v^{-1}) of acetonitrile (Sigma-Aldrich) containing 0.1% (w w^{-1}) formic acid for 180 min at a flow rate of 0.3 µL min^{-1}. Positive mode ionization and the precursor ion (MS) spectra were acquired in the 50-3000 m/z range with a 2 Hz acquisition frequency, capillary voltage at 1.5 kV, source temperature at 150 °C, 3 L min^{-1} drying gas flow and 0.2 bar nebulizer pressure. Precursor ion fragments (MS/MS) were acquired with an acquisition frequency of 4 to 16 Hz and collision energy between 23 and 65 eV.

Data files (.d) were imported into PEAKS Studio® 10 software (Bioinformatics Solution Inc., Waterloo, Canada) and MS/MS spectra were analyzed by searching the database using peaks DB, PTM and Spider (ZHANG et al., 2012). The input parameters were configured: 20 ppm precursor mass tolerance, 0.025 Da fragment mass tolerance, trypsin as specific enzyme in use, maximum three cleavage failures, Cys carbamidomethylation (+57.02 Da) as fixed modification and Met oxidation (+15.99 Da) as variable modification. False discovery rates (FDRs) for digested peptides were set at a maximum of 1%.

The possible existing correlation between the measured peptides and bioactive functionalities was determined using the BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (MINKIEWICZ et al., 2008).

2.2.6 Statistical analysis

Statistical differences between treatments data sets were determined using one-way analysis of variance (ANOVA) with Statistica® software. Tukey's significant difference post hoc test was conducted after the determination of variances ($p \geq 0.05$).

2.3 RESULTS AND DISCUSSION

2.3.1 Phycoremediation efficiently removes nutrients from wastewater

Ammonia-N and phosphate-P were both completely removed (100%) from the effluents tested (UASB and NR) (Figure 2.2AB) after 11 days. It is worth mentioning that ammonia-N removal was unikely attributed to microalgal assimilation only. In this case, bacterial-mediated nitrification and denitrification activities as reported by Mezzari et al. (2013) very likely contributed to ammonia-N removal as suggested by the presence of nitrite as byproduct of nitrification in the samples collected 3 days after the beginning of the experiments (Fig 1A). The maximum biomass concentration of 247±3.4 and 188±5.5 mg L^{-1} (DW) was obtained with the use of UASB and NR effluents, respectively (Figure 2.2AB). Overall, these data corroborate to the recent findings on phycoremediation as efficient approach for the removal of nutrients from piggery wastewaters (CHENG et al., 2019; NAGARAJAN et al., 2019).

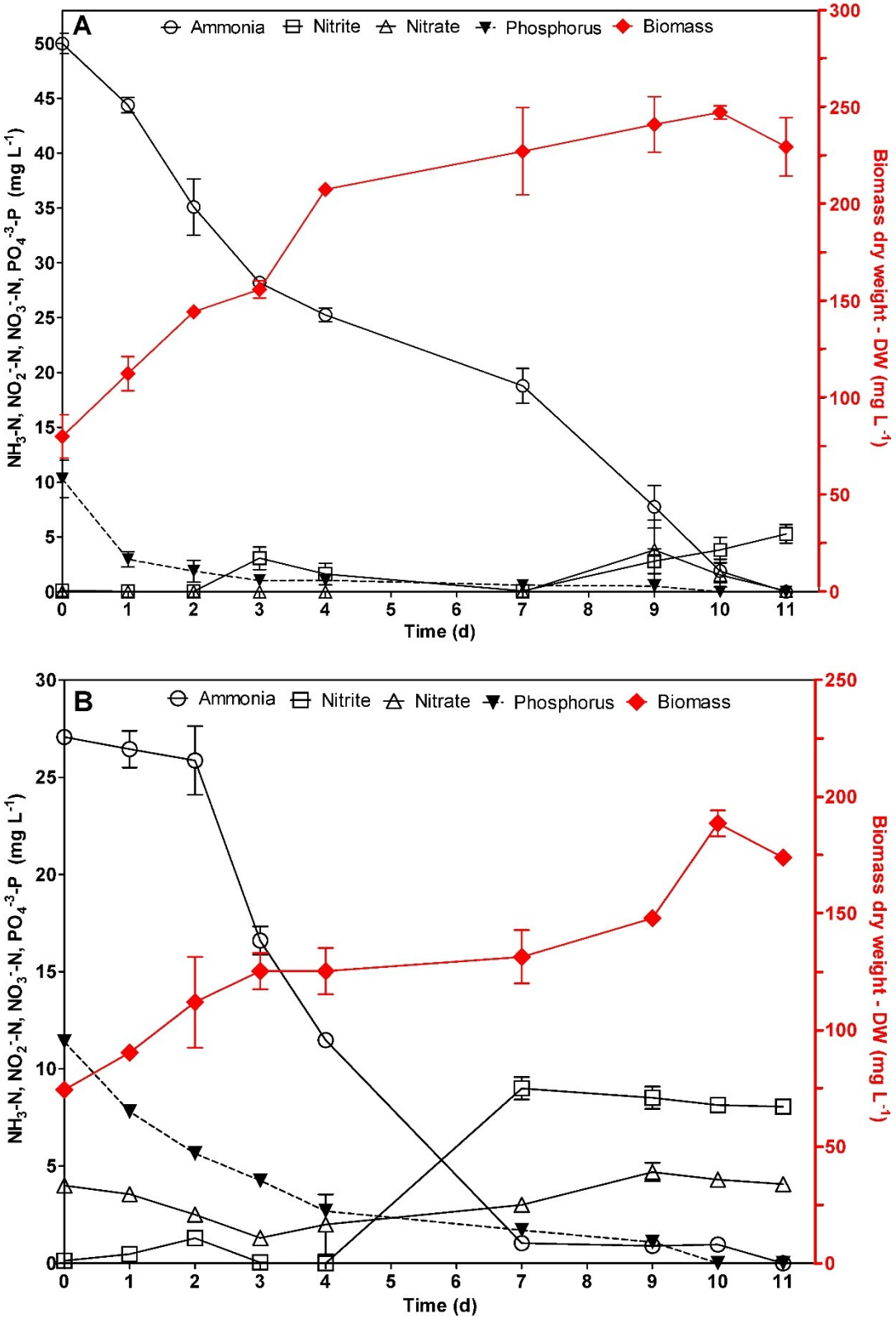


Figure 2.2. Nutrient removal and biomass concentration during phycoremediation of UASB (A) and NR (B) effluents. Bars depict standard deviation of the mean and different letters denote significant differences ($p \leq 0.05$) according to Tukey test.

2.3.2 Microalgae grown in swine wastewaters contain high protein and carbohydrate contents

The protein, carbohydrate and lipid contents measured in the microalgae biomass harvested after complete removal of nutrients from UASB and NR effluent is shown in Figure 2.3. Variations in protein and carbohydrates contents were observed as a function of the effluents tested (UASB and NR). The biomass cultivated in the UASB showed protein and carbohydrate contents of 50.1±0.7% and 34.4±0.4%, respectively. The microalgae biomass harvested from the reactor fed NR effluent had significantly lower ($p<0.05$) protein (44±0.9%), and higher carbohydrate 41.9±0.9% contents. Irrespective of the wastewater used, however, the lipid content measured in the biomass was relatively low ≤2%, suggesting that in the presence of sufficient amounts of N and P in the medium, cells preferentially store energy in the form of proteins and carbohydrates instead of lipids. Compared to UASB, the biomass grown on NR effluent had higher carbohydrate content (as short-term most favorable source of energy storage) due to lower N and P concentrations in this medium. The relatively high protein fraction observed in both microalgae biomass is likely attributable to medium composition containing adequate concentrations of nitrogen compounds (e.g., ammonia-N) known to induce protein production in microalgae (ZHU et al., 2014). Chinnasamy et al. (2010) observed similar trends in the biochemical composition of *Chlamydomonas globosa*, *Chlorella minutissima* and *Scenedesmus bijuga* cultivated in industrial wastewater (also rich in N and P), i.e., protein (53.8%), carbohydrate (15.7%) and, lipid (5.3%). Contrariwise, some other specific microalgae can significantly accumulate more lipids in the cell. For instance, under controlled laboratory-scale conditions [temperature (25±1 °C); injection air (5–6% CO_2) and continuous light irradiance (230±20 μmol m^{-2} s^{-1})], *Chlorella zofingiensis* grown in piggery wastewater can accumulate lipid by as much 43% of total cell weight (ZHU et al., 2013b).

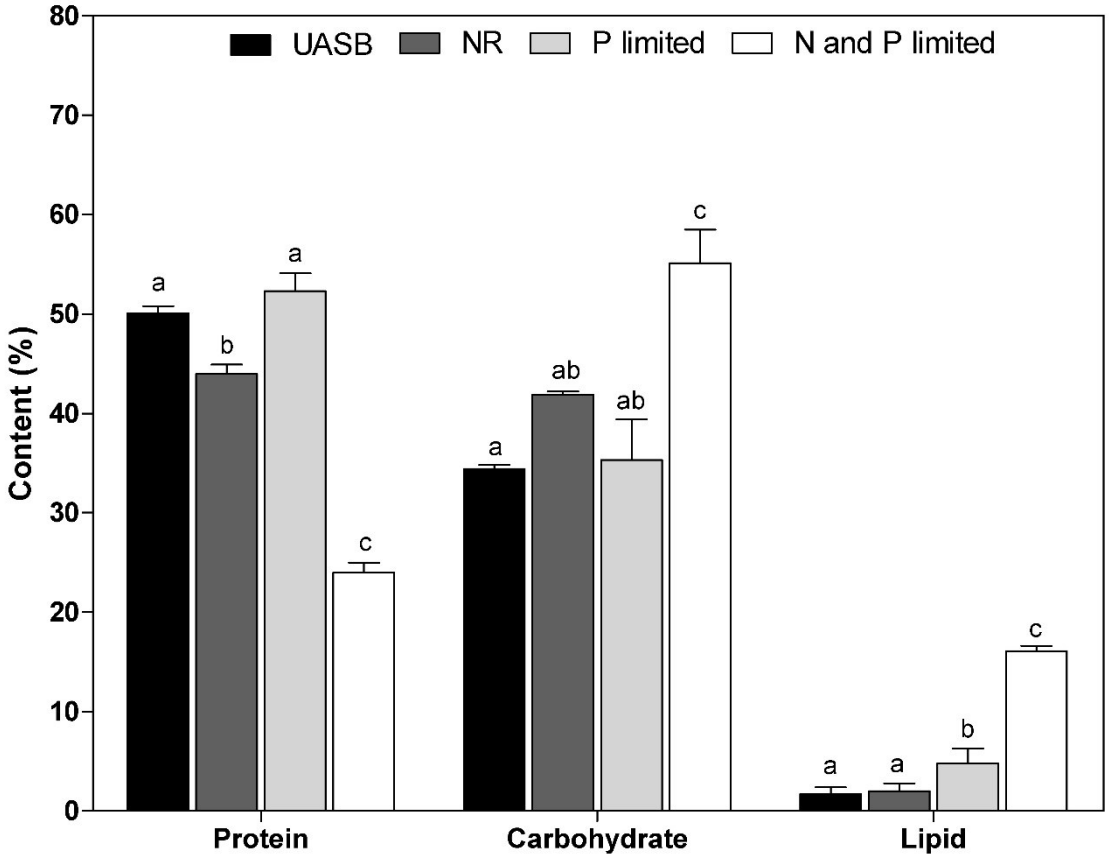


Figure 2.3. Biochemical composition (%) of the microalgae consortium harvested in different stages of growth. Bars depict standard deviation of the mean and different letters denote significant differences ($p \leq 0.05$) according to Tukey test.

The effects of N and/or P limitations on lipid storage was observed (Figure 2.3). Lipid content increased to 16.1±0.5% and 4.8±1.5% when cultivated in the absence of both N and P or just P, respectively. The increase in lipid content associated with nutrients limitations was also reported previously in cells of *Nannochloropsis oculata* and *Chlorella* spp. (CONVERTI et al., 2009). These results suggest that the fraction of proteins, carbohydrates and/or lipids can be strategically manipulated by inducing nutrients-related stress conditions (CHU et al., 2013; HO et al., 2013).

2.3.3 Microalgae grown in swine wastewaters are rich in essential aminoacids

Microalgae can synthesize essential and non-essential amino acids (SAFI et al., 2014) that can be further processed for human and animal nutrition (FAO/WHO, 1973). The AA profiles found in the microalgae consortium cultivated with UASB or NR effluents as well as under the absence of nutrients (N and/or P) is shown at Table 1. Eighteen amino acids were identified, in which 11 are essentials i.e., histidine, arginine, threonine, proline, valine,

methionine, isoleucine, leucine, phenylalanine, lysine and tryptophan. The concentration of the obtained AA (≈22% DW of total protein) was in agreement with previous studies using wastewater as growth medium for microalgae (MOHEIMANI et al., 2018). It is expected that the composition of AA is likely to change depending on the microalgae species. For example, Canizares-Villanueva et al. (CANIZARES-VILLANUEVA et al., 1995) reported that the concentrations of AA found in *Spirulina* was comparatively higher than those found in *Phormidium* when both genus of microalgae were cultivated in the same diluted swine wastewater as growth medium.

After two days of cultivation, the wastewater containing only N (but not P) produced microalgae with considerably high concentrations of AA (59.6% DW) compared to the biomass harvested (after 12 days) from wastewater depleted of N (23.3% DW). A notable difference was found in methionine, cysteine and phenylalanine contents at these different growth stages, with an incremental production (6.8±0.7%) within 8 days of cultivation. Other AA remained at similar concentrations along the entire experimental time frame. Xupeng et al. (2017) reported a 3-fold increase in phenylalanine during the early stages of microalgae growth when N was still bioavailable followed by a decrease in AA concentrations associated with the depletion of N towards the end of the experiments.

Typical swine diets need to be supplemented with the essential AA lysine, threonine, methionine, and tryptophan (HEGER; PHUNG; KŘÍŽOVÁ, 2002). Dietary deficiency of those AA may impair animal growth, immunity, increase the susceptibility to infectious diseases as well as encourage other digestive and reproductive problems (LITVAK et al., 2013). The concentration of these same AA found in the microalgae biomass (ranging from 0.2-2.5%) exceeds the minimum AA requirements (Table 2.1). Thus, whereas microalgae produced during phycoremediation of wastewaters could be later processed as source of animal nutrition supplementation (circular economy) requires further investigations.

Table 2.1. Amino acid composition (% of total protein dry weight) of the microalgae biomass grown in swine wastewater effluent and under N- and/or P-limiting conditions in comparison to typical amino acid (%) used to feed male pigs with high genetic potential.

Amino acids (%)	*Chlorella* sp. and *Scenedesmus* sp.	*Spirulina maxima*	*Phormidium* sp.	Microalgae consortium				*p value*	[Starting	Growing	Finishing]*
	Anaerobically digested piggery effluent	Aeration-stabilized swine waste		UASB	NR	P limited	N and P limited				
Aspartic acid	2.4	7.3	4.7	**4.4±0.4**^{a}	3.0±0.1^{b}	4.1±0.05^{a}	1.4±0.12^{c}	<0.001	-	-	-
Glutamic acid	3.6	12.3	5.01	**4.9±0.04**^{a}	3.3±0.2^{b}	4.9±0.05^{a}	2.0±0.03^{c}	<0.001	-	-	-
Serine	1	3.9	2.1	1.8±0.1^{a}	1.3±0.06^{b}	**2.1±0.04**^{c}	0.8±0.02^{d}	<0.001	-	-	-
Glycine	1.4	4.05	4.4	**2.8±0.3**^{a}	1.9±0.3^{b}	2.8±0.01^{a}	1.1±0.05^{c}	<0.001	-	-	-
Histidine	0.2	2.3	0.85	**0.9±1.2**^{a}	0.5±0.2^{b}	0.6±0.07^{b}	0.2±0.04^{c}	<0.001	0.4	0.3	0.3
Arginine	1.1	5.5	2.8	**3.9±2.4**	1.9±0.02	2.6±0.03	0.9±0.01	0.08	0.6	0.4	0.3
Threonine	1.1	4	2.6	1.9±0.04^{a}	1.5±0.04^{b}	**2.3±0.04**^{c}	1.1±0.03^{d}	<0.001	0.9	0.8	0.6
Alanine	2.3	5.9	3.8	2.9±0.6^{a}	2.3±0.4^{ac}	**4.1±0.01**^{b}	1.6±0.1^{c}	<0.001	-	-	-
Proline	1.3	3.9	1.1	1.9±0.09^{a}	1.3±0.05^{b}	**2.4±0.04**^{c}	0.9±0.02^{d}	<0.001	-	-	-
Tyrosine	0.7	3.2	2.6	1.2±0.4^{a}	0.8±0.3^{ab}	**1.3±0.02**^{a}	0.5±0.01^{b}	0.01	-	-	-
Valine	1.4	4.1	2.1	**2.4±0.3**^{a}	1.7±0.2^{b}	2.8±0.04^{a}	1.1±0.01^{c}	<0.001	1.0	0.8	0.6
Methionine	0.5	0.53	0.6	**2.8±3.5**	1.02±1	0.7±0.01	0.3±0.005	0.39	0.4	0.3	0.3
Cysteine	0.3	-	-	**2.9±3.8**	1.9±2.7	0.7±0.06	0.2±0.04	0.53	-	-	-
Isoleucine	0.8	3.3	2.3	1.5±0.2^{a}	1.2±0.2^{a}	**2.0±0.02**^{b}	0.8±0.05^{c}	<0.001	0.8	0.6	0.5
Leucine	2	6.7	4.1	3.4±0.1^{a}	2.5±0.01^{b}	**4.1±0.02**^{c}	1.7±0.07^{d}	<0.001	1.4	1.1	0.8
Phenylalanine	1.2	3.4	2.2	2.2±0.2^{ab}	1.6±0.3^{a}	**2.5±0.1**^{b}	0.9±0.02^{c}	<0.001	0.7	0.6	0.4
Lysine	1.2	4.1	1.8	**2.5±0.02**^{a}	1.2±0.1^{b}	2.2±0.1^{c}	0.9±0.04^{d}	<0.001	1.5	1.2	0.8
Tryptophan	0.3	-	-	**0.5±0.01**^{a}	0.2±0.01^{b}	0.3±0.01^{c}	0.2±0.005^{d}	<0.001	0.2	0.2	0.2
Reference	Moheimani et al. (2018)	Canizares-Villanueva et al. (1995)		This study							

* Nutritional requirements of whole male pigs of high genetic potential (Rostagno et al., 2017).
Data shown as means±standard deviation.
Different letters denote significant differences ($p < 0.05$) according to Tukey HSD test.

2.3.4 Microalgae grown in swine wastewater accumulates monounsaturated fatty acids

PUFAs present in microalgae are known to promote health and disease prevention (SONG et al., 2020). For instance, supplementation of PUFAs on swine diet is recognized for its beneficial physiological effects on metabolism in the intestinal mucosa, and antimicrobial, anti-inflammatory and immunomodulatory activity (ROSSI et al., 2010). Among the different omega-3 fatty acids present in microalgae, the bioactive eicosapentaenoic acid and docosahexaenoic acid are considered the most important due to its important nutritional value (BECKER, 2013). The effects of omega-3 polyunsaturated fatty acids obtained from microalgae were reported to decrease serum levels of triglycerides during swine gestation, improving swinelet birth weights (POSSER et al., 2018). Similarly, the supplementation of swine diets with omega-3 from microalgae *Schizochythrum* sp. increased the concentration of this fatty acid in swine, increasing overall meat value due to potential health benefits to consumers (SARDI et al., 2006). As discussed earlier, the accumulation of lipids increased under nutrients deprived conditions. As expected, the increase in lipid content was accompanied by an increase in monounsaturated (MUFAs, from 0.2 to 4.7%), polyunsaturated (PUFAs; from 0.6 to 5.3%), unsaturated (UFAs; from 0.9 to 10%) and saturated (SFA, from 0.3 to 5.8%) FA (Table 2.2). Comparatively, other microalgae consortium (*Chlorella*, spp., *Nannochloropsis* sp., *Scenedesmus* spp*., Chlamydomonas* spp., *Oscillatoria* sp., *Kirchnella* sp., and *Microcoleus* sp.) found in the effluent of municipal wastewater treatment system, was composed mainly by saturated FA followed by MUFA and PUFA (SHARMA et al., 2020).

Table 2.2. Fatty acids content (% of total lipids dry weight) of the microalgae biomass grown in swine wastewater effluent and under N- and/or P-limiting conditions.

Fatty acids (%)	UASB	NR	P limited	N and P limited	*p value*
Myristic Acid (C14:0)	0.01±0.006^{a}	0.008±0.002^{a}	0.02±0.005^{a}	0.09±0.005^{b}	<0.001
Myristoyl Acid (C14:1)	0.04±0.006	0.02±0.005	0.01	0,03±0.04	0.394
Pentadecanoic Acid (C15:0)	0.01±0.006	0.009±0.0005	0.009±0.0005	0.01±0.005	0.189
Palmitic Acid (C16:0)	0.25±0.04^{a}	0.35±0.04^{a}	0.86±0.03^{b}	5.48±0.36^{c}	<0.001
Palmitoleic Acid (C16:1n7)	0.17±0.23^{a}	0.04±0.005^{b}	0.02±0.01^{b}	0.04±0.005^{b}	<0.001
Margaric Acid (C17:0)	0.02±0.12^{a}	0.02±0.01^{a}	0.03±0.02^{ab}	0.05±0.005^{b}	<0.001
Estearic Acid (C18:0)	0.04±0.12^{a}	0.04±0.005^{a}	0.03±0.005^{a}	0.6±0.3^{b}	<0.001
Oleic Acid (C18:1n9c)	0.08±0.12^{a}	0.16±0.03^{a}	0.2±0.11^{a}	4.7±0.2^{b}	<0.001
Linoleic Acid (C18:2n6c)	0.15±0.04^{a}	0.2±0.02^{a}	0.2±0.08^{a}	1.5±0.32^{b}	<0.001
Linolenic Acid (C18:3n6)	0.02±0.012^{a}	0.03±0.01^{ab}	0.02±0.005^{ab}	0.04±0.005^{b}	0.05
Linolenic Acid (C18:3n3)	0.48±0.017^{a}	0.5±0.03^{a}	1.1±0.04^{b}	3.9±0.07^{c}	<0.001
Arachic acid (C20:0)	-	-	-	0.02±0.005	<0.001
Eicosatrienoic acid (20:3)	-	-	0.009±0.0005	0.02±0.005	0.104
Behenic Acid (C22:0)	-	0.009±0.001^{a}	0.02±0.01^{a}	0.04±0.005^{b}	<0.001
Erucic Acid (C22:1n9)	-	-	-	0.01±0.005	<0.001
Eicosapentaenoic Acid (C20:5n3)	-	-	-	0.02±0.01	<0.001

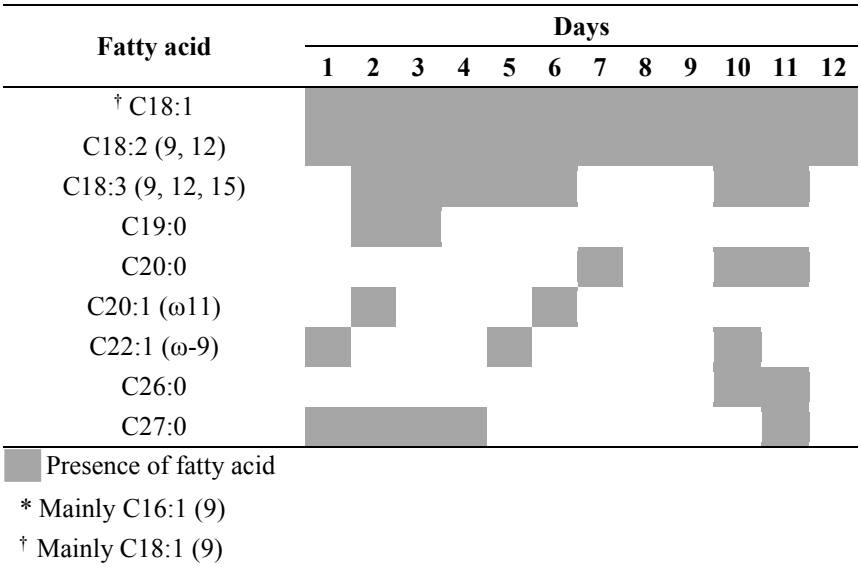
Fatty acids (%)	UASB	NR	P limited	N and P limited	*p value*
Lignoceric Acid (C24:0)	-	-	0.01	0.01±0.005	>0.05
Σ MUFAs	0.28	0.2	0.21	4.75	
Σ PUFAs	0.61	0.71	1.22	5.27	
Σ UFAs	0.92	0.91	1.45	10.03	
Σ SFAs	0.27	0.39	0.90	5.83	
Σ ω-3	0.48	0.49	1.04	3.9	
Σ ω-6	0.15	0.22	0.18	1.37	
Σ ω-9	0.09	0.14	0.21	4.7	

Monounsaturated (MUFAs), polyunsaturated (PUFAs), unsaturated (UFAs) and saturated (SFA) fatty acid.
Data shown as means±standard deviation
Different letters denote significant differences ($p<0.05$) according to Tukey HSD test.

The concentration profile of FA measured in microalgae biomass during different states of cell growth was also evaluated (Table 2.3). Long-chain FA (from 13 to 21 C) were dominant. Very long FA (22 or more C), in particular 22:1 (ω11), 26:0 and 27:0 (Table 2.3). Essential FA commonly used as supplement in animal nutrition, i.e., α-linolenic acid (C18:3) and linoleic acid (C18:2) (BENATTI et al., 2013) were also present. Overall, the FA composition is in agreement with other reported values. For instance, Gan et al. (2019) reported that *Chlorella vulgaris* produced high percentage of saturated FA, in particular palmitic acid (16:0) ≈38%. In addition, the authors described that *Chlorella vulgaris* can biosynthesize very long FA as 20:2, 20:3 and 20:4. Norashikin et al. (2018) also observed high concentrations of C:18 (2) and C:18 (3) in *Chlorella vulgaris*. The relatively high concentrations of unsaturated FA observed in the indigenous microalgae consortium and growth conditions tested in this study may be of particular interest for industries seeking alternative sources of renewable and sustainable feedstock for product manufacture (SAFI et al., 2014).

Table 2.3. Fatty acids profile in microalgae harvested from phycoremediation of UASB digestate.

Fatty acid	Days											
	1	2	3	4	5	6	7	8	9	10	11	12
C10:0												
C13:0												
C14:0												
C14:1												
C15:1												
C16:0												
* C16:1												
C16:2 (7, 10)												
C16:3 (7, 10, 13)												
C18:0												



2.3.5 Potential bioactive peptides

Peptides consist of a diverse group of oligomeric structures usually composed of protein fragments or chains of different short amino acid sequences, usually 2–20 residues. These metabolites have been reported in the regulation of a number of cellular processes such as hormonal regulation, redox homeostasis, neuronal signal, cell signaling, transduction, growth and immune response (ROMANOVA; SWEEDLER, 2015). In this study, twenty five bioactive peptides were found in the microalgae consortium, with possible multiple biofunctionalities including Angiotensin converting enzyme (ACE) Inhibitory, dipeptidyl peptidase-IV, dipeptidyl peptidase III inhibitor, anti-amnestic, antithrombotic, immunomodulating, CaMPDE inhibitor, renin inhibitor, antioxidative, activating ubiquitin-mediated proteolysis, opioid, regulating, stimulating, neuropeptide, alpha-glucosidase inhibitor, hypolipidemic, HMG-CoA reductase inhibitor, inhibitor of insulin secretion, chemotactic, anti-inflammatory, bacterial permease ligand, anticancer, hypotensive, chymotrypsin inhibitor and dipeptidyl carboxypeptidase inhibitor (Table 2.4). Most functional bioactive peptides are from the ribulose-1,5-bisphosphate carboxylase/oxygense (RuBisCO) protein complex responsible for the conversion of atmospheric carbon dioxide into organic carbon through the Calvin cycle (SELVARAJ et al., 2017) and to a lower extent related to 50S Ribosomal Protein L7/L12, phosphoglycerate kinase, ATP Synthase Subunit Beta and Heat Shock Protein 70 (TEJANO et al., 2019). It is worth noting that most of the peptides determined in microalgae biomass were dipeptides or tripeptides with diverse biological activities. To illustrate, a pepsin-hydrolyzed peptide with important antioxidant activities were obtained from *Chlorella vulgaris* and *Chlorella ellipsoidea* (SHEIH; WU; FANG, 2009). Similarly, bioactive peptides with antihypertensive, anticancer and ACE inhibitory activities were isolated from *Chlorella* spp.

and *Spirulina platensis* (KO et al., 2012; SHEIH et al., 2010; SHEIH; FANG; WU, 2009; ZHANG; ZHANG, 2013).

Table 2.4. Bioactive peptides identified in the microalgae consortium grown in UASB digestate using BIOPEP's "profiles of potential biological activities" tool.

Bioactive peptides	Days							
	1	3	5	6	7	9	11	12
ACE Inhibitory	■	■	■	■	■	■	■	■
Dipeptidyl peptidase-IV	■	■	■	■	■	■	■	■
Dipeptidyl peptidase III inhibitor	■	■	■	■	■	■	■	■
Anti-amnestic	■	■	■	■	■	■	■	■
Antithrombotic	■	■	■	■	■	■	■	■
Immunomodulating	■	■	■	■	■	■	■	■
CaMPDE inhibitor	■	■	■	■	■	■	■	■
Renin inhibitor	■	■	■	■	■	■	■	■
Antioxidative	■	■	■	■	■	■	■	■
Activating ubiquitin-mediated proteolysis	■	■	■	■	■	■	■	■
Opioid	■	■	■	■	■	■	■	■
Regulating*	■	■	■	■	■	■	■	■
Stimulating**	■	■	■	■	■	■	■	■
Neuropeptide	■	■	■	■	■	■	■	■
Alpha-glucosidase inhibitor	■	■	■	■	■	■	■	■
Hypolipidemic	■	■	■	■	■	■	■	■
HMG-CoA reductase inhibitor	■	■	■	■	■			
Inhibitor of insulin secretion			■	■	■	■	■	■
Chemotactic			■	■	■	■	■	■
Anti-inflammatory			■	■	■	■	■	■
Bacterial permease ligand	■			■	■			
Anticancer	■	■	■					
Hypotensive	■	■	■					
Chymotrypsin inhibitor			■					
Dipeptidyl carboxypeptidase inhibitor								■

■ Presence peptides

* Peptide regulating ion flow or peptide regulating the stomach mucosal membrane activity

** Stimulating vasoactive substance release or glucose uptake stimulating peptide

Overall, the microalgae biomass obtained from the phycoremediation of swine wastewaters seems to contain significant concentrations of important AA (e.g., arginine, lysine, tryptophan, proline), FA (e.g., conjugated linoleic acids and EPA), and peptides with remarkable biological properties. Whereas the use of this source of feedstock biomass is feasible for exploration of these compounds remains unclear and requires further investigations.

2.4 CONCLUSIONS

Phycoremediation efficiently removed ammonia-N and P (100%) from swine wastewaters effluents. Significant accumulation of lipids in microalgae biomass was observed in nutrients deprived growth conditions. The most abundant essential AA found were lysine, leucine, threonine, methionine and tryptophan. FA was mainly composed by linolenic acid (ω-3 and 6) and oleic acid (ω-9). It was identified, 25 peptides that are associated with relevant biological functions. The concept of biorefinery herein contribute to advancing our understanding of technological arrangements combining wastewater treatment with production of biomass rich in metabolites with a broad range of biotechnological applications.

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- CHAPTER 3 -

3 MICROALGAE PRODUCED DURING PHYCOREMEDIATION OF SWINE WASTEWATER CONTAINS EFFECTIVE BACTERIOSTATIC COMPOUNDS AGAINST ANTIBIOTIC-RESISTANT BACTERIA [3]

Abstract: Studies on the antimicrobial effects of microalgae extracts are commonly reported using algae biomass grown in sterile synthetic mineral medium and controlled laboratory conditions. However, variations in environmental conditions and culture medium composition are known to alter microalgae biochemical structure possibly affecting the type and concentrations of bioactive compounds with antimicrobial properties. In this work, solvent extracts of the microalgae *Chlorella* spp. were tested for antimicrobial effects against gram-positive and multidrug resistant pathogenic bacteria *Staphylococcus hyicus*, *Enterococcus faecalis* and *Streptococcus suis*. Microalgae was cultivated at field scale open pond reactor using raw swine wastewater as growth substrate. Dichloromethane or methanol were used to obtain the microalgae extracts. Characterization of the extracts by ultra-high performance liquid chromatography-quadrupole mass spectrometry revealed the presence of 23 phytochemicals with recognized antimicrobial properties. Bacteriostatic activity was observed in plating assays by formation of inhibition zones ranging from 7 to 18 mm in diameter. Only dichloromethane extracts were inhibitory to all three model bacteria. The minimum inhibitory concentration assessed for dichloromethane extracts were 0.5 mg mL^{-1} for *Staphylococcus hyicus* and *Enterococcus faecalis* and 0.2 mg mL^{-1} for *Streptococcus suis*. Bactericidal effects were not observed using solvent-extracts at 2 or 5 mg L^{-1}. To the best of author's knowledge, this is the first report on the antimicrobial effects of *Chlorella* spp. extracts against *Staphylococcus hyicus* and *Streptococcus suis*. Overall, *Chlorella* spp. grown on swine wastewater contains several phytochemicals that could be further explored for the treatment of infections caused by antibiotic-resistant bacteria pathogens.

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3.1 INTRODUCTION

The use of antibiotics is essential in large-scale animal production, however its continuous application [i.e., misuse and/or abuse (LIPSITCH; SINGER; LEVIN, 2002)] has been associated with selection and spreading of resistant pathogenic bacteria (CHANG et al., 2014; BAQUERO et al., 2008). The increasing resistance of *Staphylococcus hyicus* (*S. hyicus*) (OSMAN et al., 2016; PARK et al., 2013a), *Streptococcus suis* (*S. suis*) (HERNANDEZ-GARCIA et al., 2017) and *Enterococcus faecalis* (*E. faecalis*) (ŠEPUTIENE et al., 2012) became a problem not only for treating and controlling diseases in animal production systems but also of concern as an emerging zoonotic agent in humans (GOYETTE-DESJARDINS et al., 2014). Therefore, alternative practices and medications are constantly being investigated in attempt to minimize or even replace antibiotics use.

Microalgae has been extensively explored for its potential as renewable feedstock for food and energy production (JACOB-LOPES et al., 2019; SUDHAKAR et al., 2019) as well as a source of novel molecules with a wide range of biotechnological applications (MAYER et al., 2013; DANTAS et al., 2019; GUZMÁN et al., 2019; HUSSEIN et al., 2020). For instance, compounds with antimicrobial properties were previously identified in microalgae thriving in oceans (CHANG et al., 1993; OHTA et al., 1993; RAMOS et al., 2015), freshwaters (Bhagavathy et al., 2011; Santoyo et al., 2009), and soils (SAFONOVA; REISSER, 2005). These bioactive compounds are typically extracted with solvents (SHANNON; ABU-GHANNAM, 2016) and include phenolic compounds (WANG et al., 2009), fatty acids (ZHENG et al., 2005), protein/peptides (NGUYEN; HANEY; VOGEL, 2011; RAMOS et al., 2015), terpenes (RODRIGUES et al., 2015) and/or polysaccharides (HE et al., 2010). The exact mechanisms of bacteria inhibition are still not completely understood (SHANNON; ABU-GHANNAM, 2016) but know to be bacteria-specific (CEPAS et al., 2019). To illustrate, extracts from *Chlorella* spp. had no inhibitory effect on *Enterobacter* sp., *Proteus* sp., *Escherichia coli*, *Klebsiella* sp., *Staphylococcus aureus*, *Lactobacillus acidophilus* and *Streptococcus pyogenes* (HUSSEIN; NAJI; AL-KHAFAJI, 2018). Yet, bactericidal, or bacteriostatic effects were reported on *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Serratia marcescens* (SANTHOSH et al., 2019).

Variations in the culture medium and environmental conditions are known to alter microalgae biochemical composition (AREMU et al., 2015), ultimately affecting the type, concentration and effectiveness of bioactive compounds (AREMU et al., 2014, 2015; DUVAL;

SHETTY; THOMAS, 1999). Most studies on microalgae-extracts were conducted in controlled conditions using sterile synthetic mineral medium (PLAZA et al., 2012; SANTHOSH et al., 2019) (Table 3.1). However, the continuous supply of nutrients for microalgae production at scale is known to affect industry profitability as result of increased operational costs (JEBALI et al., 2015). In this regard, wastewater rich in nutrients have been considered alternatively as source of nutrients for the cultivation of microalgae (ZHANG et al., 2016) while decreasing water footprint. Nonetheless, information on the effects and characterization of biocides with antimicrobial properties from microalgae biomass grown in wastewater is scarce or unavailable. Further studies are thus needed to advance current knowledge on these value-added bioactive products from algae-grown wastewater.

Therefore, this study aimed to: i) determine if extracts of *Chlorella* spp. grown in raw swine wastewater simulating field scale phycoremediation have antimicrobial effects on three industrially relevant antibiotic resistant bacteria i.e., *Streptococcus suis*, *Enterococcus faecalis* and *Staphylococcus hyicus* and, ii) characterize the extracts for the presence of bioactive compounds using Ultra High-Pressure Liquid Chromatography (UHPLC).

Table 3.1. Antibacterial activity of microalgae extracts against several pathogenic bacteria.

Microalgae	Culturing media	Solvent-extracts	Targeted bacteria	Inhibition zone size (mm)	References
Chlorella variabilis	Synthetic	Diethyl ether and methanol	*Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis* and *Staphylococcus aureus*	7.5–15	Gee et al., 2020
Chlorella sorokiniana	Synthetic	Hexane, diethyl ether, chloroform, dichloromethane, ethyl acetate, methanol/dichloromethane (1:1), acetone, ethanol, methanol and water	*Pseudomonas aeruginosa Schroeter, Escherichia coli, Salmonella enterica subsp. Enterica ex Kauffmann and Edwards. Le Minor and Popoff and Proteus mirabilis*	0–10.7±1.1	Navarro et al., 2017
Isochrysis galbana, Scenedesmus sp., and Chlorella sp.	Synthetic	Ethyl acetate, ethanol and methanol in water	*Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Enterococcus faecalis, Clavibacter michiganensis, Escherichia coli , Salmonella typhoid, Pseudomonas syringae, and Proteus vulgaris*	0-20	Alsenani et al., 2020
Scenedesmus obliquus	Synthetic	Methanol, ethanol, acetone, chloroform, diethyl ether, ethyl acetate and hexane	*Bacillus cereus, Staphylococcus aureus, Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae*	0–18.5±2.18	Marrez et al., 2019
Chlorella vulgaris	Synthetic	Methanol and hexane	*Staphylococcus aureus*	0–9	Ali, 2016
Chlorella vulgaris	Synthetic	Methanol, chloroform and diethyl ether	*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis*	0–28.6±0.6	Alwathnani and Perveen, 2017
Chlorella vulgaris	Synthetic	Methanol	*Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*	12–17.5	Jayshree et al., 2016
Chlorella spp.	Synthetic	Ethyl acetate and ethanol	*Staphylococcus aureus* and *Escherichia coli*	0–14±2	Li et al., 2016
Chlorella spp.	Synthetic	Methanol, ethyl acetate, chloroform and hexane	*Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Serratia marcescens*	0–26±0.6	Santhosh et al., 2019
Chlorella spp.	Non-sterile swine digestate	Dichloromethane and methanol	*Staphylococcus hyicus, Streptococcus suis* and *Enterococcus faecalis*	0–18±1	**This study**

3.2 MATERIAL AND METHODS

Figure 3.1 shows a schematic diagram of the material and methods. Steps are described in the sections below.

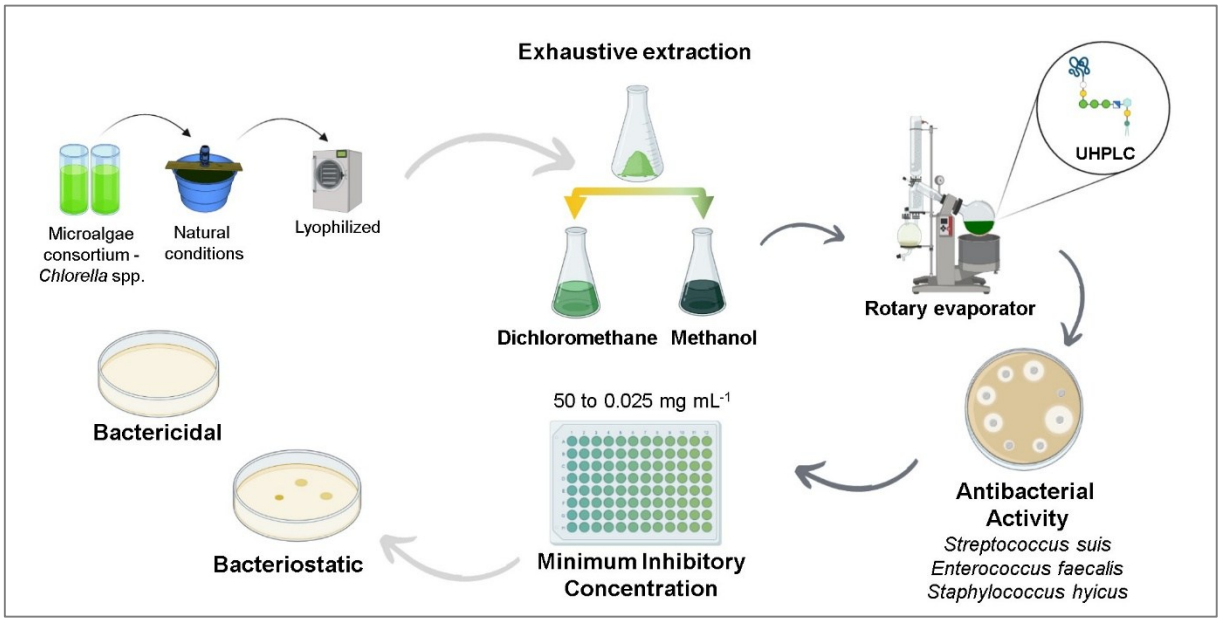

Figure 3.1. Schematic diagram of methodology

3.2.1 Microalgae cultivation

In this study the microalgae *Chlorella* spp. previously isolated from a field scale open pond designed as tertiary process for the treatment of swine wastewater (Brazilian Agricultural Research Corporation, Santa Catarina, Brazil) was utilized as inoculum (MICHELON et al., 2015) (Supplementary material I). Cells were grown in 500 L open reservoir placed inside a greenhouse under temperature-controlled conditions (25 °C) and exposed to natural sun light (photosynthetic photon flux density of 321.5±411.4 μmol m^{-2} s^{-1}). The growth medium consisted of 30 L non-sterile swine wastewater effluent digestate collected from an upflow anaerobic sludge blanket (UASB) diluted into 320 L of chlorine-free tap water. Dilution of digestate was necessary to decrease turbidity allowing sufficient light penetration for microalgae growth. The final medium composition was (in mg L^{-1}): total organic carbon (110±4.4), biological oxygen demand (BOD_5 89.8±1.2), alkalinity as $CaCO_3$ (201±9.2), total nitrogen (53.3±5.9), ammonia-N (49.1±1.7) and phosphate-P (11.5±5.5). The medium was continuously stirred using a submersible pump at 1,200 L h^{-1} (Sarlobetter®, S300, Brazil). A microalgae dry weight (DW) concentration of 70±0.2 mg_{DW} L^{-1} (30% v v^{-1}) was used as inoculum. The pH of the medium at time zero was 8 increasing to 11 after 11 days of cultivation. After 11 days following inoculation, the microalgae biomass was harvested by centrifugation at 3,000 ×g (EVODOS T10, Netherlands). A total biomass fresh weight of 0.3±0.1 g L^{-1} was

obtained. The collected biomass was then immediately frozen (-20 °C) and lyophilized (Model 030-JJ LJI Scientific, Brazil) for further analysis.

3.2.2 Analytical methods

The concentration of phosphate-P was determined by the ascorbic acid colorimetric method [4500-P, Standard Methods for the Examination of Water and Wastewater (APHA, 2012)]. The reagent solution was prepared using 50 mL sulfuric acid (5 N) (Sigma-Aldrich, USA), 5 mL antimony potassium tartrate solution (Sigma-Aldrich, USA), 15 mL ammonium molybdate solution (Synth, China), and 30 mL ascorbic acid solution (Synth, China). Then, 0.8 mL of this solution was added to 5 mL of previously filtered samples (0.45 μm membrane filter, Millipore, USA). After 10 min, the absorbance of each sample was measured in a UV-Visible spectrophotometer (Cary® 50 UV-Vis, Varian, USA) at wavelength of 880 nm. Standard curves were prepared by serial dilution of a stock phosphate-P solution (0.05–0.2 mg-P L^{-1}) (Merck, Germany).

Alkalinity (measured as $CaCO_3$ L^{-1}) was determined by automatic titration (Metrohm 848 Titrino Plus, Switzerland) using sulfuric acid (0.1 M, Merck, Germany) as titrant. Alkalinity was determined as $CaCO_3$ L^{-1}: [(M×A×10,000)/V]; where M is molarity of standardized acid (M); A is the acid volume dispensed to reduce sample pH to 4.5 (mL) and V is total sample volume (mL).

Nitrite-N, nitrate-N, and ammonia-N concentrations were determined by series of colorimetric assays performed automatically by a flow injection analysis system (FIAlab 2500 system, USA) equipped with a cadmium column (0.5 mm ID × 5 cm long) (APHA, 2012). Briefly, samples (10 mL) were filtered using a 0.45 μm membrane filter (Millipore, USA) then dispensed in the autosampler. The FIA parameters were set as follows: sample injection volume of 6 mL, carrier flow rate of 2 mL min^{-1}, flow-cell volume of 30 μL and run time of 50 s. Nitrite-N and nitrate-N were measured at wavelength of 550 nm. Ammonia-N was measured at wavelength of 650 nm. Calibration curves were prepared by serial dilution of nitrite-N (0.1–2.0 mg-N L^{-1}, Merck, Germany), nitrate-N (0.1–3.0 mg-N L^{-1}, Merck, Germany), and ammonia-N (2–10 mg-N L^{-1}, Merck, Germany) stock solutions.

Total organic carbon was determined by thermal catalytic oxidation using a total carbon/total nitrogen (TC/TN analyzer, Multi C/N 2100, Analytik Jena, Germany). Oxygen was used as carrier at flow rate of 160 mL min^{-1}. Temperature was set at 900 °C. Briefly, the samples were filtered using 0.45 μm membrane filters (Millipore, USA), acidified with phosphoric acid

(40% w w^{-1}) (Sigma-Aldrich, EUA) and injected (250 μL) directly into analyzer. Calibration curves were prepared by serial dilution of a stock 1 g L^{-1} biphthalate (Synth, Brazil) solution.

Light intensity and pH were measured using a Luximeter (DX-100, Japan) and pH meter (Hanna Instruments, HI8424, USA), respectively.

3.2.3 Extraction of bioactive compounds from microalgae

The lyophilized biomass (20 g) was washed with dichloromethane (≥99.7%; Sigma-Aldrich, USA) or methanol (≥99.9%; J.T. Baker, USA) at 1:5 (g:mL^{-1}) ratio. The solvent containing the extracts was then dried in a rotary evaporator (Fisaton 803, Brazil) under vacuum and at 50 °C. The obtained yields were 5.7 and 4.9% for methanol and dichloromethane, respectively. The dried extracts were resuspended in 100 mg mL^{-1} dimethyl sulfoxide (≥99.7%; Sigma-Aldrich, USA) for further analysis.

3.2.4 Pathogenic bacteria and antibiotic resistant

The antimicrobial effects of microalgae extracts were assessed against three model gram-positive bacteria: *Streptococcus suis* (BRMSA 1410), *Staphylococcus hyicus* (CEDISA 634/15), and *Enterococcus faecalis*. These bacteria are recognized to cause major problems in the swine industry worldwide (HERNANDEZ-GARCIA et al., 2017; OSMAN et al., 2016; ŠEPUTIENE et al., 2012), leading to animal morbidity and mortality of 10–90% and 5–90%, respectively (AASMÄE et al., 2019; BA et al., 2019; PAN et al., 2019; PARK et al., 2018). *S. suis* is resistant to doxycycline, enrofloxacin, lincomycin and penicillin (EUCAST, 2020). *S. hyicus* is resistant to enrofloxacin, norfloxacin, ampicillin, penicillin, amoxicillin, ciprofloxacin, lincomycin and, spectinomycin (EUCAST, 2020). *E. faecalis* is resistant to amoxicillin (EUCAST, 2020).

3.2.5 Assessment of antibacterial effects

The Kirby Bauer disc diffusion method (LENNETTE; BALOWS, 1985) was used to investigate the antibacterial activity of the microalgae extracts. Sterile blank paper discs (diameter of 6 mm) were impregnated with either 20 or 50 μL of each dichloromethane or methanol extracts and then placed on the center of Mueller-Hinton agar plates [except for *S. suis*, which was cultured in sheep blood (OLIVEIRA, 2000)]. Plates were incubated at 37 °C for 24 h in dark. Sterile disks impregnated with florfenicol, at concentration known to have minimum inhibitory effects in antibiogram assays (30 μg mL^{-1}; Shin et al., 2005), were used as

positive control. Negative controls were prepared by impregnation of sterile disks with 20 or 50 μL dimethyl sulfoxide (≥99.7%; Sigma-Aldrich, USA) without microalgae solvent-extracts. The formation of inhibition zone surrounding the outer diameter of the impregnated discs was measured in millimeters. The larger the diameter of inhibition zone diameter (halo), the higher the inhibitory activity. All tests were performed in triplicate.

3.2.6 Minimum Inhibitory Concentration (MIC) and Minimal Bacterial Concentration

The MIC was determined by dilutions of the extracts in 96-well plate (EUCAST, 2019). Plates were prepared using Mueller-Hinton growth medium (Kasvi, Brazil) and inoculated with 5×10^5 CFU mL^{-1} of *S. hyicus*, *E. faecalis* or *S. suis*. Different concentrations of dichloromethane or methanol extracts were tested (in mg mL^{-1}): (50; 25; 12.5; 6.0; 3.0; 1.5; 1.0; 0.5; 0.2; 0.1; 0.05; or 0.025) in each of individual wells. After 24 h of incubation at 37 °C, 20 μL of 0.5% w v^{-1} of triphenyltetrazolium chloride (≥98%; Sigma-Aldrich, USA) was added to each well as indicator of bacterial growth. The MIC was determined visually by checking samples for the absence of color as indicative of no bacteria growth. The bactericidal effects of the extracts were further determined by streaking 25 μL of samples that showed no color change onto Trypticase Soy Agar (TSA, Kasvi, Brazil) plates. After 24 h incubation at 36 °C the plates were assessed for colony-forming units (CFU).

3.2.7 Phytochemical screening

The compounds present in the microalgae extracts were characterized by UHPLC (Shimadzu, Nexera X2, Japan) equipped with Acquity UPLC HSS T3 C18 column (1.7 μm, 2.1×100 mm, Waters, USA,). All solvents were UHPLC grade (>99%, J.T. Baker, Phillipsburg, USA or Sigma-Aldrich, St. Louis, USA). The separation gradient was composed of solutions A (water with 0.1% formic acid, v:v) and B (acetonitrile with 0.1% formic acid, v:v). Flow was set using 5% B (0–1 min), 70% B (1–10 min), 98% B (12–20 min) and 5% B (20–25 min). Temperature and flow rate were set at 40 °C and 0.250 mL min^{-1}, respectively.

The eluted compounds were analyzed by a quadrupole high resolution mass spectrometer (MS, Impact II, Bruker Daltonics Corporation, Germany) equipped with an electrospray ionization source (ESI) in positive mode. MS parameters were: capillary voltage 4,500 V (potential plate end of -500 V), gas flow of 8 L min^{-1} at 180 °C, nebulization gas pressure of 4 bar. Data acquisition was monitored at mass/charge (m/z) ranges between 50 to

1,300 at 5 Hz. The five most intense ions were selected for automatic tandem mass spectrometry (AutoMS/MS). Data acquisition, process and report were performed using Hystar Application software version 3.2 and Qtof Control (Bruker Daltonics Corporation, Germany) (SILVA et al., 2020).

3.3 RESULTS AND DISCUSSION

3.3.1 Microalgae extracts have antibacterial activity

This work investigated the antimicrobial effects of microalgae extracts obtained from *Chlorella* spp. biomass cultivated in swine digestate effluent at conditions resembling phycoremediation of wastewaters. Variations in environmental conditions and wastewater composition expected at field scale can play an important role on microalgae biochemical composition (GALLOWAY; WINDER, 2015; JUNEJA; CEBALLOS; MURTHY, 2013; PIGNOLET et al., 2013) possibly altering the type and concentration of bioactive compounds with antimicrobial properties (ABU-GHANNAM; RAJAURIA, 2013; AREMU et al., 2015). Very few information (if any) is available on the antimicrobial effects of *Chlorella* spp. extracts cultivated in wastewater.

Bactericides seems to have advantages in the treatment of infections caused by gram-positive bacteria (PANKEY; SABATH, 2004). As a matter of fact, the majority of antibiotics currently applied for gram-positive bacteria are bacteriostatic not bactericidal (PANKEY; SABATH, 2004). Bacteria inhibition was verified at varying degree depending on the type of solvent used and the concentrations tested. Only the extracts obtained with dichloromethane either at 2 and 5 mg L^{-1} were inhibitory to all three bacteria (Table 3.2), as observed by the formation of inhibition zone (halo diameter) of 15±1 mm for *S. hyicus*, 18±1 mm for *S. suis*, and 12±1 mm for *E. faecalis* (Table 3.2). Extracts obtained with methanol was only inhibitory to *E. faecalis* and *S. suis* at 5 mg L^{-1} (Table 3.2) with a measured halo of 16±1 and 9±1 mm, respectively. The inhibitory effects of *Chlorella* spp. extracts on *E. faecalis* were previously described using either dichloromethane or methanol (Table 3.1). However, to the best of authors knowledge, this is the first report on the inhibitory effects of *Chlorella* spp. extracts against the antibiotic resistant *S. hyicus* and *S. suis*. This information serves to broaden current knowledge on the potential antimicrobial effects of *Chlorella* spp. extracts. Neither dichloromethane nor methanol extracts showed bactericidal effects (Table 3.3).

Table 3.2. Antimicrobial activity of microalgae *Chlorella* spp. extracts against antibiotic resistant pathogenic bacteria. Florfenicol (30 μg mL^{-1}) and dimethyl sulfoxide (4 mg mL^{-1}) were used as positive and negative controls, respectively. Results presented as mean±standard deviation.

Bacteria	Zone of inhibition (diameter in mm)				Concentration tested (mg mL^{-1})
	Negative control	Positive control	Dichloromethane	Methanol	
Staphylococcus hyicus	ND	24	15±1	ND	5
	ND		12±1	ND	2
Enterococcus faecalis	ND	26	18±1	16±1	5
	ND		10±1	9±1	2
Streptococcus suis	ND	20	12±1	9±1	5
	ND		9±1	7±1	2

ND not detected

3.3.2 Bacteriostatic effects of extracts and the minimum inhibitory concentration

The MIC obtained with dichloromethane extracts were verified at ≥0.5 mg mL^{-1} for the gram-positive *S. hyicus* and *E. faecalis*, and ≥0.2 mg mL^{-1} for *S. suis* (Table 3.3). Methanol extract showed a MIC of 1.5 and 1.0 mg mL^{-1} for *E. faecalis* and *S. suis*, respectively. These results were in the lower MIC range found for other algae and bacteria. For example, MIC measured using *Dunaliella salina* extracts against *Escherichia coli*, *Shigella sonnei*, *Salmonella enteritidis* and *Listeria monocytogenes* was 2.5 and 5 mg mL^{-1} for methanol and dichloromethane, respectively (CAKMAK; KAYA; ASAN-OZUSAGLAM, 2014). Methanol and dichloromethane extracts of *Ecklonia cava* exhibited a MIC value of 0.3 and 0.5 mg mL^{-1} against *Listeria monocytogenes*, respectively (NSHIMIYUMUKIZA et al., 2015). Methanol extracts of *Chlorella variabilis* showed MIC of 0.3 mg mL^{-1} against *Enterococcus faecalis* and *Staphylococcus aureus* (GEE et al., 2020). Polyphenols extracts isolated from brown seaweeds (*Ascophyllum nodosum* and *Fucus serratus*) showed a MIC range of 0.8–3.1 mg mL^{-1} for *Escherichia coli* O157, *Salmonella agona*, and *Streptococcus suis* (FORD et al., 2020). The higher degree of potency as determined by MIC is likely attributed to intracellular production of specifically active bioactive compounds with superior antimicrobial properties.

Table 3.3. Minimum inhibitory concentration (MIC) and qualitative assessment of the potential effects of dichloromethane and methanol extracted compounds as bacteriostatic and bactericidal.

Test organism	MIC (mg mL^{-1})		Bacteriostatic	Bactericidal
	Dichloromethane	Methanol		
Staphylococcus hyicus	0.5	-	+	-
Enteroccoccus faecalis	0.5	1.5	+	-
Streptococcus suis	0.2	1.0	+	-

3.3.3 Extracts contain bioactive compounds associated with antimicrobial activity

In attempt to identify the compounds responsible for the observed antimicrobial activity, the extracts were characterized by UHPLC-ESI-MS. Compounds were detected by MS and MS/MS spectra and then putatively identified by comparison with literature and public databases such as KEGG (OGATA et al., 1999), CHEBI (DEGTYARENKO et al., 2007) and, PubChem (KIM et al., 2016). Results showed the presence of 23 bioactive compounds (Table 3.4 and supplementary material II) associated with antimicrobial activities (BHAGAVATHY; SUMATHI; BELL, 2011; FALAISE et al., 2016; MICHALAK; CHOJNACKA, 2015). Interestingly, some of these compounds with possible antimicrobial activity have only been described for vascular plants. For instance, methyl linoleate extracted from *Jacaranda acutifolia* exhibited antibacterial activity against both gram-positive and gram-negative bacteria (SINGAB et al., 2014). Tobacco extracts containing sclareolide had antimicrobial activity *against Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (halo of 14.4–24.4 mm) (STOJANOVIC et al., 2000). 9(S)-HODE isolated from chili seeds showed antimicrobial activity against fungi (*Colletotrichum gleosporoides*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*) and pathogenic bacteria (*Xanthamonas campestris*, *Erwinia* sp., and *Pseudomonas* sp.) (SUCHARITHA; DEVI, 2010). Lipophilic metabolites such as pinolenic acid and linoleoyl glycerol from Five-Needle Pines, *Pinus armandii* and *Pinus kwangtungensis* exhibited antibacterial activity against *Bacillus subtilis* and *Serratia marcescens* (SHPATOV et al., 2020).

Table 3.4. Characterization of the microalgae extracts by UHPLC–MS/MS.

ID	Compound	Class	Molecular formula	$[M+H]^+$ Measuared	$[M-H_2O]^+$ Measuared	$[M+H]^+/[M-H_2O]^+$ Theoretical	Mass error (ppm)	Solvent
1	Glycerophosphocholine	Fatty acid	$C_8H_{20}NO_6P$	258.109	-	258.110	-3.10	Dichloromethane/Methanol
2	Niacinamide	Vitamin	$C_6H_6N_2O$	123.055	-	123.055	-1.54	Dichloromethane/Methanol
3	13(S)-HpOTrE	Fatty acid	$C_{18}H_{30}O_4$	-	293.211	293.211	-3.82	Dichloromethane/Methanol
4	Methyl linoleate	Fatty acid	$C_{19}H_{32}O_2$	293.247	-	293.248	-2.75	Dichloromethane/Methanol
5	Sclareolide	Terpenoid	$C_{16}H_{26}O_2$	251.200	-	251.201	-2.62	Dichloromethane
6	14-HDoHE	Fatty acid	$C_{22}H_{32}O_3$	-	327.231	327.231	-2.62	Dichloromethane
7	9(S)-HODE	Fatty acid	$C_{18}H_{32}O_3$	-	279.231	279.231	-2.71	Dichloromethane
8	Pinolenic acid	Fatty acid	$C_{18}H_{30}O_2$	279.231	-	279.232	-3.07	Dichloromethane
9	Stearidonic acid	Fatty acid	$C_{18}H_{28}O_2$	277.215	-	277.216	-5.08	Dichloromethane
10	Adenosine	Nucleoside	$C_{10}H_{13}N_5O_4$	268.103	-	268.104	-2.72	Methanol
11	L-Phenylalanine	Amino acid	$C_9H_{11}NO_2$	166.086	-	166.086	-2.74	Methanol
12	L-Leucine	Amino acid	$C_6H_{13}NO_2$	132.102	-	132.102	-2.31	Methanol
13	2,3-Dinor Prostaglandin E1	Fatty acid	$C_{18}H_{30}O_5$	-	309.205	309.206	-3.03	Methanol
14	3b-Hydroxy-5-cholenoic acid	Fatty acid	$C_{24}H_{38}O_3$	-	357.278	357.279	-1.98	Methanol
15	Nutriacholic acid	Steroid	$C_{24}H_{38}O_4$	391.283	-	391.284	-4.05	Methanol
16	2-Linoleoyl glycerol	Fatty acid	$C_{21}H_{38}O_4$	355.283	-	355.284	-2.78	Methanol
17	1-Heptadecanoyl-sn-glycero-3-phosphocholine	Fatty acid	$C_{25}H_{52}NO_7P$	510.353	-	510.355	-3.95	Methanol
18	11beta-Hydroxy-5alpha-androstan-17-one	Steroid	$C_{19}H_{30}O_2$	291.231	-	291.232	-2.94	Methanol
19	PAF (C16)	Fatty acid	$C_{26}H_{54}NO_7P$	524.369	-	524.371	-3.18	Methanol
20	Glyceryl monooleate	Fatty acid	$C_{21}H_{40}O_4$	357.299	-	357.300	-3.18	Methanol
21	1-hexadecyl-2-butyryl-sn-glycero-3-phosphocholine	Fatty acid	$C_{28}H_{58}NO_7P$	552.400	-	552.402	-3.56	Methanol
22	Linoleic acid	Fatty acid	$C_{18}H_{32}O_2$	281.246	-	281.248	-4.56	Methanol
23	Protoporphyrin IX	Tetrapyrroles	$C_{34}H_{34}N_4O_4$	563.264	-	563.265	-2.99	Methanol

It is known that amino acids and fatty acids constitute the largest group of primary metabolites identified in algal species with recognized antibacterial properties (ABU-GHANNAM; RAJAURIA, 2013; YADAVALLI et al., 2018). There are several mechanisms in which amino acids, fatty acids and steroid compounds can affect bacteria: i) it can bind to cell surfaces damaging membrane and/or cell wall disruption, and/ or ii) it can affect enzymes activity and induce substrate deprivation (GUEDES et al., 2011; PINA-PÉREZ et al., 2017; PRADHAN; DAS; DAS, 2014). Polyunsaturated and unsaturated fatty acids extracted from *Chlorococcum* strain HS-101, *Dunaliella primolecta* and *Phaeodactylum tricornutum* showed antimicrobial activity against the methicillin-resistant *Staphylococcus aureus* (DESBOIS; MEARNS-SPRAGG; SMITH, 2009; OHTA et al., 1995). Antimicrobial activity of fatty acids extracted from *Chlorella vulgaris* was observed in *Escherichia coli* and *Staphylococcus aureus* (PLAZA et al., 2012). The effectiveness of these compounds seem to be dependent on the degree of fatty acid unsaturation and chain length (GUEDES et al., 2011). Similarly, fatty acids isolated from different species of algae were proven to be effective against many gram-positive or negative bacteria (AREMU et al., 2015; MENDIOLA et al., 2007; SHOUBAKY; SALEM, 2014). In addition, antimicrobial activities of stearidonic and linolenic acids from the green seaweed *Enteromorpha linza* against several oral pathogenic bacteria were reported (Park et al., 2013). Isolated amino acids and steroid compounds extracted from *Ulva* sp. indicated antimicrobial activity against gram-positive and gram-negative bacteria (PRINCEL; DHANARAJU, 2017). Lukáč et al. (2010) reported the antimicrobial properties of phenylalanine on *Escherichia coli* and *Staphylococcus aureus*. However, antimicrobial activity on *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* was attributed to leucine (PERINELLI et al., 2019).

Overall, the synthesis of specific bioactive compounds in microalgae is largely dependent on the algae strain as well as cultivation conditions. Unlike previous studies using synthetic medium and/ or laboratory-controlled conditions, this work explored the antimicrobial properties of microalgae obtained from a field-scale pilot reactor simulating phycoremediation of raw wastewaters. Several bioactive compounds associated with antimicrobial activity were found in the biomass. Regarding waste valorization, the application of microalgae as remediation strategy to remove nutrients from wastewaters (tertiary treatment or polishing) can produce biomass of biotechnology interest including the manufacturing of pharmacologically active compounds Further studies are still needed to elucidate how the complex wastewater physical-chemical characteristics and changes in environmental conditions can ultimately affect the type and concentrations of bioactive compounds present in microalgae.

3.4 CONCLUSIONS

The antibacterial effects of *Chlorella* spp. solvent-extracts on three antibiotic resistant pathogenic bacteria were demonstrated. Dichloromethane extracts was bacteriostatic to all bacteria tested. Neither dichloromethane nor methanol were bactericidal. Twenty-three different compounds mainly amino acids, fatty acids, steroid and terpenoid were detected in the microalgae extracts. Antimicrobial compounds reported from vascular plants extracts (methyl linoleate, 9(S)-HODE, pinolenic acid, linoleoyl glycerol and sclareolide) were identified in *Chlorella* spp. This is the first study on the antimicrobial effects of microalgae biomass obtained from cultivation system resembling swine wastewater tertiary treatment process. Moreover, the findings contribute to current knowledge on the applicability potential of microalgae extracts as bacteriostatic against *S. suis*, *S. hyicus* and, *E. faecalis* known to play an important role in animal diseases and food industry.

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- CHAPTER 4 -

4 VIRUCIDAL ACTIVITY OF MICROALGAE EXTRACTS HARVESTED DURING PHYCOREMEDIATION OF SWINE WASTEWATER [4]

Abstract: Phycoremediation of swine wastewater is a promising treatment since it efficiently removes nutrients and contaminants and, simultaneously, its biomass can be harvested and used to obtain a wide range of valuable compounds and metabolites. In this context, microalgae were investigated for the phycoremediation of swine wastewater, and biomass were evaluated for their virucidal effect against enveloped and non-enveloped viruses. Microalgae were cultivated at pilot scale bioreactor fed with swine wastewater as the growth substrate. Hexane, dichloromethane and methanol were used to obtain the microalgae extracts. Extracts were tested for virucidal potential against HSV-1 and HAdV-5. Virucidal assays were conducted at temperatures that emulate environmental conditions (21 ºC) and body temperature (37 ºC). The maximum production of microalgae biomass reached a concentration of 318.5±23.6 $mg_{DW}\ L^{-1}$. The results showed that phycoremediation removed 100% of ammonia-N and phosphate-P, with rates (k_1) of 0.218±0.013 and 0.501±0.038 (d^{-1}), respectively. All microalgae extracts reduced 100% of the infectious capacity of HSV-1. The microalgae extracts obtained with dichloromethane and methanol showed inhibition activities at the lowest concentration (3.125 μg mL^{-1}). Virucidal assays against HAdV-5 using microalgae extract of hexane and methanol inhibited the infectious capacity of the virus by 70% at all concentrations tested at 37 ºC. At a concentration of 12.5 μg mL^{-1}, the dichloromethane microalgae extract reduced 50-80% of the infectious capacity of HAdV-5, also at 37 ºC. Overall, the results suggest that swine wastewater can be used to grow microalgae and an attractive source of feedstock biomass for the exploration of alternative virucidal compounds.

4.1 INTRODUCTION

The nutrient-rich wastewater produced in the swine farm industry is conventionally treated by anaerobic digestion to reduce carbon loads followed by a tertiary treatment system, such as phycoremediation, to remove nitrogenous and phosphate compounds (CHENG et al., 2019). Phycoremediation have gained considerable attention, mainly for their efficiency in

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bioremediation and the potential for generating valuable raw material for various products of biotechnological importance, such as bioenergy production, nutrition and pharmacological (MOHD UDAIYAPPAN et al., 2017). Pharmacological compounds are of particular interest, and efforts have been focused on the potential of bioactive compounds for the control of microorganisms, since the worldwide scenario of diseases caused by microorganisms has been worsening (FALAISE et al., 2016).

For the past 50 years, algae have been studied for their antiviral potential, with successful results in experiments conducted under controlled laboratory conditions and with synthetic culture medium (PAGARETE et al., 2021). The exact mechanisms of virus inhibition by microalgae extracts are still not completely understood (JOSEPH et al., 2020) but could be associated with the presence of polysaccharide (HASUI et al., 1995; LEE et al., 2006), protein (EMAD; SANAA; VIKRAMJIT, 2010), fatty acid (KAMAT et al., 1992) and terpene (CIRNE-SANTOS et al., 2020; PEREIRA et al., 2005) compounds. However, it is well known that depending on the culture medium composition, the microalgae can change their metabolites and compounds production, changing the antimicrobial effect (AREMU et al., 2015).

In recent years, microalgae have been grown using swine wastewater as a base for the culture medium, a complex matrix that can influence cellular biochemical profile (MICHELON et al., 2021). However, as far as we know, no study has been applied to evaluate the virucidal activity of microalgae extracts obtained from the biomass harvested from swine wastewater treatment systems.

Viral diseases have caused public health concern worldwide, since non-envoloped viruses are responsible for more than 500,000 deaths per year (WHO, 2017); while enveloped viruses are responsible for the largest recorded pandemics, with the number of deaths increasing every day (WIGGINTON; BOEHM, 2020). Viral diseases are of particular concern because viruses present infections at low doses, which can vary from 10^1 to 10^3 particles (GIBSON, 2014), and long periods of survival in environmental matrices (BARARDI et al., 2012).

In this sense, research has focused on finding low-cost alternatives or those associated with the simultaneous removal of other contaminants, for the inactivation of viruses. For that, some viruses have been used as a research model, such as the human *Herpes Virus Simplex* type 1 (HSV-1), which are enveloped and double-stranded DNA viruses (SILVA et al., 2010); and *Human Adenovirus* type 5 (HAdV-5), which are non-enveloped and double-stranded DNA viruses (RAFIE et al., 2021).

The aim of this study was to investigate the phycoremediation applied to the treatment of swine wastewater for obtaining biomass extracts, using different solvents, to assess the virucidal potential against enveloped and non-enveloped viral models.

4.2 MATERIAL AND METHODS

Figure 4.1 shows a schematic diagram of the material and methods. Steps are described in the sections below.

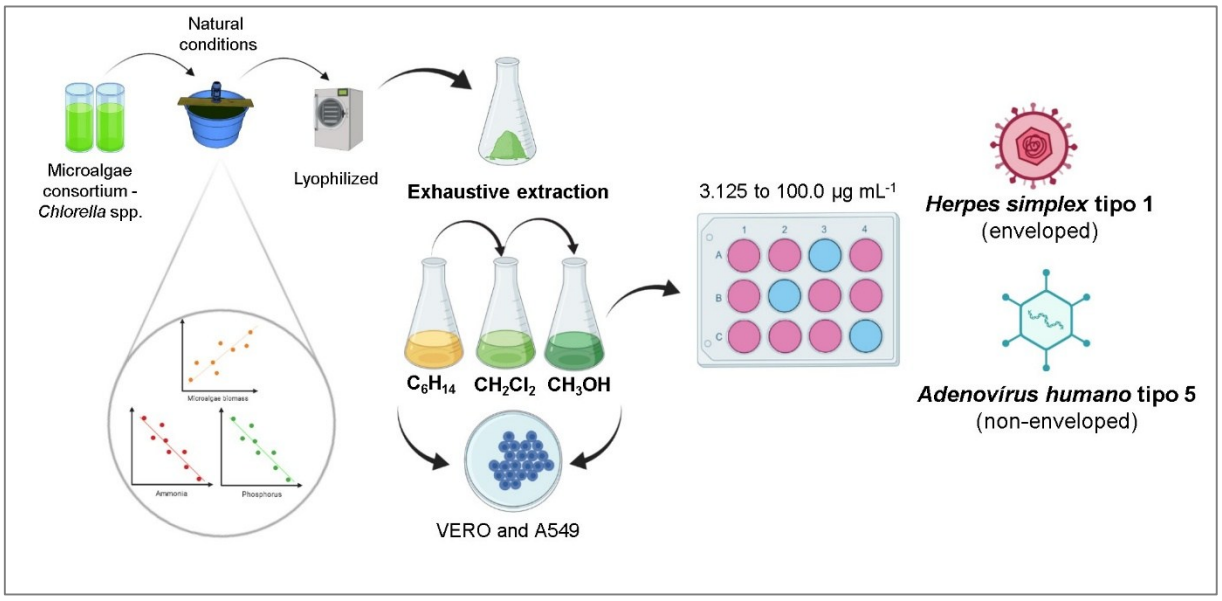

Figure 4.1. Schematic diagram of methodology

4.2.1 Consortium of microalgae and phycoremediation of swine wastewater

The microalgae consortium (predominantly *Chlorella* spp.) used in this study was characterized and identified previously in Michelon et al. (2015) (Appendix I). The microalgae were grown at pilot scale in an open 500 L bioreactor (121.2 cm Ø i.d.; 58.4 cm height), installed in a greenhouse with controlled temperature (25 °C) and exposed to natural sunlight (photosynthetic photon flux density of 336.5±410.4 µmol m^{-2} s^{-1}). The culture medium was prepared with 50 L of swine wastewater digestate effluent diluted in 300 L of tap water. The composition of the medium was (mg L^{-1}): total organic carbon (120±3.4), alkalinity as $CaCO_3$ (200±9.2), ammonia-N (141.6±12.7) and phosphate-P (12.9±2.7). pH was 7.9±0.9.

The bioreactor was inoculated with 150 L of inoculum, with a dry weight (DW) concentration of 60±5.1 mg_{DW} L^{-1} of biomass. The agitation was kept by means of mechanical pumps at 1,200 L h^{-1} (Sarlobetter®, S300, Brazil). After 11 days of inoculation, biomass was harvested by centrifugation at 3,000 ×g (EVODOS T10, Netherlands), immediately frozen (-20 °C) and then lyophilized (Model 030-JJ LJI, Scientific, Brazil) for further assays.

4.2.2 Wastewater chemical analysis

Total organic carbon was measured by thermal catalytic oxidation using a TC/TN analyzer (Multi C/N 2100, Analytik Jena, Germany). Temperature was set at 900 °C. Oxygen was used as carrier at flow rate of 160 mL min^{-1}. The samples were filtered using 0.45 μm membrane filters (Millipore, USA) acidified with phosphoric acid (40% w w^{-1}) (Sigma-Aldrich, EUA) and injected (250 μL) directly into analyzer. Calibration curves were prepared by serial dilution of a stock solution of 1,000 mg L^{-1} biphthalate (Synth, Brazil).

Alkalinity (measured as $CaCO_3$ L^{-1}) was determined by automatic titration (Metrohm 848 Titrino Plus, Switzerland) using sulfuric acid (0.1 mol L^{-1}, Merck, Germany) as titrant. Ammonia-N concentration was determined by a series of colorimetric assays performed automatically by a flow injection analysis system (FIAlab 2500 system, USA) (APHA, 2012). Briefly, samples (10 mL) were filtered using a 0.45 μm membrane filter (Millipore, USA) then dispensed in the autosampler. Ammonia-N was measured at a wavelength of 650 nm. Calibration curves were prepared by a serial dilution of ammonia-N stock solution (2–10 mg L^{-1}, Merck, Germany).

The concentration of phosphate-P was determined by the ascorbic acid colorimetric method (4500-P) (APHA, 2012). The reagent solution was prepared using 50 mL sulfuric acid (5 N; Sigma-Aldrich, USA), 5 mL antimony potassium tartrate solution (Sigma-Aldrich, USA), 15 mL ammonium molybdate solution (Synth, China), and 30 mL ascorbic acid solution (Synth, China). Then, 0.8 mL of this solution was added to 5 mL of previously filtered samples (0.45 μm membrane filters, Millipore, USA). After 10 min, the absorbance of each sample was measured on a UV-visible spectrophotometer (Varian, Cary® 50 UV-Vis, USA) at 880 nm. Standard curves were prepared by a serial dilution of a phosphate-P stock solution (0.05–0.2 mg L^{-1}) (Merck, Germany).

A satisfactory correlation (r^2=0.97) between gravimetric assay (APHA, 2012) and optical density (OD_{750}) (mg_{DW} L^{-1}=241.88×OD_{750}nm–36.522) was determined for the dry matter biomass concentration. The growth of microalgae over time was measured using a spectrophotometer (Varin, Cary® 50 UV-Vis, USA) at 750 nm. Light intensity and pH were measured using a Luximeter (DX-100, Japan) and pHmeter (Hanna Instruments, HI8424, USA), respectively.

4.2.3 Solvent extraction

A quantity of 20 g lyophilized biomass was successively extracted by a serial of exhaustive extraction with hexane (≥97.0%; Sigma-Aldrich, USA), dichloromethane (≥99.7%; Sigma-Aldrich, USA) and methanol (≥99.9%; J.T. Baker, USA) at concentration ratio of 1:5 (g:mL). Extracts were dried using a rotatory evaporator (Fisaton 803, Brazil) kept under vacuum at 50 °C to eliminate any residual concentrations of the solvents. The extracts were then resuspended in dimethyl sulphoxide (DMSO) (≥99.7%; Sigma-Aldrich, USA) at 50 mg mL^{-1}.

4.2.4 Application on viral models

4.2.4.1 Viruses and cell line

The cell lines used were Kidney Epithelial Derived from African Green Monkey (VERO cells) (ATCC: CCL81) and Adenocarcinomic Human Alveolar Basal Epithelial (A549 cells) (ATCC: CCL-185), grown in Minimum Essential Medium (MEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Viral stock of *Herpes simplex virus* type 1 (KOS strain; Faculty of Pharmacy, University of Rennes, France) was propagated on VERO cells, and the viral titers were determined by standard plaque assay (BURLESON; CHAMBERS; WIEDBRAUK, 1992) and stored at -80 °C. The *Human adenovirus* type 5 viral stock was propagated on A549 cells, titrated as described by Rigotto et al. (2011) and stored at -80 °C.

4.2.4.2 Cytotoxicity assay

VERO and A549 cells were seeded (2.5 10^4 $well^{-1}$ in 96-well plates) and, after 24 h, the confluent cells were exposed for 48 h to different concentrations, ranging from 0.488 µg mL^{-1} to 500 µg mL^{-1}, of microalgae-extract samples. After incubation, cell viability was assessed by sulforhodamine B (Sigma-Aldrich, USA) assay, that measure total protein mass, which is related to cell viability (VICHAI; KIRTIKARA, 2006). The percentages of viable cells were plotted against each sample concentration, and the cytotoxic concentration CC_{50} values (concentration that inhibited cell viability by 50% when compared to untreated controls) were determined based on concentration-response curves using Graphpad Prism 6.0 (GraphPad software, La Jolla, CA).

4.2.4.3 Virucidal assay

The virucidal assay followed the procedures described by Silva et al. (2010), where mixtures of the samples at six different concentrations (3.125, 6.25, 12.5, 25.0, 50.0 and 100.0 μg mL^{-1}) and 4×10^4 PFU of HSV-1 or HAdV-5 in serum-free minimum essential medium eagle (MEM; Gibco, USA) or serum-free dulbecco's modified eagle medium (DMEM; Gibco, USA), respectively, were co-incubated during 15 min at room temperature (21 °C) or at 37 °C. These temperatures were chosen aiming to emulate the environmental average temperature (21 °C) and body temperature (37 °C).

The samples were then diluted to non-inhibitory concentrations (1:100) in MEM and the residual infectivity was determined by plaque number reduction assay. For this, 400 μL of each dilution was adsorbed for 1 h at 37 °C on VERO cells or A549 cells. Cell cultures were then overlaid with MEM 2× containing 1.5% carboxymethylcellulose (CMC; Sigma-Aldrich, USA) or DMEM 2× supplemented with 4% fetal bovine serum (FBS; Gibco, USA), 2% penicillin and streptomycin (PS; Gibco, USA), 2% magnesium chloride (1M; Sigma-Aldrich, Brazil) and 2% pyruvic acid (100 mM; Sigma-Aldrich, USA) containing 0.6% bacteriological agar (BD – Becton, Dickinson and Company Sparks, MD 21152 USA) for 2 or 7 days at 37 °C. Cells were fixed and stained with naphthol blue-black (Sigma-Aldrich, USA) and viral plaques were counted by using a stereomicroscope.

4.3 RESULTS AND DISCUSSION

4.3.1 Phycoremediation of swine wastewater and biomass production

The results of the phycoremediation applied in the removal of ammonia-N and phosphate-P are shown in Figure 4.2A. It is possible to observe a 100% removal with an exponential profile in 7 and 11 days for ammonia-N and phosphate-P, respectively. The removal of ammonia-N and phosphate-P by phycoremediation process adjusted to the pseudo-first-order kinetics (Figure 4.2B). The calculated pseudo-first-order kinetic rates (k_1) were 0.218±0.013 and 0.501±0.038 (d^{-1}) for ammonia-N and phosphate-P, respectively. Overall, these data corroborate what has been reported in the literature about phycoremediation to be efficient for the removal of ammonia-N and phosphate-P from wastewater (APANDI et al., 2019; ASLAN; KAPDAN, 2006; PRANDINI et al., 2015).

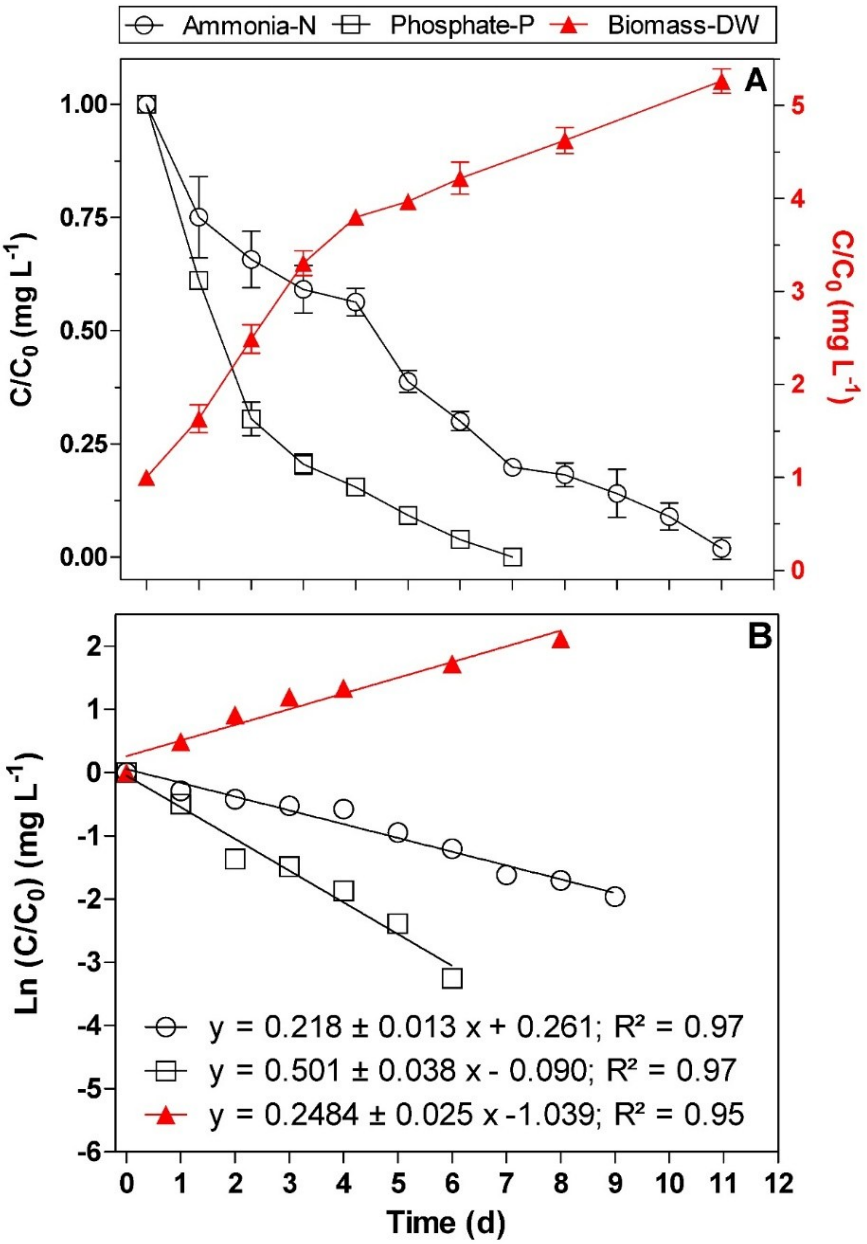

Figure 4.2. Nutrient removal and biomass concentration during swine wastewater phycoremediation (A); values of k_1 to kinetics of nutrients removal, and kinetics of biomass growth (B). Experimental conditions: $[\text{ammonia-N}]_0$= 41.5±1.2 mg L^{-1}, $[\text{phosphate-P}]_0$= 12.9±1.5 mg L^{-1}, $[\text{Biomass-DW}]_0$= 60±5.1 mg_{DW} L^{-1}, T= 25 °C and light intensity= 336.5±410.4 µmol m^{-2} s^{-1}.

The maximum concentration of microalgae biomass reached 318.5±23.6 mg_{DW} L^{-1} after 11 days of phycoremediation (Figure 4.2A). Similar results with microalgae grown from swine wastewater effluent have been reported previously (ABOU-SHANAB et al., 2013; LUO et al., 2016). The estimated specific growth rate of 0.248±0.025 d^{-1} (Figure 4.2B) was within typical values reported for microalgae growth in swine wastewaters (JI et al., 2013; LUO et al., 2016).

4.3.2 Yield of biomass extracts

The yields obtained from the extracts, applying the hexane, dichloromethane and methanol extractors, were 0.4%, 4.8% and 5.6% of dry weight, respectively. Santoyo et al. (2009) reported similar yield values when applying hexane extractor in a pressurized liquid extraction. The obtaining of different bioactive compounds is closely related to the extractor used, mainly due to the polarity interaction between the compounds and solvent. The polarity of the solvents used in the present study increase as follows: hexane < dichloromethane < methanol. In this context, considering that hexane is an apolar solvent, it is possible to infer that it would extract lipophilic compounds from the biomass. Additionaly, the low lipid content observed in microalgae grown with swine wastewater (MICHELON et al., 2015) may explain the lower yield observed by hexane when compared to the other solvents. Methanol has polar characteristics and therefore interacts with hydrophilic compounds (MÄKI-ARVELA; HACHEMI; MURZIN, 2014).

A theoretical scenario was created to simulate a route to evaluate the potential for producing microalgae extracts from swine wastewater. The layout of a cultivation system was considered according to Michelon et al. (2021), and the biomass production and extract yields observed in the present study. The results showed a possible estimate of annual extract production of 0.17, 2.0 and 2.40 ton ha^{-1} $year^{-1}$ using hexane, dichloromethane and methanol, respectively. This can be an attractive model of circular economy integrated with the current agribusiness scenario (ROBLES et al., 2020).

4.3.3 Cytotoxicity evaluation

According to the results presented in Table 4.1, none of the samples showed cytotoxicity in VERO cells and only the hexane extract was not toxic to A549 cells.

Table 4.1. Cytotoxicity of microalgae extracts grown in swine wastewater.

Concentration tested (μg mL^{-1})	**VERO**		**A549**	
	CC_{50}[a]	$(CI_{95\%})$[b]	CC_{50}[a]	$(CI_{95\%})$[b]
Hexane	384.8	212.9 to 695.4	233.1	202.6 to 268.1
Dichloromethane	239.9	162.3 to 354.6	52.0	38.31 to 70.57
Methanol	219.3	136.5 to 352.2	30.5	23.42 to 39.66

[a]CC_{50}: inhibitory concentration of 50% cell growth was calculated through a nonlinear fit-curve (log of sample concentration versus normalized response- variable slope)
[b]$CI_{95\%}$: 95% confidence interval

4.3.4 Virucidal activity

The extracts with dichloromethane and methanol reduced 100% of the infectious capacity of HSV-1 at the lowest concentration tested (3.125 µg mL^{-1}) when compared to the untreated control, at both temperatures tested (Figure 4.3AB). The hexane extract was able to inactivate up to 100% of HSV-1, presenting a concentration-response profile regardless of temperature (21 or 37 °C).

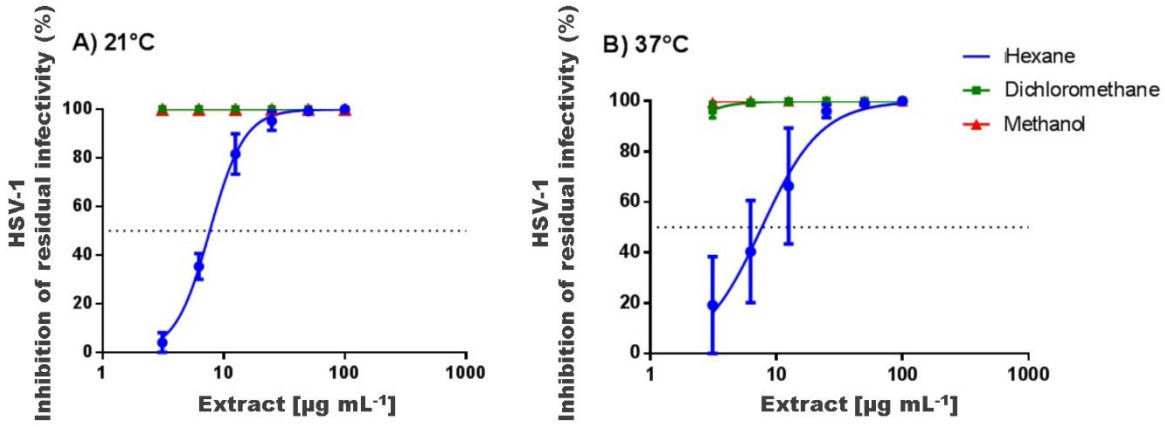

Figure 4.3. Inhibition of HSV-1 infectivity after exposition to microalgae biomass extracts at 21 °C (A) and 37 °C (B).

The virucidal test against HAdV-5 at 21 °C showed that the extracts of microalgae in hexane and methanol, at 3.82 and 3.125 µg mL^{-1} respectively, reduced by more than 50% out of the infectious capacity of the virus. The microalgae extract in dichloromethane reached a maximum of 60% inhibition of viral infectivity in 100 µg mL^{-1} at 21 °C (Figure 4.4A). The virucidal test performed at 37 °C using hexane and methanol extracts inhibited above 60% the virus infectious capacity in all tested concentrations (Figure 4.4B). In addition, the microalgae extracted in dichloromethane at 12.5 µg mL^{-1} reduced between 50 to 80% of the infectious capacity of the HAdV-5 virus at 37 °C.

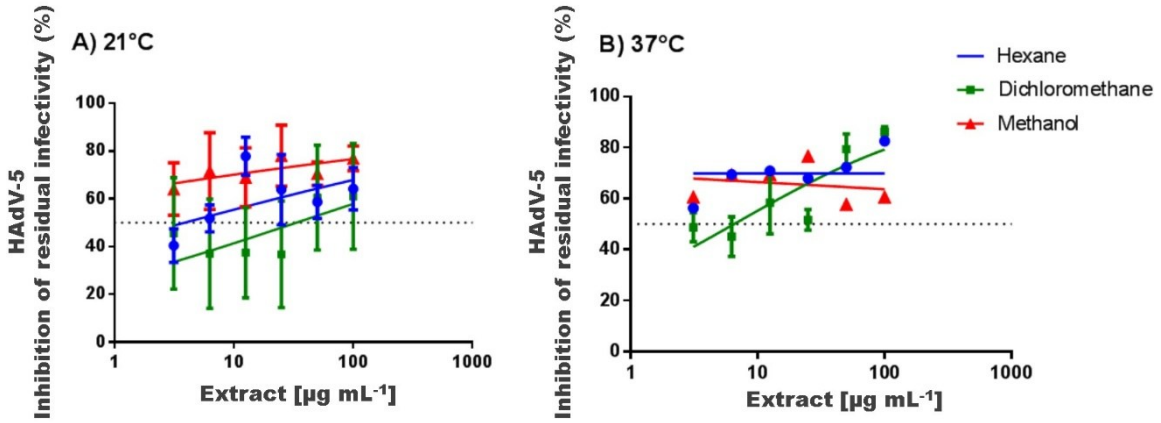

Figure 4.4. Inhibition of HAdV-5 infectivity after exposition to microalgae biomass extracts at 21 °C (A) and 37 °C (B).

Different solvents can be used to extract different compounds from microalgae with virucidal properties. Santoyo et al. (2010) investigated the use of different solvents (acetone, ethanol, and water) on *Chlorella vulgaris* compounds extraction against HSV-1. Ethanol and water extracts were efficient to inhibit the *in vitro* virus replication, demonstrating $IC_{50\%}$ values of 80.2 and 61 µg mL^{-1}, respectively. The antiherpetic properties were correlated with the presence of polysaccharides (SANTOYO et al., 2010). Abdo et al. (2012) investigated water and methanol extracts of five freshwater microalgae (*Anabaena sphaerica*, *Chroococcus turgidus*, *Oscillatoria limnetica*, and *Spirulina platensis* and *Cosmarium leave*) on non-cytotoxic concentrations of 2 mg mL^{-1}, against *Human adenovirus* type 40 and verified that only extracts from *Spirulina platensis* were efficient on the reduction of this virus (23.3% and 50% using water and methanol, respectively). Methanol extract of *Spirulina platensis* at 6.8 µg mL^{-1} showed antiherpetic activity (CHIRASUWAN et al., 2009).

Other studies tested microalgae extracts against HSV-1, and reported inhibitory activity related to attachment, adsorption, penetration or replication (OHTA et al., 1998; SANTOYO et al., 2010, 2012). The antiviral activity against non-enveloped viruses also have shown successful results, as reported by Afify et al. (2018), that tested *Scenedesmus obliquus* extracts against *Coxsackie* B3 virus, and the mechanism involved was the inhibition of the attachment, penetration and adsorption of the viral particles.

In summary, the production of bioactive compounds integrated with other applications such as wastewater treatment, CO_2 biofixation and bioenergy, highlight the circular economy concept strongly associated with phycoremediation (CHU; PHANG, 2019). The low citotoxicity and high virucidal activity of some microalgae compounds reinforce their potential

against viruses, playing an important role in the production of nutraceuticals, and in human and animal diseases.

4.4 CONCLUSIONS

Phycoremediation was efficient in removing ammonia-N and phosphate-P from swine wastewater. The best yields of the extracts were obtained with methanol and dichloromethane solvents. The virucidal effects of microalgae extracts obtained with methanol and dichloromethane showed greater efficiency in controlling enveloped than non-enveloped viruses.

The main limitation for large-scale commercial production of microalgae is the cost associated with the culture medium. In this way, we reinforce in this study that the microalgae biomass can be successfully cultivated with swine wastewater, and then harvested for the exploration of pharmaceuticals products; in this sense, becoming an option for the valorization of waste. The search and development of new virucidal agents is very encouraging, as it is an expanding market with real possibilities for application, especially considering the current scenario of the spread of viral diseases, causing social and economic losses for the population and the industry.

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- CHAPTER 5 -

5 PRODUCTION OF BIO-HERBICIDES FROM BIOMASS OF *CHLORELLA* spp. AND *TRICHODERMA KONINGIOPSIS* [5]

Abstract: The use of microalgae as a polishing treatment for swine wastewater is promising, as they are able to tolerate high concentrations of nutrients (mainly N and P). The biomass produced at the end of the treatment has received special attention, as they serve as feedstock for several products of biotechnological interest. In addition, fungal biomass has been used for over a century as versatile cell factories and is enzyme- and antibiotic- highly productive. In order to evaluate its product valorization, the biomass of microalgae harvested in the phycoremediation process, associated with the cost reduction of culture media in fermentative processes, was investigated for its potential to produce bio-extracts by submerged fermentation, using *Trichoderma koningiopsis*, as a bio-herbicide agent for weeds. Tests were carried out using fresh and lyophilized biomass, with and without pre-treatment by ultrasound and production of enzymes (peroxidase, cellulase, lipase and amylase). The weeds selected were *Bidens pilosa*, *Conyza bonariensis*, *Urochloa plantaginea* and *Euphorbia heterophylla*. Peroxidase was the most produced enzyme by both fresh and lyophilized biomass associated with *T. koningiopsis*, with a maximum activity of 16,200 U mL^{-1}. The produced bio-extracts showed phytotoxic damage of up to 80% in the first 7 days and 100% after 15 days of treatment. The integration of bioprocesses in enzymatic production is very promising, especially in the synthesis of compounds with high added value, which enables the use of these compounds in an ecofriendly product such as bio-herbicides.

5.1 INTRODUCTION

Losses in agricultural production due to weeds continue to reduce the availability of food and crop yields worldwide (OERKE, 2006). Weed management strategies can vary and depend mainly on the use of synthetic herbicides. The intensive use of synthetic herbicides in the last fifty years has increased productivity considerably, but with impressive environmental and ecological impacts (THILL et al., 1999; STOATE et al., 2009).

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[5] Partial results of this chapter were published in **Frontiers in Sustainable Food Systems**.
STEFANSKI, F. S. et al. Potential use of biological herbicides in a circular economy context: A sustainable approach. **Frontiers in Sustainable Food Systems**, n. 4, p. 195, 2020.
https://doi.org/10.3389/fsufs.2020.521102

The dependence on synthetic herbicides to control weeds has been questioned for many years, since the problems caused today are significant. In this way, the long-term uses of chemical herbicides favor the occurrence of phenological adaptations and selection of resistant genotypes (CHAUVEL et al., 2012). In addition, chemical herbicides also contaminate water and soil, which causes toxicological effects on living organisms, including humans (LECHENET et al., 2014). The increase in herbicide-resistant weeds reduces crop yields and new ecologically effective methods are needed to control weeds. In this way, the global interest in organic agriculture supports alternative methods that do not use chemical herbicides and prevent the development of herbicide-resistant genes (DUKE, 2012).

Bio-herbicides are products that originate naturally from living organisms or their natural metabolites that are used to control weed populations without degrading the environment (HOAGLAND et al., 2007). Bio-herbicides were first introduced to commercial markets in 1980 and farmers in the USA, Canada, Ukraine and Europe were the only users of the products (BAILEY, 2014, CORDEAU et al., 2016).

Bio-herbicides are categorized as specific for the host and not specific for weed control. Hoagland et al. (2007) reviewed several species of bacteria and fungi to show their specific bio-herbicidal activities in susceptible weed populations. The specificity of the target and the rapid environmental degradation of bio-herbicide products guarantee more attention to the development of commercial products that are alternatives to chemical herbicides (CORDEAU et al., 2016).

The microalgae biomass recovered after tertiary treatment of swine wastewater is promising in biotechnological processes, as well as bio-herbicides, as they aim to minimize the exploitation of natural resources and also their costs, seeking the development of natural and ecofriendly correct products in the global market. Therefore, the aim of this study was to integrate bioprocesses that produce bio-herbicide extract into a submerged fermentation process using biomass from recycled microalgae as a substrate.

5.2 MATERIAL AND METHODS

Figure 5.1 shows a schematic diagram of the material and methods. Steps are described in the sections below.

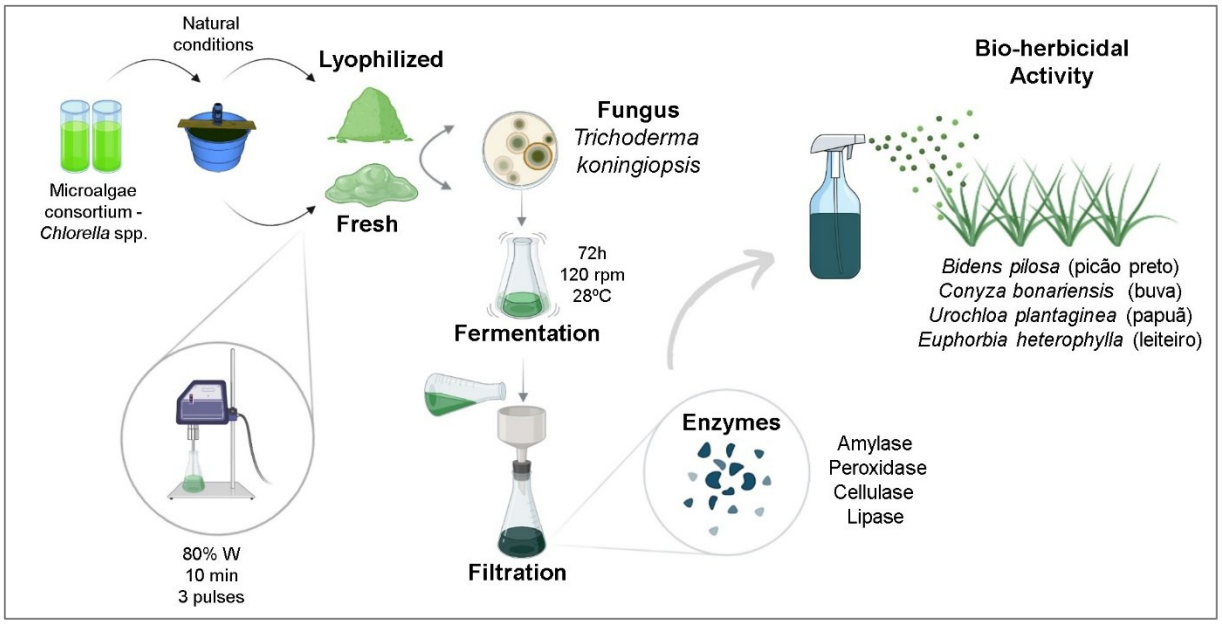

Figure 5.1. Schematic diagram of methodology

5.2.1 Microalgae cultivation

The microalgae consortium (predominantly *Chlorella* spp.) used in this study was characterized and identified previously in Michelon et al. (2015) (Supplementary material I). The microalgae were grown on a pilot scale in an open 500 L bioreactor (121.2 cm Ø i.d.; 58.4 cm height), installed in a greenhouse with controlled temperature (25 °C) and exposed to natural sunlight (photosynthetic photon flux density of 336.5±410.4 µmol m^{-2} s^{-1}). The culture medium was prepared with 50 L of swine wastewater digestate effluent diluted in 300 L of tap water. The composition of the medium was (mg L^{-1}): total organic carbon (120±3.4), alkalinity as $CaCO_3$ (200±9.2), ammonia-N (141.6±12.7) and phosphate-P (12.9±2.7). pH was 7.9±0.9.

The bioreactor was inoculated with 150 L of inoculum, with a dry weight (DW) concentration of 60±5.1 mg_{DW} L^{-1} of biomass. The agitation was kept by means of mechanical pumps at 1,200 L h^{-1} (Sarlobetter®, S300, Brazil). After 11 days of inoculation, biomass was harvested by centrifugation at 3,000 ×g (EVODOS T10, Netherlands), immediately frozen (-20 °C) and then lyophilized (Model 030-JJ LJI, Scientific, Brazil) for further assays.

5.2.2 Pre-treatment

The fresh and lyophilized biomass were pre-treated with an ultrasound probe (80% power, time 10 min and 3 pulses).

5.2.3 Fermentation process

The microorganism used for the production of the bio-extracts corresponds to the species *Trichoderma koningiopsis*, obtained from the microorganisms' bank of the Agroecology Laboratory of the Federal University of the Fronteira Sul - Campus Erechim / RS. The fungus used was selected because it presented satisfactory results in the control of weeds in preliminary tests and was isolated from the weed *Digitaria horizontalis* (REICHERT JÚNIOR, 2019).

The fermentation process was carried out in 300 mL flasks with an effective volume of 150 mL. The amount of microalgal biomass used for the cultivation of *T. koningiopsis*, was biochemically related to synthetic culture media already optimized and the fermentation was carried out with 72 h, stirring at 120 rpm and temperatures of 28 ºC (BORDIN et al. 2018).

5.2.3.1 Enzymatic activities and fungal biomass

To evaluate the enzymatic activity present in the extracts, four enzymes were selected as reported in the literature that act in the biocontrol of weeds including lipase, cellulase, amylase and peroxidase (BORDIN et al., 2018; REICHERT JÚNIOR et al., 2019).

5.2.3.2 Lipase

For the lipase quantification, the methodology proposed by Treichel et al. (2017) was used. Briefly, a 10% olive oil emulsion (w v^{-1}) and 5% Arabic gum diluted in 90% (v v^{-1}) of 100 mM pH 6 sodium phosphate buffer solution were prepared. Each sample presented 9 mL of this emulsion to which 1 ml of the fermented extract was added. Later, for each sample, 10 mL of acetone/ethanol solution (1:1; v:v) was added to stop the reaction and the samples were submitted to a titrimetric process using 0.050 M NaOH as the titrant until reaching pH 11.

5.2.3.3 Celulase

To quantification of this enzyme it was according to Ghose (1987). Briefly, 50 mg Whatman filter paper nº1 is used as a cellulose source. The 50 mg was added in test tubes containing 2 mL of 0.2 M acetate buffer and pH 5.5. Total reducing sugars was evaluated by the DNS (MILLER, 1959).

5.2.3.4 Amilase

The quantification of amylase enzymes present in the fermented extract was based on Fuwa (1954) and Pongsawasdi and Yagisawa (1987). Briefly, soluble starch was used as a substrate diluted in the proportion 1:100 (w:v) in 100 mM acetate buffer (pH 5.0). The samples were taken in a thermostatic bath for a period of 10 minutes and at a temperature of 38 ºC. Enzymatic activity was determined using the DNS method (MILLER, 1959).

5.2.3.5 Peroxidase

The peroxidase enzyme was determined according to Devaiah and Shetty (2009). Briefly, 1.5 mL of 5 mM phosphate buffer (pH 5), 2 mL of distilled water, 0.5 mL of 1% guaiacol and 1 mL of 0.08 % hydrogen peroxide were used. The guaiacol reaction was evaluated using a UV-VIS 470 nm spectrophotometer (KHAN; ROBINSON, 1994).

5.2.3.6 Fungal Biomass

Biomass was quantified according to Gern (2005). Briefly, the samples were filtered on Whatman paper nº1 and subsequently dried the material containing the cells retained for 24 h at 60 ºC.

5.2.3.7 Evaluation of bio-herbicidal activity on weeds

To evaluate the bio-herbicidal activity, it was according to the Brazilian Society of Weed Science (SBCPD) (1995). Briefly, the application was carried out when the plants had 2 to 4 leaves, with the aid of a spray bottle, directly on the leaf area of the plants. Three doses were applied (i.e the product was applied and, after drying, reapplied). After the end of the application, the irrigation was kept off for 24 h. The plants selected for the study comprised those that have difficulty controlling by chemicals: *Bidens pilosa* (picão preto), *Conyza bonariensis* (buva), *Urochloa plantaginea* (papuã) and *Euphorbia heterophylla* (leiteiro).

5.3 RESULTS AND DISCUSSION

5.3.1 Bio-herbicidal production

5.3.1.1 Enzymatic activities

Table 5.1 shows the values of enzymatic activities and biomass found. Tests that presented the greatest responses were with 66.6 g pre and non-treated + *T. koningiopsis* assay (fresh biomass) and all with lyophilized biomass, including fermentation without the presence of *T. koningiopsis*. Peroxidase enzyme was the most produced for both fresh and lyophilized

biomass, with activity in U mL^{-1}: 667 (33.3 g pre-treated + *T. koningiopsis);* 767 (66.6 g + *T. koningiopsis)*; 9,200 (lyophilized microalgae biomass without fungus); 2,966 (33.3 g + *T. koningiopsis*); 133.3 (33.3 g pre-treated + *T. koningiopsis*); 15,400 (66.6 g pre-treated + *T. koningiopsis*) and 16,200 (66.6 g + *T. koningiopsis*). The largest fungal biomasses obtained were (in g L^{-1}) 7.7; 9.5; 10.6 and 10.3 for 33.3 g + *T. koningiopsis*, 33.3 g pre-treated + *T. koningiopsis*, 66.6 g pre-treated + *T. koningiopsis* and 66.6 g + *T. koningiopsis*, respectively. The increases in activity applying fungi can also be associated with the lyophilization process, since the respective pre-treatment can facilitate the assimilation of nutrients by the fungus and the concomitant production of peroxidases and biomass.

Table 5.1. Enzymatic activity and biomass production of fermentative extracts using *T. koningiopsis* and fresh and lyophilized microalgae under different conditions.

Enzymes (U mL^{-1})	Fresh biomass					Lyophilized biomass				
	Microalgae without fungus	33.3 g + *T. koningiopsis*	33.3 g pre-treated + *T. koningiopsis*	66.6 g pre-treated + *T. koningiopsis*	66.6 g + *T. koningiopsis*	Microalgae without fungus	33.3 g + *T. koningiopsis*	33.3 g pre-treated + *T. koningiopsis*	66.6 g pre-treated + *T. koningiopsis*	66.6 g + *T. koningiopsis*
Peroxidase	ND	ND	ND	667	767	9,200	2,966	133.3	15,400	16,200
Cellulase	ND	0,5	ND	ND	0.25	3.62	ND	ND	4	6
Lipase	ND	ND	ND	0.75	1.6	0.8	ND	ND	ND	ND
Amylase	ND	ND	ND	2.25	ND	1	ND	ND	ND	7
Fungal biomass (g L^{-1})	0	0	0	1.85	1.82	0	7.7	9.5	10.6	10.3

ND - not detected

The tests with 66.6 g pre and non-treated + *T. koningiopsis* showed a higher production of cellulase and biomass. Cellulose is the main carbohydrate in the cell wall of microalgae and is disposed to enzymatic degradation, while starch, a substrate of amylase, is found in plastids (intracellular) (CHU et al., 1982; SANDER; MURTHY, 2009; VELAZQUEZ-LUCIO et al., 2018). The production of amylase was only verified with the presence of peroxidase with lipase (66.6 g pre-treated + *T. koningiopsis*) and peroxidase with cellulase (66.6 g + *T. koningiopsis*) suggesting that there is possibly a synergism between these enzymes in the breakdown of the polysaccharides present in the microalgae cell wall making carbohydrates such as starch for fungal growth that is present intracellularly (VELAZQUEZ-LUCIO et al., 2018). Exogenous enzymes like these have already been reported to significantly disrupt microalgae cell walls by releasing trapped nutrients with important nutritional value (COELHO et al., 2019).

Tests showed higher production of fungal biomass; demonstrating that the microalgae biomass produced from swine manure, can be applied as a culture medium for the fungus *T. koningiopsis* in parallel with the production of peroxidase. This is due to the composition of microalgae, as they have a high concentration of carbon, amino acids and nitrogen necessary for the growth of the microorganism (NOLL et al., 2016). Other studies have reported the positive effect of nitrogen sources (yeast extract and peptone), emphasizing that high concentrations of these products cause an increase in biomass production (BORDIN et al., 2018). In the production of mycelial biomass, it was found that peptone and maltose generate a significant positive effect, with the effect of peptone concentration being more important than maltose (ZHAI and HAN, 2016).

5.3.1.2 Bio-herbicidal activity

Species of *Trichoderma* spp. can produce several secondary metabolites, such as: alcohols, ketones, alkanes, furans, acids, among others, which are related to the environmental conditions of growth of the fungus, for example the composition of the culture medium and can inhibit the production of some enzymes (STOPPACHER et al., 2010, VELÁZQUEZ-ROBLEDO et al., 2011). Bordin et al. (2018) reported that performance of the enzyme peroxidase on the lignin present in the cell wall of plants influences the occurrence of phytotoxic damage.

The best fungus cultivation results using as substrate fresh and lyophilized microalga (66.6 g + *T. koningiopsis*) with a higher concentration of peroxidase, were applied to the plants leaf surface. It was selective for the species *Conyza bonariensis*, seeing up to 80% phytotoxicity

in the first 7 days and 100% after 15 days of treatment (Table 5.2). This species showed complete damage of the leaf structure at the end of the evaluation, showing the compound can act effectively in the biological control of this species.

Table 5.2. Phytotoxicity assessment of bio-extracts from fungus grown with microalgae biomass in weeds.

Species	Response to foliar damage (%)			
	Fresh biomass		Lyophilized biomass	
	7th day	15th day	7th day	15th day
B. pilosa	0	1	5	5
C. bonariensis	70	100	80	40
E. heterophylla	2	3	30	20
U. plantaginea	0	0	5	5

When bio-extract was applied for the species *E. heterophylla* was observed on the 7 day a 30% leaf damage. However, when applied only microalgal biomass, it was observed effect only for the species *C. bonariensis*, with leaf damage of 50 and 85% in 7 and 15 days, respectively (Table 5.23). Additionally, after application of the bio-extract microalgae showed minimal phytotoxic activity for *B. pilosa*, *E. heterophylla* and *U. plantaginea*. This shows an advantage, as it has selective action only to combat this weed species, such as some synthetic herbicides.

Table 5.3. Phytotoxicity assessment of bio-extracts from microalgae in weeds.

Species	Response to foliar damage (%)	
	7th day	15th day
B. pilosa	5	2
C. bonariensis	50	85
E. heterophylla	10	0
U. plantaginea	1	0

Studies reported that effects caused by the action of the fungus *Diaporthe* sp. was leaf lesions and, in some cases, the plants yellowing. It was observed that plants did not resist the compound application, presenting necrosis in a large part of the leaf tissue (DE SOUZA et al., 2015). Bio-extracts produced from 39 phytopathogenic fungi were isolated from weeds in rice culture, in order to carry out the biological control of the plants. Out of the fungi strains isolated, 28 showed some phytopathogenic potential in the weeds (DE SOUZA et al., 2017).

Trichoderma spp. use hydrolytic enzymes (such as cellulase, lipase, amylase) to promote cell wall degradation of the target plant, thus enabling the microorganism to act

intensively as a biocontrol (CONTRERAS-CORNEJO et al., 2018; VELÁZQUEZ-ROBLEDO et al., 2011). Therefore, the peroxidase enzymes induce the accumulation of hydrogen peroxide in the plant leaves, which consequently causes cellular damage and are considered oxidoreductases, so they may be responsible for the lignin degradation, part of the plants cellular structure (MOEDER et al., 2002, MEDINA et al., 2017).

Studies have reported the relationship between peroxidase (superoxide dismutase and catalase) and the target plant, in which the action of enzymes with antioxidant potential was observed when applied to plants, resulting in leaf damage (MORRA et al., 2018; YU; ZHANG, 2013). Monteiro et al. (2010) combined the production of different enzymes of the fungus *Trichoderma* spp., concluding that interaction mechanism between the elements signals as synergism, in which, the hydrolytic enzymes act by degrading the plant cell wall. In addition, *Trichoderma* is an excellent biological control agent, as it similarly acts on the plant's nutritional competition (ALMEIDA et al., 2007).

One of the mechanisms used by plants to overcome this situation is based on enzymatic (catalase, peroxidase, superoxide dismutase, glutathione reductase) and non-enzymatic defense systems, that act synergistically and interactively to neutralize the free radicals formed and eliminate ROS (APEL; HIRT, 2004; BENAVIDES et al., 2005; HUANG et al., 2019). When there is an imbalance in the cells between the elimination systems and the ROS levels, the cells arrive an oxidative state that can lead to their death (HUANG et al., 2019). The presence of high peroxidase enzyme concentrations in the bio-extract can have caused stress in the plant, due these enzymes act on the structure of the polysaccharide cell wall.

5.4 CONCLUSIONS

The biomass of *Chlorella* spp. cultivated in the effluent of swine treatment has potential to be used as a substrate for the *T. koningiopsis* cultivation. Peroxidase enzyme was the most produced enzyme for both microalgae fresh and lyophilized biomass. The bio-extracts produced with only microalgae biomass reached levels of up to 85% in biocontrol with the species *C. bonariensis*. Bio-extract application, after cultivation of *T. koningiopsis* using biomass as substrate, caused phytotoxic activity in *C. bonariensis* and *E. heterophylla*. The bioprocesses integration of phycoremediation associated with *T. koningiopsis* cultivation and enzymes production, is a promising source of feedstock for the byproducts production such as bio-herbicides.

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- CHAPTER 6 -

6 REMOVAL OF VETERINARY ANTIBIOTICS IN SWINE WASTEWATER USING MICROALGAE-BASED PROCESS [6]

Abstract: Phycoremediation of swine wastewater is an attractive treatment to remove contaminants and simultaneously produce valuable feedstock biomass. However, there is a lack of information about the application of phycoremediation on veterinary antibiotic removal. Thus, this research investigated the degradation of tetracycline, oxytetracycline, chlortetracycline and doxycycline in swine wastewater treated with phycoremediation. The tetracyclines degradation kinetics was adjusted to the pseudo-first-order kinetics model, being 0.36 > 0.27 > 0.19 > 0.18 (d^{-1}) for tetracycline, doxycycline, oxytetracycline and chlortetracycline, respectively. The maximum concentration of microalgae biomass (342.4±20.3 mg L^{-1}) was obtained after 11 days of cultivation, when tetracycline was completely removed. Chlortetracycline concentration decreased, generating iso-chlortetracycline and 4-epi-iso-chlortetracycline. Microalgae biomass harvested after antibiotics removal presented a carbohydrate-rich content of 52.7±8.1, 50.1±3.3, 51.4±5.4 and 57.4±10.4 (%) when cultured with tetracycline, oxytetracycline, chlortetracycline and doxycycline, respectively, which could be a valuable source for bioenergy conversion.

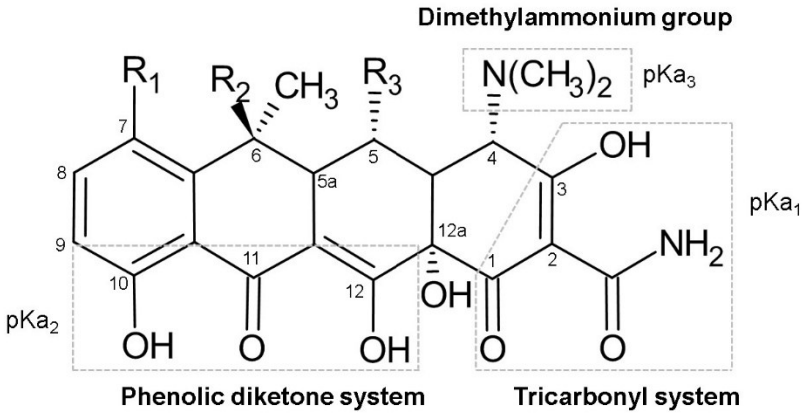
6.1 INTRODUCTION

Swine wastewater contains a high concentration of organic matter and nutrients (nitrogen and phosphorus), as well as heavy metals and veterinary drug residues that can affect public and environmental health (NAGARAJAN et al., 2019). Among the residues of veterinary drugs, there is a special concern with antibiotics, as they are extensively used in swine production and have long persistence in the environment (RICHARDSON; TERNES, 2011). The global consumption of veterinary antibiotics in 2016 was 97,784 tonnes; mostly in Asia, the Far East and Oceania (61,170 tonnes), followed by the Americas (24,035 tonnes) (OIE, 2020). Nevertheless, 70 to 90% of the administered antibiotics are excreted by the swine, which results in significant amounts of antibiotic micrograms per liter of wastewater (ZHOU et al., 2020).

[6] This chapter is submitted to the journal **Environmental Research**.

In the last decades, the use of veterinary antibiotics for the preservation of animal health (including growth promoters at the sub-therapeutic level) has increased considerably (LEES et al., 2020), as they are essential to modern intensive agricultural production. However, their continuous administration (i.e., misuse and/or abuse) has been associated with the selection and spreading of antibiotic resistance genes (ARGs) (CHANG et al., 2015). The major classes reported for use in animals are the tetracyclines (35.3%), followed by penicillin (16.4%), macrolides (10.9%) and sulfonamides (4.8%) (OIE, 2020).

Tetracyclines have been used since the 1940s and exhibit activity against a wide range of microorganisms (CHOPRA; ROBERTS, 2001), besides being the cheapest available antibiotic class (CHOPRA; ROBERTS, 2001). Different concentrations of this antibiotic class have been detected in swine wastewater: tetracycline (TC), oxytetracycline (OT), chlortetracycline (CT) and doxycycline (DC) (Figure 6.1) at concentrations of 388.7, 387.1, 138.8 and 685.6 (μg L^{-1}), respectively (CHEN et al., 2012; PAN et al., 2011; WANG et al., 2016). However, even at these concentrations, these residues can be toxic or induce ARGs expression (ZHANG; ZHANG; FANG, 2009).



Antibiotic	R			pKa		
	R_1	R_2	R_3	pKa_1	pKa_2	pKa_3
Tetracycline	-H	-OH	-H	3.3	7.68	9.69
Chlortetracycline	-Cl	-OH	-H	3.3	7.44	9.27
Oxytetracycline	-H	-OH	-OH	3.27	7.32	9.11
Doxycycline	-H	-H	-OH	3.02	7.97	9.15

Figure 6.1. Chemical structures and pKa of tetracycline antibiotics

Many treatment processes such as adsorption, advanced oxidation (AOPs) and biological treatment have been studied to remove TCs from wastewater matrices (GAO et al., 2012; REYES et al., 2006; YAHIAT et al., 2011; ZHANG et al., 2019). Adsorption processes are commonly expensive and highly adsorbent-dependent (AHMED et al., 2015). AOPs are generally effective but require expensive chemical agents or catalysts (JIANG et al., 2018) and

may produce a more toxic byproduct than the parental compound (KANG; LIM; KWON, 2012). On the other hand, conventional biological treatment, such as aerobic (activated sewage sludge) and anaerobic (digesters) treatments, cannot effectively remove TCs, due to their antibacterial activity, which can act by killing or inhibiting the microbial community of the system (KIM et al., 2005; MONTES et al., 2015; ZHOU et al., 2020).

Among swine wastewater treatment alternatives, microalgae-based processes (phycoremediation) are promising due to the low-cost, high-efficiency potential use of biomass and generation of valuable byproducts (LENG et al., 2020; NAGARAJAN et al., 2019). In this scenario, it is important to investigate the use of phycoremediation on the removal of antibiotics and identify the byproducts of the process to understand how this technology can help mitigate environmental impact. Considering all the advantages of this process, the present study aimed to evaluate the efficiency of removal and transformation of the four tetracyclines most used in swine breeding by an indigenous microalgae consortium biomass (dominated mainly by *Chlorella* spp.) grown in swine wastewater.

6.2 MATERIAL AND METHODS

Figure 6.2 shows a schematic diagram of the material and methods. Steps are described in the sections below.

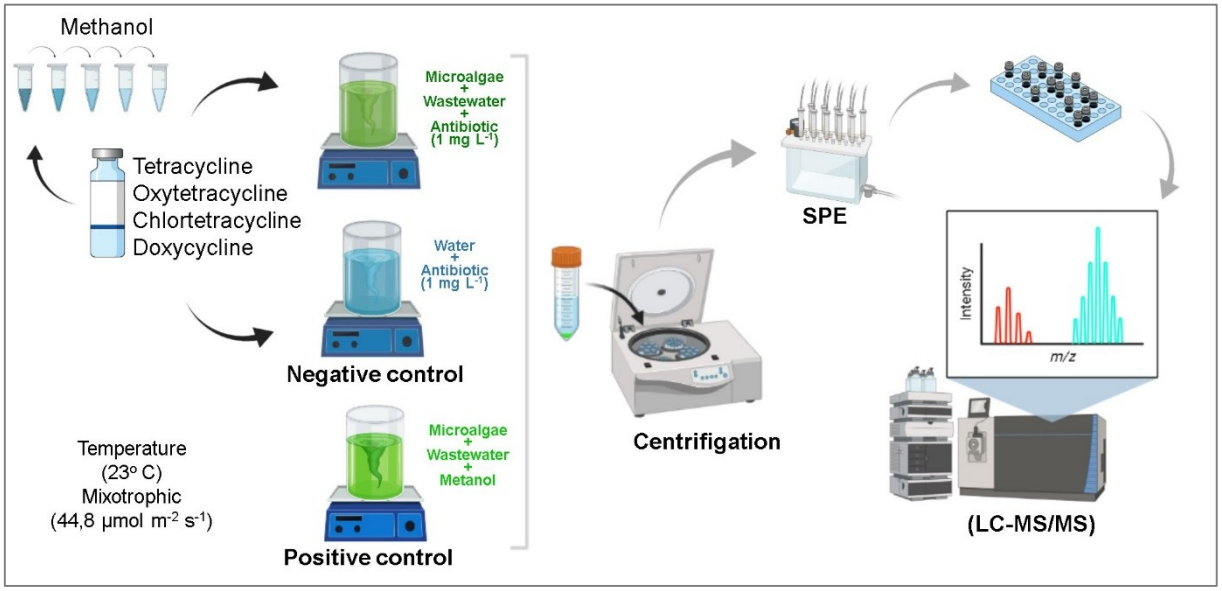


Figure 6.2. Schematic diagram of methodology

6.2.1 Microalgae inoculum acclimation

The microalgae consortium (predominance of *Chlorella* spp.) used as inoculum in this study was previously obtained from a field-scale facultative open pond used in a tertiary treatment process of swine wastewater (Brazilian Agricultural Research Corporation, EMBRAPA, Concordia, Brazil) (MICHELON et al., 2015) (Supplementary material I). The collected microalgae inoculum was then acclimated in 12 L glass photobioreactors (PBRs; 20 cm Ø ID), filled with water containing 6% v v^{-1} of non-sterile digestate from an Upflow Anaerobic Sludge Blanket (UASB) treating swine wastewater. PBRs were kept at room temperature (23 ºC) under mixotrophic conditions (12h:12h; light:dark) using red emitting diode light (PGL-RBC 2500, Parus) at 630 nm [photosynthetic photon flux density (PPFD) of 148.5 μmol m^{-2} s^{-1}] and continuous agitation using recirculation mechanical pumps (Sarlobetter brand).

6.2.2 Wastewater characterization

The chemical composition of the diluted UASB effluent, prior to inoculation (time zero), was (in mg L^{-1}): phosphate-P (11.5±3.6), biological oxygen demand (BOD_5 90.8±0.9), total organic carbon (103±4.2), alkalinity as $CaCO_3$ (180±20), ammonia-N (55.1±1.7) and total nitrogen (50.1±2.0). The pH was 7.9±0.6. The biomass was collected by centrifugation at 3,000 ×*g* (EVODOS, T10, Netherlands) eleven days after inoculation and immediately frozen (-20 °C) and lyophilized (Model 030-JJ LJI Scientific) for further analyses.

Phosphate-P was measured by the ascorbic acid colorimetric method (APHA, 2012). Nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$) and ammonia ($N\text{-}NH_3$) were quantified by flow injection analysis (FIAlab – 2500) (APHA, 2012). Alkalinity (as mg $CaCO_3$) was determined by automatic titration (Metrohm 848 Titrino Plus). Total organic carbon (TOC) was measured using a TOC analyzer (TOC-LCPH/CPN, Shimadzu, Kyoto, Japan). The pH was monitored using a pH meter (pH–mV, Hanna Instruments, Inc.). Light intensity was measured with a Luximeter (DX-100, Japan). A satisfactory correlation (r^2=0.96) between dry matter biomass concentration was determined by suspended solids (APHA, 2012) and optical density (OD_{750}) (mg_{DW} L^{-1}=238.93×OD_{750}nm–37.432). Therefore, microalgae growth over time was assessed using a spectrophotometer (Varian, Inc. Cary® 50) analysis by absorbance at 750 nm.

6.2.3 Experimental procedure – TCs removal

Experiments were conducted using 4 L reactors exposed to white light [PPFD of 99 μmol m^{-2} s^{-1}], mixotrophic conditions (12 h:12 h; light:dark), 500 rpm agitation (IKA Color

Squid White), and room temperature (23 °C). Reactors were operated in fed-batch mode using effluents from an UASB treatment, which was diluted by adding 0.24 L into 1.04 L of chlorine-free tap water. Reactors were inoculated with 67±0.6 mg_{DW} microalgae L^{-1}.

The TCs were added and analyzed separately and all reactors were exposed to the same experimental conditions. Each TC standard solution was prepared with methanol in a 10 mg L^{-1} concentration, and 4 mL were added to each reactor of microalgae culture, yielding a final concentration of 1 mg L^{-1}. Reactors containing microalgae cultures with 4 mL of methanol and no TCs were used for positive control. Negative controls consisted of solutions with water and TCs, and were performed to evaluate TCs photodegradation.

6.2.4 Antibiotic measurement

6.2.4.1 Chemicals and materials

The analytical standards tetracycline hydrochloride, chlortetracycline hydrochloride, oxytetracycline hydrochloride and doxycycline hydrochloride were all purchased from Sigma-Aldrich (St. Louis, USA) with a quality level of 100 (QL 100). Formic acid Optima LC-MS grade was obtained from Fisher Chemical (USA). All solvents were HPLC grade and acquired from Panreac (Darmstadt, Germany) and J.T. Baker (USA). An ultrapure water purification system provided by Millipore (Advantage A10) system was used to obtain ultrapure water. Solid phase extraction cartridges (Oasis HLB, 60 mg, 30 cc) were purchased from Waters (Milford, MA, USA).

6.2.4.2 Standard solutions

Antibiotics solutions were mixed and diluted, with ultrapure water (Milli-Q, Millipore, EUA), to a final concentration of 10 mg L^{-1} of each antibiotic becoming the working solution for spiking the calibration curve samples. Serial dilution was performed to generate standard curve as described on 2.4.3 section.

6.2.4.3 Extraction procedure

The target compounds were extracted from culture samples by solid-phase extraction (SPE) experiments using the 60 mg Oasis HLB cartridge with flow at atmospheric pressure. HLB cartridges were conditioned and equilibrated immediately prior to extraction, using a vacuum manifold, with 3 mL of methanol followed by 3 mL of ultrapure water (Milli-Q, Millipore, EUA). The supernatant (3 mL) of the centrifuged culture sample was loaded through the cartridge, and after extraction, HLB cartridges were flushed with 3 mL of ultrapure water

followed by 1 min of vacuuming to help remove interferences. Tetracyclines were removed from the cartridges using 3 mL of methanol and the extracts evaporated under nitrogen steam at less than 40 ºC, and then re-dissolved in methanol for the liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

6.2.4.4 LC-MS/MS analysis

LC separation was performed using a Thermo Survayor Plus with a Kinetex C18 100 Å analytical column (4.6 mm i.d. × 100 mm, particle size 5 μm) combined with a C18 guard column (2.0 mm, 3 μm), both from Phenomenex® (Torrance, CA, USA). The autosampler was held at 10 ºC and the column temperature oven at 30 ºC. The mobile phase was composed of eluent A (0.1% formic acid in ultrapure water), and eluent B (methanol with 0.1% formic acid), at a flow rate of 1 mL min^{-1}. The best separation was achieved with a gradient starting with 95% of eluent A for 1 min, which was increased linearly over 9 min to 100% of eluent B then was kept in this proportion for 3 min. It was then gradually returned to its initial conditions over 1 min, remaining at a proportion of 95% of eluent A for another 2 min, for a total 16 min run. Injection volume was 10 μL.

The flow from the LC column was transferred to a triple quadrupole mass spectrometer (Thermo TSQ Quantum Access Max) equipped with an electrospray (ESI) source. The analysis was performed in positive polarity mode for all compounds. Instrument control, data acquisition and evaluation were done with the XCalibur software, Version 2.1. Determination was performed by SRM (selected reaction monitoring) mode transitions. To confirm the identity of the antibiotics in the samples, specific precursor-to-product ion for each antibiotic was selected. For quantification, the most abundant transition was used and the second more abundant was used for confirmation. The SRM dynamics were given in the Table 1. Mass spectrometer parameters were: sheath gas at 40 psi, auxiliary gas at 55 psi, spray voltage at 3.5 kV, vaporized temperature of 350 °C and capillary temperature of 242 ºC. In the collision cell the experiments were performed using high purity argon as the collision gas at 2.1 mTorr.

To quantify tetracyclines in samples, the respective antibiotic analytical curve was performed with antibiotic free (blank) culture samples fortified at final levels of 10, 50, 100, 250, 500, 750 and 1000 (ηg L^{-1}) of each antibiotic prior to SPE extraction. The analytical curves linearity and correlation coefficients (R^2) over the calibration range are described in Table 6.1. Retention times for tetracycline, oxytetracycline, chlortetracycline and doxycycline were 6.1, 6.2, 7.1 and 7.9 (min) respectively.

Table 6.1. Mass parameters, multiple reaction monitoring settings, method linearity, detection limits (LOD) and quantitation limits (LOQ) for tetracyclines.

Antimicrobial	**Molecular Mass**	**Precursor ion $[M+H]^+$**	**Quantitation product ion (CE)**	**Confirmation product ion (CE)**	**Equation**	**R^2**	**LOD (µg L^{-1})**	**LOQ (µg L^{-1})**
Tetracycline	444.4	*m/z* 445.1	*m/z* 410.3 (18 eV)	*m/z* 154.2 (26 eV)	Y=492.8x+30914	0.9967	1.5	5.0
Chlortetracycline	478.9	*m/z* 479.1	*m/z* 444.3 (19 eV)	*m/z* 462.3 (16 eV)	Y=189.8x+3861	0.9925	1.5	5.0
Oxytetracycline	460.4	*m/z* 461.1	*m/z* 426.3 (17 eV)	*m/z* 443.3 (11 eV)	Y=1130x-404.4	0.9965	1.5	5.0
Doxycycline	444.4	*m/z* 445.1	*m/z* 428.3 (17 eV)	*m/z* 321.2 (30 eV)	Y=2257.1x-5877	0.9903	1.5	5.0

m/z – mass/charge ratio

CE - collision energy in electronvolts (eV)

6.2.5 Biochemical composition of biomass from phycoremediation

The cellular composition of the microalgae biomass was determined as described by Michelon et al. (2015) for the characterization of lipid, carbohydrates, protein and mineral material content.

6.2.6 Statistical Analysis

Statistical differences among treatment data sets were determined using one-way analysis of variance (ANOVA) with Statistica® 8.0 software (STATSOFT trial version). Tukey's significant difference test was conducted after the determination of variances ($p \leq 0.05$).

6.3 RESULTS AND DISCUSSION

6.3.1 Effect of TCs on the biomass of consortium

Microalgae growth inhibition caused by TCs is an important aspect to consider in the improvement and development of microalgae-based processes. The effect of 1 mg L^{-1} of TC, CT, OT and DC on the growth of the microalgae consortium cultivated with swine wastewater is shown in Figure 6.3. The biomass growth curves at different TCs concentration revealed a final microalgal biomass of 342.4±20.3, 334.1±32.1, 371.1±37.16, 368,7±30.4 mg_{DW} L^{-1} for TC, CT, OT and DC, respectively, while the positive control (without TCs) was of 318.6±60.8 mg_{DW} L^{-1}. A faster growth was obtained after 2 days of the experiment, which then reached a plateau. Biomass growth did not present significant differences between the experiments and the positive control ($p>0.05$), showing that different concentrations of TC, CT, OT and DC do not affect growth rate.

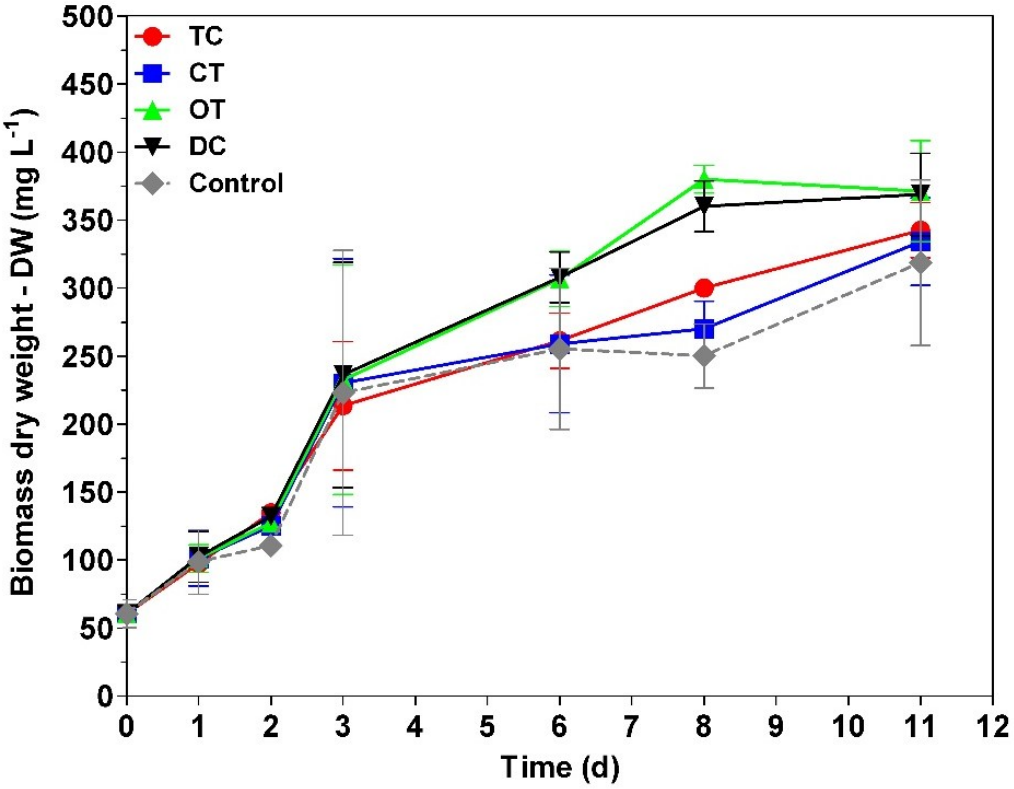


Figure 6.3. Effect of tetracycline (TC), chlortetracycline (CT), oxytetracycline (OT) and doxycycline (DC) on growth of microalgae cultivated with swine wastewater.

Different results were reported by Li et al (2016) that found *Chlamydomonas reinhardtii* reaches its maximum inhibition rate when exposed to 0.25 mg L^{-1} of TC in synthetic effluent from wastewater treatment plants. TC concentrations of 0.1-0.2 mg L^{-1} inhibited *Microcystis aeruginosa* and *Selenastrum capricornutum* biomass growth, while a concentration of <0.05 mg L^{-1} stimulated the growth of *Microcystis aeruginosa*, due to the improvement of the enzymatic activities in the physiological and biochemical processes of cells (YANG et al., 2013). These differences could be associated with the microalgae species (YANG et al., 2013), showing that the microalgae consortium from the present study tolerated higher amounts of TCs.

Taşkan (2016) observed that exposure of a microalgae-bacteria consortium to high TC, OT and CT concentrations (30 mg L^{-1}) caused a decrease of 21 and 36 (%) on removal efficiencies of total nitrogen and phosphorus, respectively. Nutrient removal rates decreased when the microalgae-bacteria consortium was exposed to TCs (TC, OT and CT) in concentrations above 1 mg L^{-1} (TAŞKAN, 2016). The main toxicity mechanism to affect microalgae biomass in this case was the oxidative stress caused by reactive oxygen species production (hydroxyl radicals, hydrogen peroxide, superoxide radicals, free radicals and nonradical forms), which can promote changes in the

superoxide dismutase, peroxidase, and catalase activities (LI et al., 2016); denaturating proteins, DNA, and causing cell death (LI et al., 2015; VIANCELLI et al., 2020).

6.3.2 Antibiotic removal

Microalgae growth conditions such as pH, light, temperature, CO_2, nutrient concentration, and cultivation methods (autotrophic or heterotrophic) are critical to antibiotic removal efficiency (NORVILL; SHILTON; GUIEYSSE, 2016). pH variation during the experiments is shown in Figure 6.4. The pH of the culture medium with different TCs ranged from 8.3±0.2 to 10±0.2 and showed no significant difference when compared to the positive control ($p>0.05$). On the other hand, the pH of the negative control (water and TCs) reactors remained at 8.5±0.1 (data not shown), being significantly different from the experiments which reached pH 10.

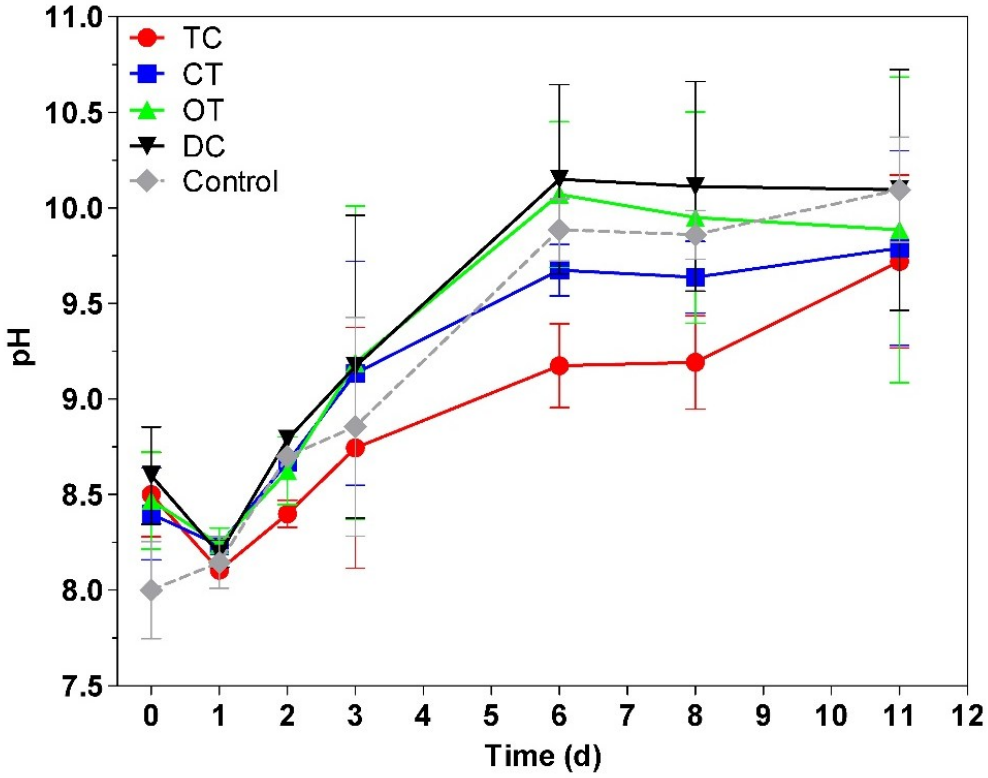


Figure 6.4. pH variation during swine wastewater phycoremediation in the presence of tetracycline (TC), chlortetracycline (CT), oxytetracycline (OT) and doxycycline (DC).

In general, elevated photosynthesis rates of microalgae can increase pH, causing hydrolysis of some ionic antibiotics, such as TCs; therefore, pH control is essential to efficiently remove them. In strong acidic and alkaline conditions, TCs stability is affected. At pH 2 to 6, C-4 suffers epimerization to form 4-epi-TCs, anhydro-TCs and 4-epi-

anhydroTCs; in alkaline conditions, C-11 suffers epimerization, leading to the formation of iso-TCs and 4-epi-iso-TCs (Figure 6.5) (KENNEDY et al., 1998; SØEBORG; INGERSLEV; HALLING-SØRENSEN, 2004).

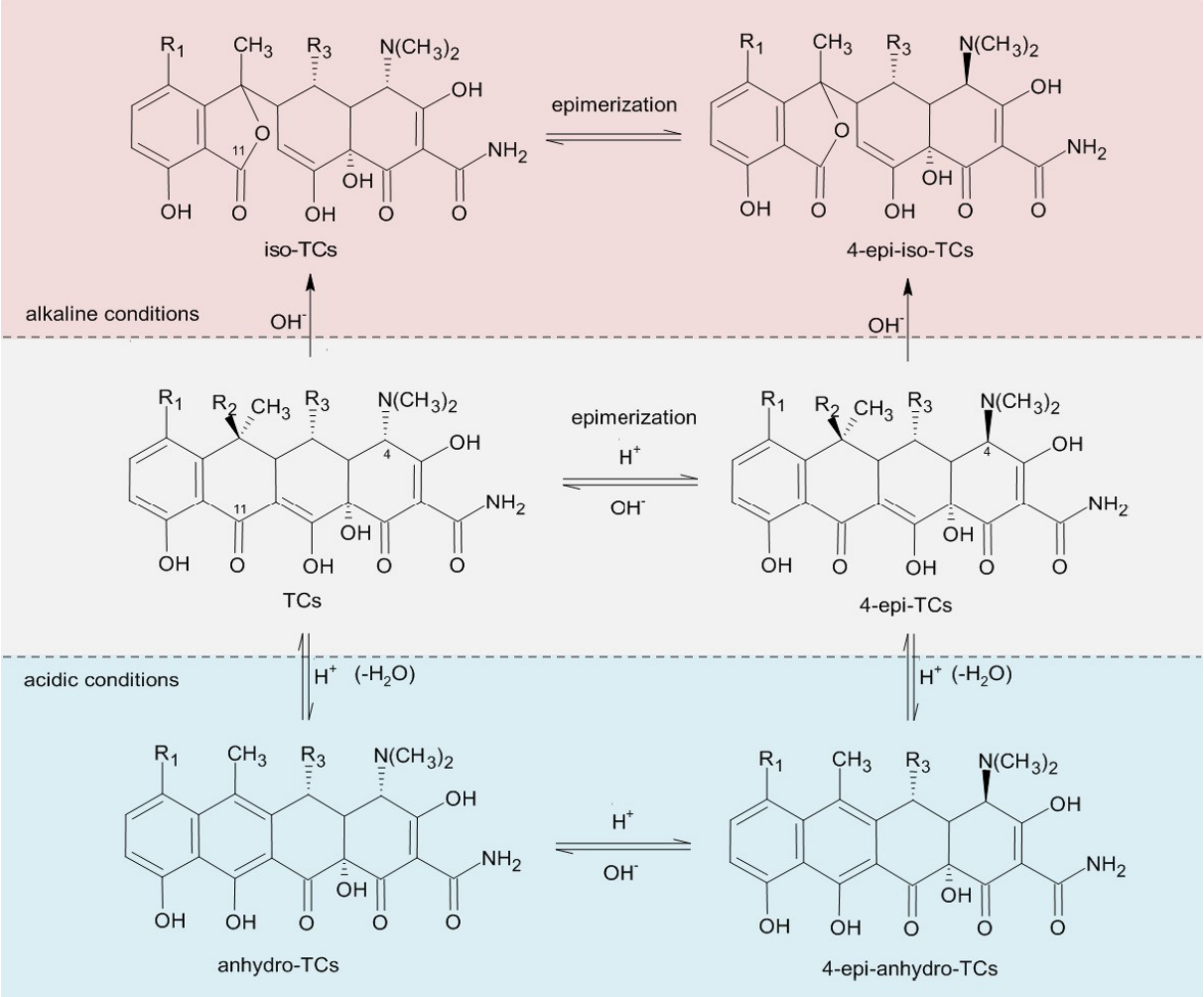


Figure 6.5. Chemical stability of tetracyclines under acidic and alkaline conditions.

6.3.2.1 Kinetics of antibiotics removal

TCs degradation by the microalgae-based process in our experiment fitted pseudo-first-order kinetics (Eq 1), corroborating previous studies (DE GODOS; MUÑOZ; GUIEYSSE, 2012; NORVILL et al., 2017).

$$\text{Ln}([TCs]/[TCs]_0) = -k_1 t \quad \text{(Eq 1)}$$

Where k_1 is the observed pseudo-first-order rate constant (d^{-1}). $[TCs]_0$ and $[TCs]$ represent TCs concentration at time 0 and t, respectively.

The calculated pseudo-first-order kinetic rates (k_1) for TC, CT, OT and DC were 0.367±0.08, 0.182±0.1, 0.192±0.05 and 0.277±0.01 (d^{-1}), respectively. In contrast, the

negative control presented k_1 values for TC, CT, OT and DC of 0.172±0.03, 0.304±0.11, 0.212±0.03 and 0.128±0.006 (d^{-1}), respectively (Table 6.2). Thus, in the negative control it was possible to note that TCs were degraded by direct photolysis. In the experiments, the rate of TC and DC photodegradation was 2-fold greater in the presence of microalgae biomass, which indicates that phycoremediation processes contribute to the removal of these antibiotics by indirect photodegradation. The effect observed in the removal of CT and OT were direct photodegradation, once that the k_1 value from CT and OT were similar to the negative control.

It is important to highlight that in swine manure treatment systems the organic load and concentration of total solids generally leaves the matrix with high turbidity, which makes it difficult for light to penetrate for effective direct degradation.

Table 6.2. The values of k_1 to removal kinetics of tetracycline (TC), chlortetracycline (CT), oxytetracycline (OT) and doxycycline (DC) by microalgae-based process.

Antibiotic	**K_1 (d^{-1})**	**R^2**	**K_1 (d^{-1}) (control)**	**R^2 (control)**
TC	0.367 ± 0.08^a	0.986	0.172 ± 0.03^b	0.978
CT	0.182 ± 0.1	0.956	0.304 ± 0.11	0.797
OT	0.192 ± 0.05	0.971	0.212 ± 0.03	0.912
DC	0.277 ± 0.01^a	0.942	0.128 ± 0.006^b	0.964

Experimental conditions: $[TC, CT, OT \text{ and } DC]_0$= 1 mg L^{-1}, T= 23 °C and light intensity = 99 μmol m^{-2} s^{-1}. Different letters denote significant differences ($p<0.05$) according to Tukey test.

6.3.2.2 Tetracycline

Microalgae-based antibiotic removal depends mainly on the classes and concentrations of the drugs. Most antibiotics present inhibition on microalgal growth when the concentration is close to or higher than the microalgae tolerable dose/concentration (LENG et al., 2020). As shown in Figure 6.6A, TC removal reached 100% after 11 days, when compared to the negative control (Figure 6.6B), which degradation was of 68%. As stated by Oka et al. (1989), this phenomenon could be due to antibiotics instability in an aqueous solution and direct photolysis (photodegradation); they reported that TC in an aqueous solution decreased around 50% when exposed to sunlight condition. De Godos et al (2012) identified that photodegradation and biosorption were mechanisms of TC degradation (92–98% after 43 h) from synthetic wastewater treated by *Chlorella vulgaris*. In a microalgae-bacteria based high rate pond, TC removal by indirect photodegradation was over 93% (NORVILL et al., 2017).

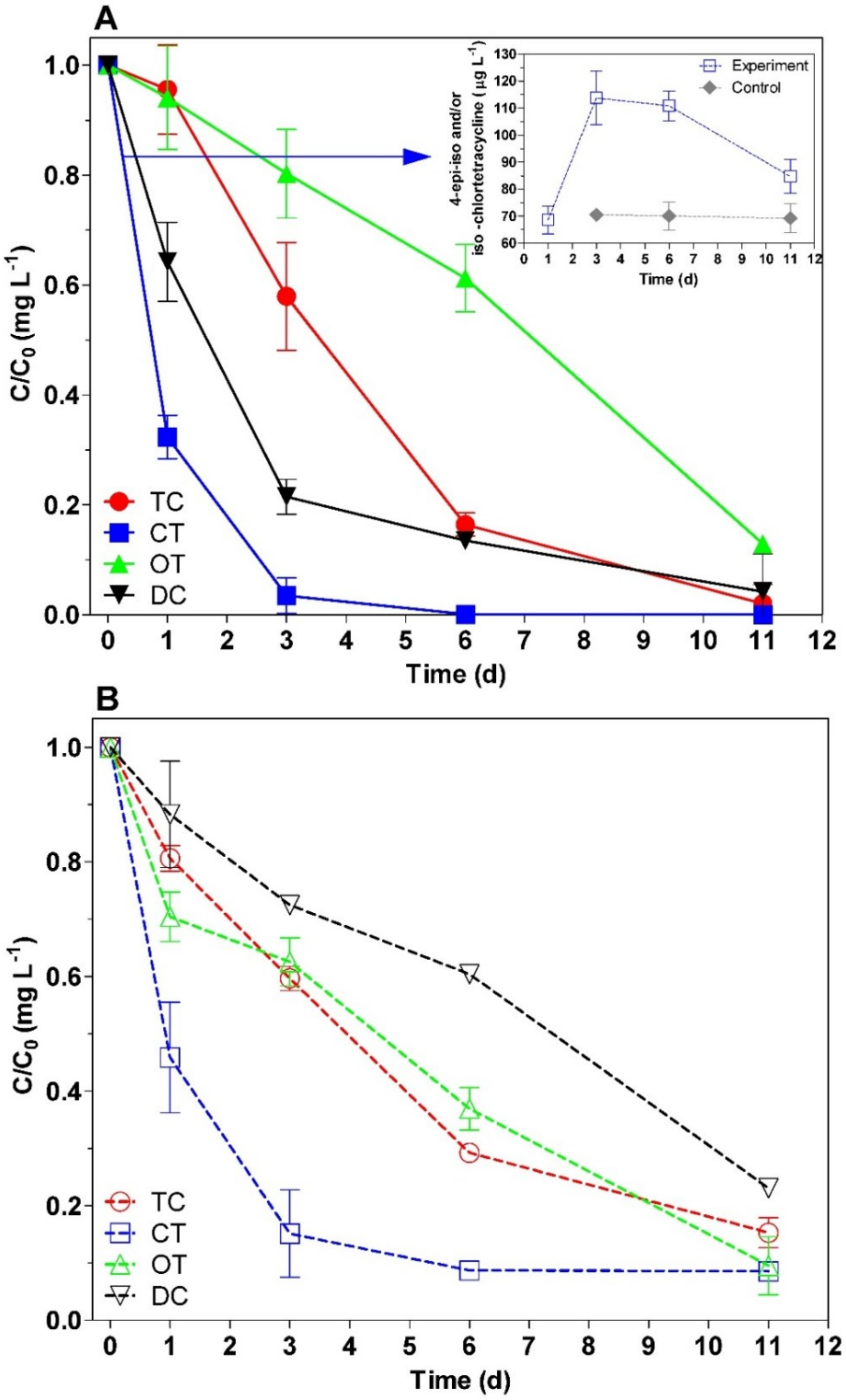


Figure 6.6. Tetracycline (TC), chlortetracycline (CT), oxytetracycline (OT) and doxycycline (DC) removal during swine wastewater phycoremediation process (a) and negative control (water and tetracyclines) (b). Bars depict standard deviation of the mean, and different letters denote significant differences ($p \leq 0.05$) according to Tukey test.

6.3.2.3 Chlortetracycline

As shown in Figure 6.6A, CT was completely removed and/or transformed after 6 days of swine wastewater phycoremediation while around 10% of CT remained in the reactor in the negative control. The fast CT decrease occurred concomitantly with the formation of an unidentified compound, which was observed by an additional peak in the liquid chromatography chromatogram (data not shown). This unidentified compound remained in the culture medium. In an aqueous solution, the parent CT demonstrated limited chemical stability, resulting in its transformation into epimers/isomers and degradation products (SØEBORG; INGERSLEV; HALLING-SØRENSEN, 2004).

Further investigations led us to suppose that the unidentified compound might be iso-CT or 4-epi-iso-CT (KENNEDY et al., 1998), once under the ESI conditions used, iso-CT and 4-epi-iso-CT form an $[M+H]^+$ ion at *m/z* 479, which is the same for CT. Iso-CT and 4-epi-iso-CT lose ammonia to form an ion at *m/z* 462, which is the same mass ion used for CT confirmation (Table 6.1). However, neither iso-CT nor 4-epi-iso-CT can lose water to form a fragment at *m/z* 444, which was not found as a fragment of this unknown peak. The co-elution of iso-CT and 4-epi-iso-CT in liquid chromatography is very common; therefore, the differentiation of these epimers was not possible to confirm.

Similar transformation results were reported by Zhou et al. (2021) when evaluating the use of *Spirulina platensis* on the removal of CT, where the mechanism of degradation was hydrolation and side-chain breakdown. The authors also reported that CT concentrations above 1 mg L^{-1} can initiate an oxidative stress mechanism on microalgae cells. However, it is important to highlight that environmental factors such as light, pH, temperature and matrix can influence CT stability (SØEBORG; INGERSLEV; HALLING-SØRENSEN, 2004).

CT removal results observed in the present study were similar to those found by Zhao et al. (2020), where the *Chlamydomonas reinhardtii* microalgae was highly efficient in degrading chlortetracycline (99% after 24 h), when compared to *Selenastrum capricornutum* and *Scenedesmus obliquus* species, which presented degradation efficiencies of 62 and 70 (%), respectively; additionally, the 4-epi-chlortetracycline and iso-chlortetracycline were accumulated in the presence of *Chlamydomonas reinhardtii*. Tian et al. (2019) indicated that the presence of *Chlorella vulgaris* extracellular organic matters contributed to 93% of chlortetracycline indirect photodegradation in aqueous solutions.

6.3.2.4 Oxytetracycline

OT concentration decreased exponentially throughout the experiment (Figure 6.6A). There was no significant difference ($p>0.05$) between the experiments with microalgae biomass and the negative control (Figure 6.6B). Santaeufemia et al. (2016) found that, under lighting conditions without biomass, OT concentration also decreased exponentially; around 21% was degraded through direct photodegradation process. Sunlight photodegradation of OT in aquaculture wastewater was 3.9-fold higher than in deionized water, as photodegradation of OT can be influenced by the matrix characteristics (suspended solids, inorganic compounds and dissolved organic matter) (LEAL; ESTEVES; SANTOS, 2016). Living and dead biomass of *Phaeodactylum tricornutum* were efficient for OT bioremediation up to 97% (2.5 mg L^{-1} in 11h) and 29.18 mg g^{-1} in seawater, respectively (SANTAEUFEMIA et al., 2016).

The effect of OT on photochemistry and photosynthetic apparatus was investigated by Siedlewicz et al. (2020) with different microalgae species: *Chlorella vulgaris*, *Phaeodactylum tricornutum*, *Nodularia spumigena* and *Microcystis aeruginosa*. The study showed that changes in physiology caused by damage to photosystem II (PSII) were observed after 72 h in OT concentrations of 4 and 8 mg L^{-1}, together with inhibition of pigment synthesis (SIEDLEWICZ et al., 2020).

6.3.2.5 Doxycycline

DC degradation by microalgae-based processes is not fully explored in the literature, even considering the amount of DC concentration on wastewater. Figure 6.6A shows DC degradation after 11 days of phycoremediation. In the presence of microalgae biomass, there was higher DC removal (91%) when compared to the negative control (72.2%) (Figure 6.5B) ($p<0.05$). Yuan et al. (2011) reported a fast removal of doxycycline in water in the presence of UV light; however, the authors suggest a biological post-treatment, since oxidation via UV is not necessarily followed by total mineralization of doxycycline.

Doxycycline, at 0.33 mg L^{-1}, caused a 50% toxicity to *Pseudokirchneriella subcapitata* growth (FU et al., 2017). On the other hand, 22 mg L^{-1} of it caused a 50% growth inhibition in *Tetraselmis chuii*, and in the presence of microplastic, sensitivity to the antibiotic increased 100% (PRATA et al., 2018). For *Microcystis aeruginosa*, DC at 1 mg L^{-1} caused a 55% growth inhibition (WU et al., 2014). However, in this study, no growth inhibition was observed at a concentration of 1 mg L^{-1} of DC when compared to

the positive control (Figure 6.3), as described in section 6.3.1. These results highlighted the difference on removal efficiency of different microalgae species.

Wu et al (2014) reported that growth inhibition of *Microcystis aeruginosa* exposed to DC could be related to the increase of oxidative stress affecting the production of antioxidant enzymes (mainly through catalase), the lipid peroxidation and the 1.8-fold increase in the production of malondialdehyde (peroxidation product of fatty acids) (WU et al., 2014).

6.3.3 Biochemical composition of biomass from phycoremediation and theoretical bioenergy production

The lipid, protein and carbohydrate content determined in the microalgae biomass harvested after swine wastewater phycoremediation exposed to different TCs is shown at Fig 4c. Significant difference in carbohydrate content was observed as a function of TCs investigated when compared to the positive control (Figure 6.7). The biomass cultivated in the TC, CT, OT and DC presence showed carbohydrate contents of 52.7±8.1, 50.1±3.3, 51.4±5.4 and 57.4±10.4 (%), respectively. The microalgae biomass harvested from the reactor with OT and CT had significantly higher ($p<0.05$) lipid (5.1±1.3%) and mineral matter (8.9±1.9%) content than the positive control (Fig 4c). Protein content did not present significant difference after phycoremediation. Many studies have reported that *Chlorella* spp. has a high carbohydrate content, ranging from 36% to 55% of its dry weight (CHENG et al., 2013; HO et al., 2013; MICHELON et al., 2019).

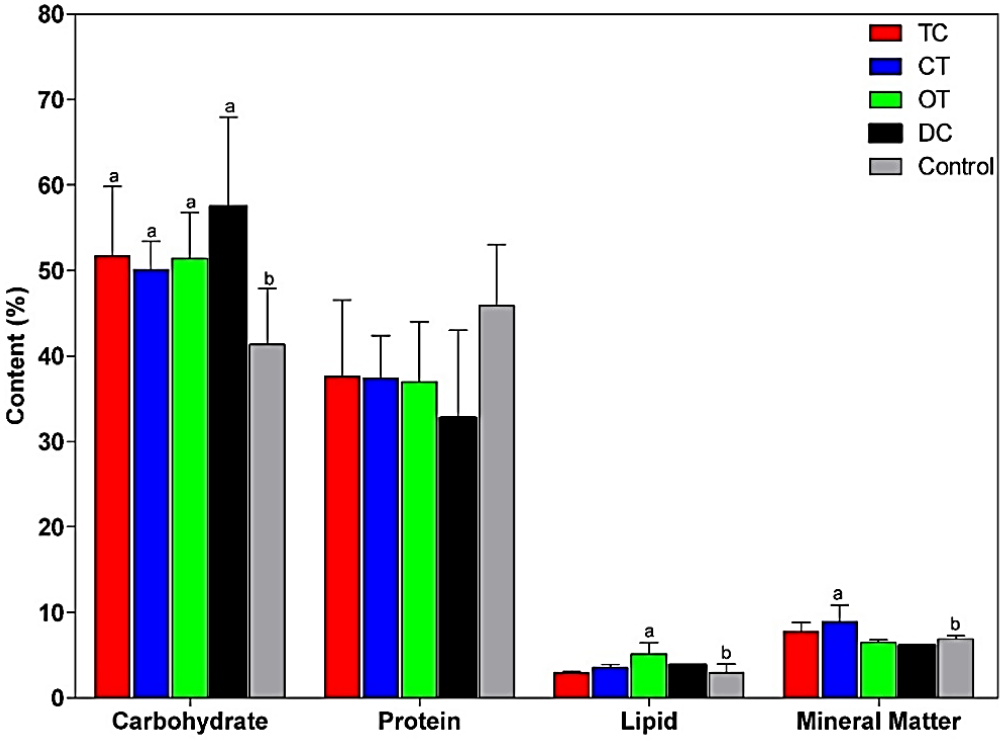


Figure 6.7. Biochemical cellular composition of microalgae cultured in swine wastewater with tetracycline (TC), chlortetracycline (CT), oxytetracycline (OT) and doxycycline (DC). Bars depict standard deviation of the mean, and different letters denote significant differences ($p \leq 0.05$) according to Tukey test.

Recovered microalgae biomass from swine wastewater phycoremediation may contain residual antibiotics, raising concerns about the safety of its use (MARKOU et al., 2018). However, obtaining a large amount of carbohydrate-rich microalgae biomass, it can be used as feedstock for bioethanol production at a low-cost (HO et al., 2013) and/or applied to biomethane production (PERAZZOLI et al., 2016), an approach of particular interest in the context of circular economy. For instance, a production of swine wastewater estimated by Michelon et al. (2019) treated by microalgae-based process could lead to microalgae biomass yield of approximately 46.5 ton ha^{-1} $year^{-1}$, considering a yield of 324 L bioethanol ton $biomass^{-1}$ [with an average of 50% of carbohydrate content (SILVA; BERTUCCO, 2019)], would result in an estimated production of 15,051 L bioethanol ha^{-1} $year^{-1}$. Alternatively, using the biomass to produce biomethane could lead to a total of 2,044,000 L_N biomethane ha^{-1} $year^{-1}$ [estimated using 44±2.5 L-CH_4 (kg biomass)$^{-1}$, according to values obtained by Perazzoli et al. (2016)]. In this case, bioenergy production can be improved considering the integration of substrates (i.e microalgae and swine manure) and microalgae cellular composition changes (MICHELON et al., 2015; PERAZZOLI et al., 2016).

In general, phycoremediation from swine wastewater using microalgae consortium including *Chlorella* spp. can be used for veterinary antibiotic removal, since the concentration found in wastewater (about 1 mg L^{-1}) does not cause growth inhibition or increased hydraulic retention time (around 11 days).

6.4 CONCLUSIONS

Microalgae-based processes are a useful tool for the removal of tetracyclines from swine wastewater. Tetracycline was much more efficiently removed than chlortetracycline, oxytetracycline and doxycycline. The fast 4-epi-iso-chlortetracycline and/or iso-chlortetracycline formation occurred concomitantly with the chlortetracycline decrease. The microalgae biomass harvested after tetracyclines removal presented a carbohydrate-rich content (≥50%), indicating that the treatment of swine wastewater, containing antibiotic residues, using microalgae-based processes could be an environmental-friendly alternative to providing feedstock for bioethanol and biomethane production.

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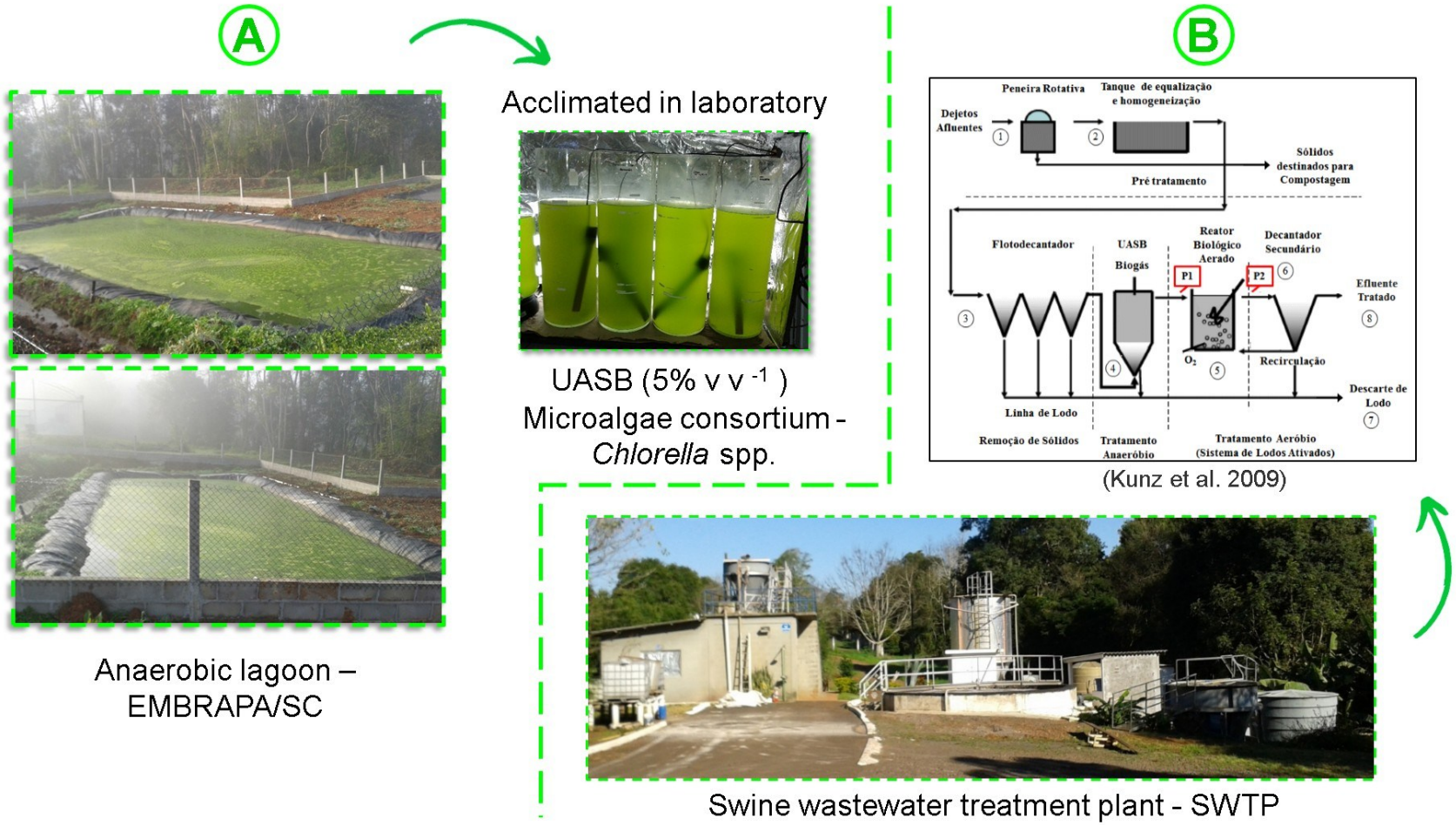
OVERVIEW

Wastewater treatment using microalgae has been reported since the 1978s; however this process has not yet been expanded to a large-scale. Since the production of microalgae still has some limitations such as water footprint, contamination by microalgae predators and other species of microorganisms, but possibly the most critical bottleneck is the economic viability associated with high nutrient costs. In this way, mixotrophic cultivation (mainly in open systems), with good tolerance to environmental changes and growth capacity in wastewater, is an essential criterion to achieve maximum biomass and byproducts production. In this context, the present study showed the successful isolation and use of microalgae consortium from environments such as wastewater, shows the advantage of these organisms being already selected by natural conditions of these matrices.

To solve the high cost related to nutrients, we reported the use of swine wastewater, which after anaerobic digestion treatment, rich in nutrients such as nitrogen and phosphorus, is a suitable medium for cultivation microalgae, and simultaneously remove these and other compounds from the wastewater. Additionally, in the use of microalgae consortium, associated or not with bacteria to treat wastewater, has the advantage not be necessary to sterilize the wastewater before its use, which is an important step to consider in a large-scale application.

Overall, phycoremediation was an effective tertiary treatment approach for the removal of nutrients and residual veterinary antibiotics from swine wastewater. The biomass concomitantly produced in the bioprocess contains valuable metabolites worth exploring as feedstock for numerous industry applications from nutraceuticals to biofuels. Wastewater engineering operations, such as controlling the loading of inputs and reactor hydraulic retention time, can be used to interfere in the biochemical composition of the microalgae biomass produced to improve the specific metabolites that best meet the interests and demands of the market.

Supplementary material I



Microalgae inoculum was obtained from an anaerobic lagoon and acclimated in a photobioreactor on a lab scale (A). Swine wastewater was obtained from a swine wastewater treatment plant (B).

Supplementary material II –

Ions spectra of metabolites obtained by UHPL-MS/MS from the extract of microalgae biomass obtained under different solvents.

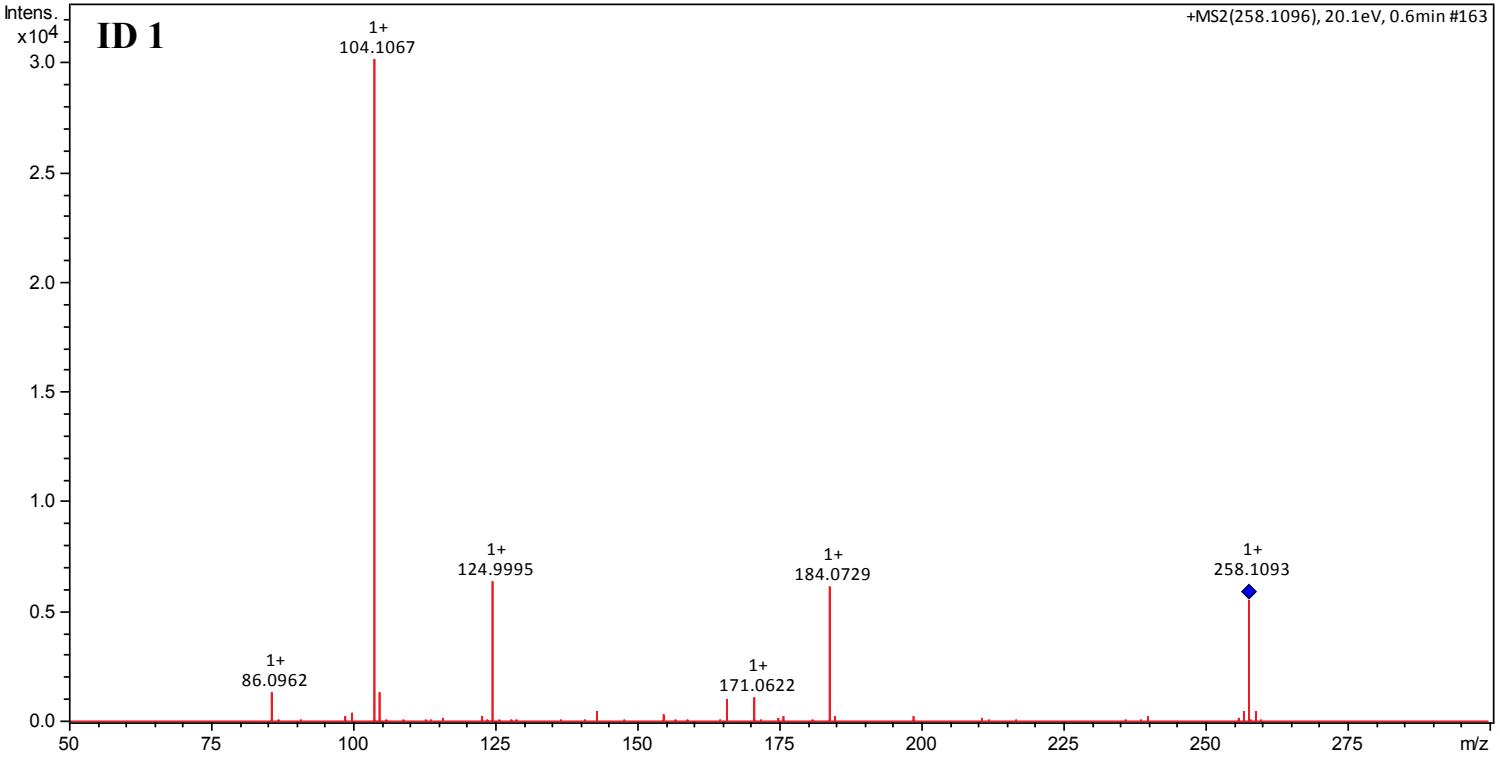


Fig S1 Glycerophosphocholine ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane and methanol.

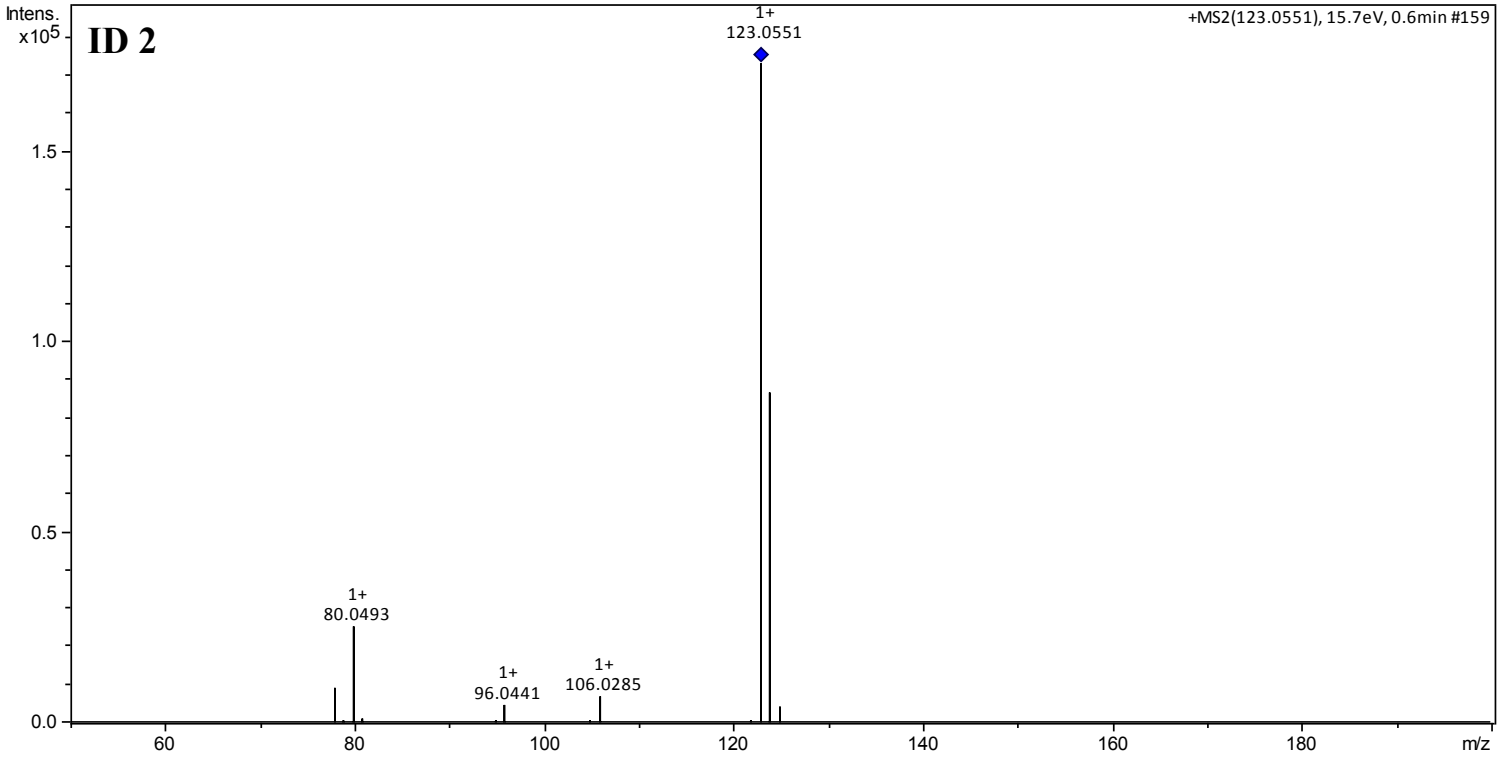


Fig S2 Niacinamide ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane and methanol.

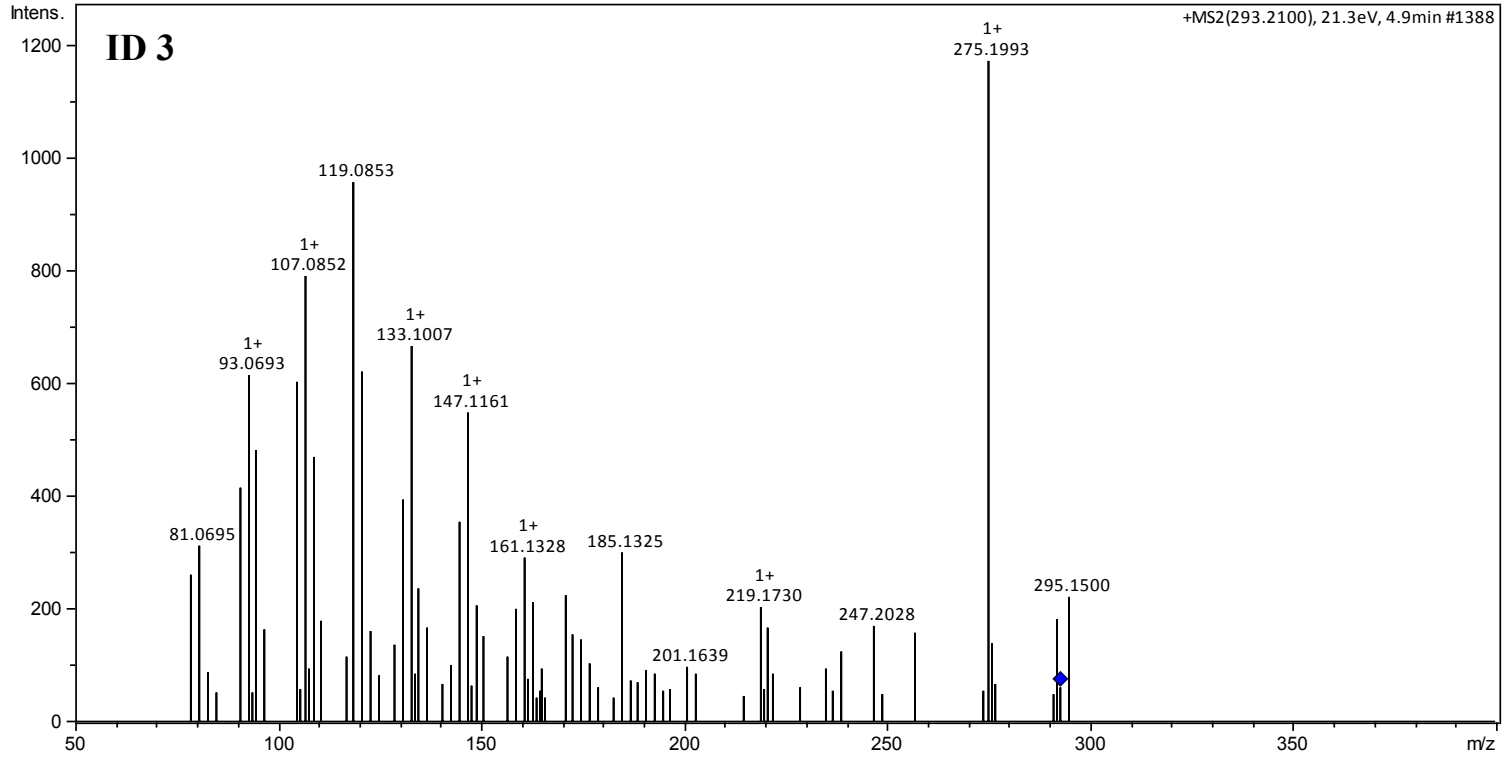


Fig S3 13(S)-HpOTrE ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane and methanol.

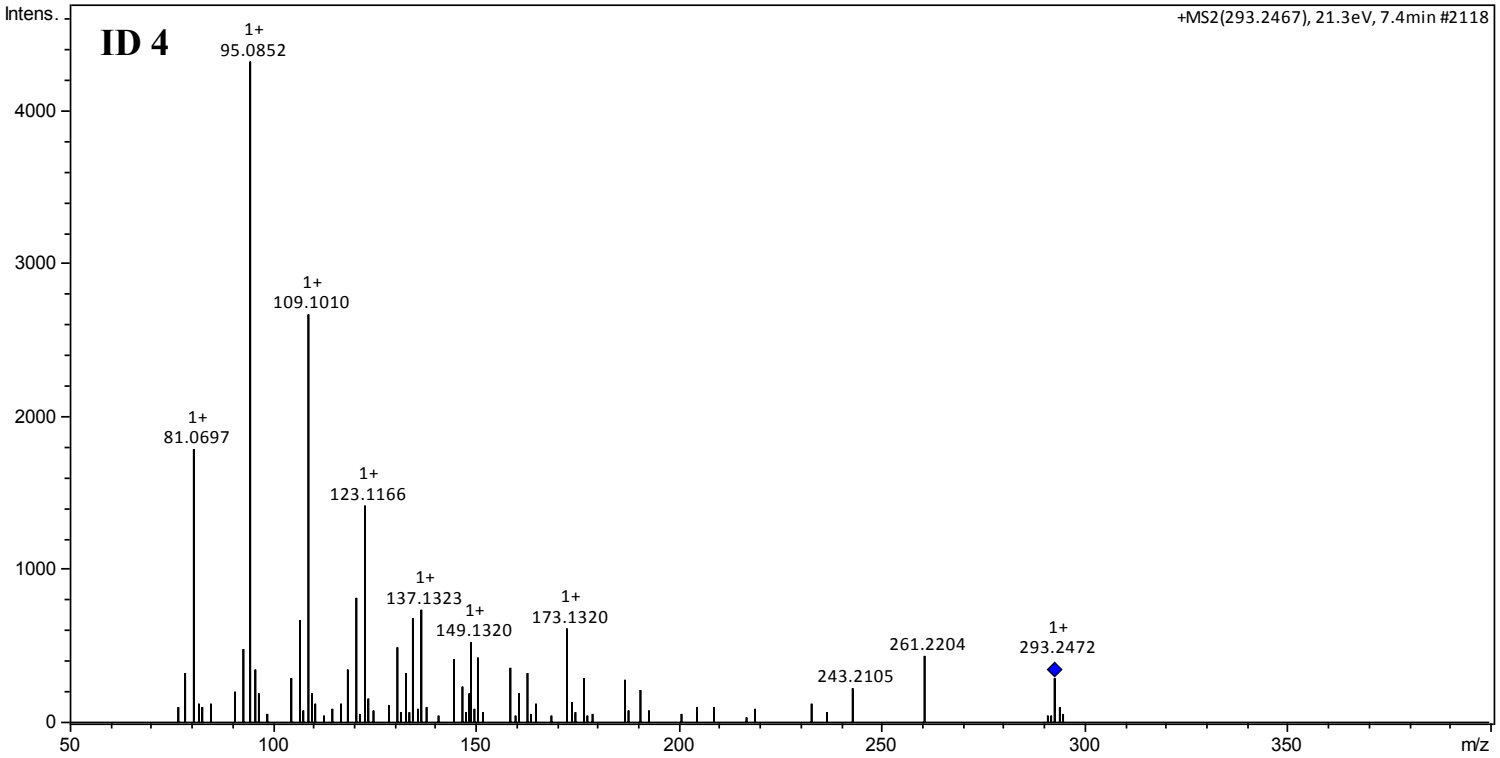


Fig S4 Methyl linolenate ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane and methanol.

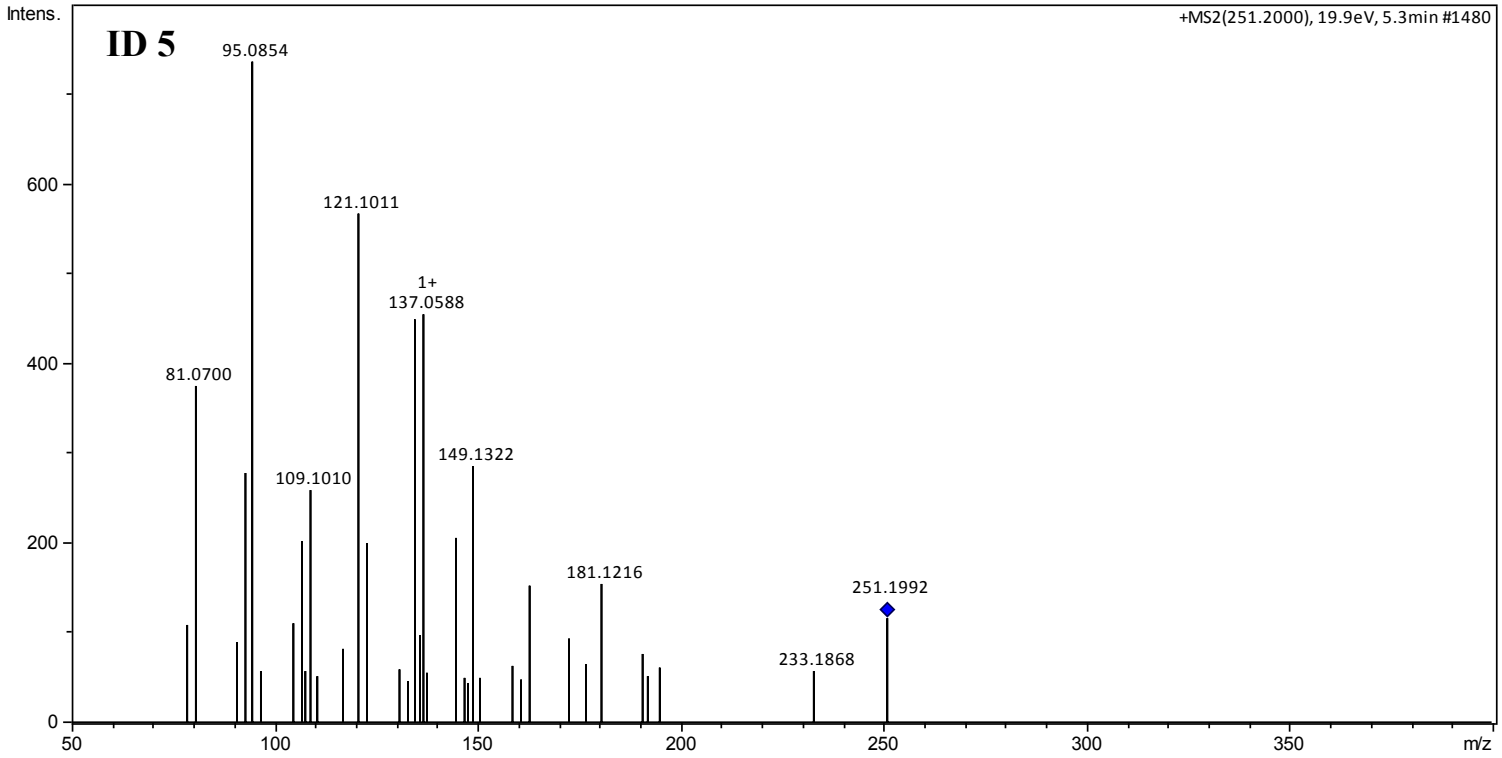


Fig S5 Sclareolide ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane.

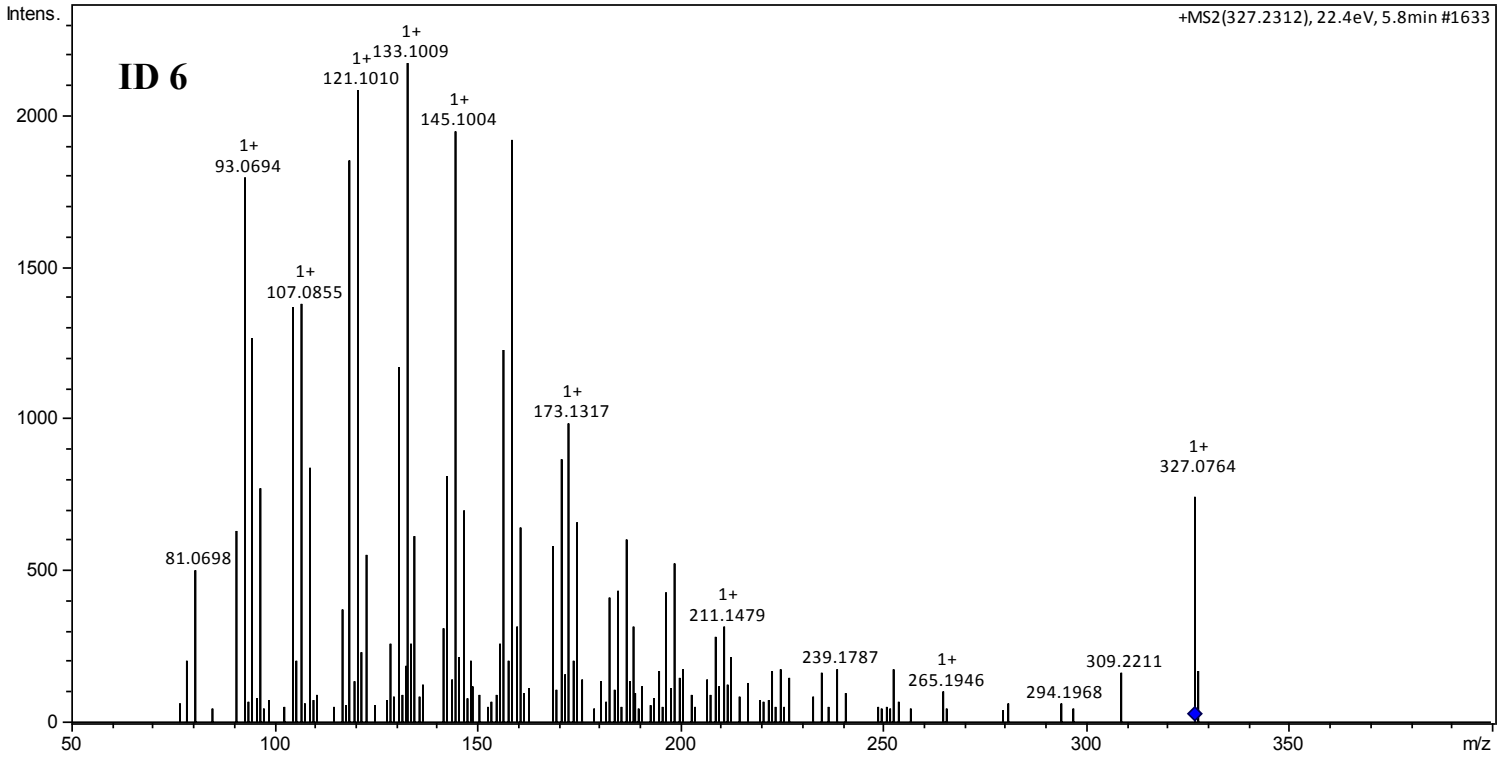


Fig S6 14-HDoHE ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane.

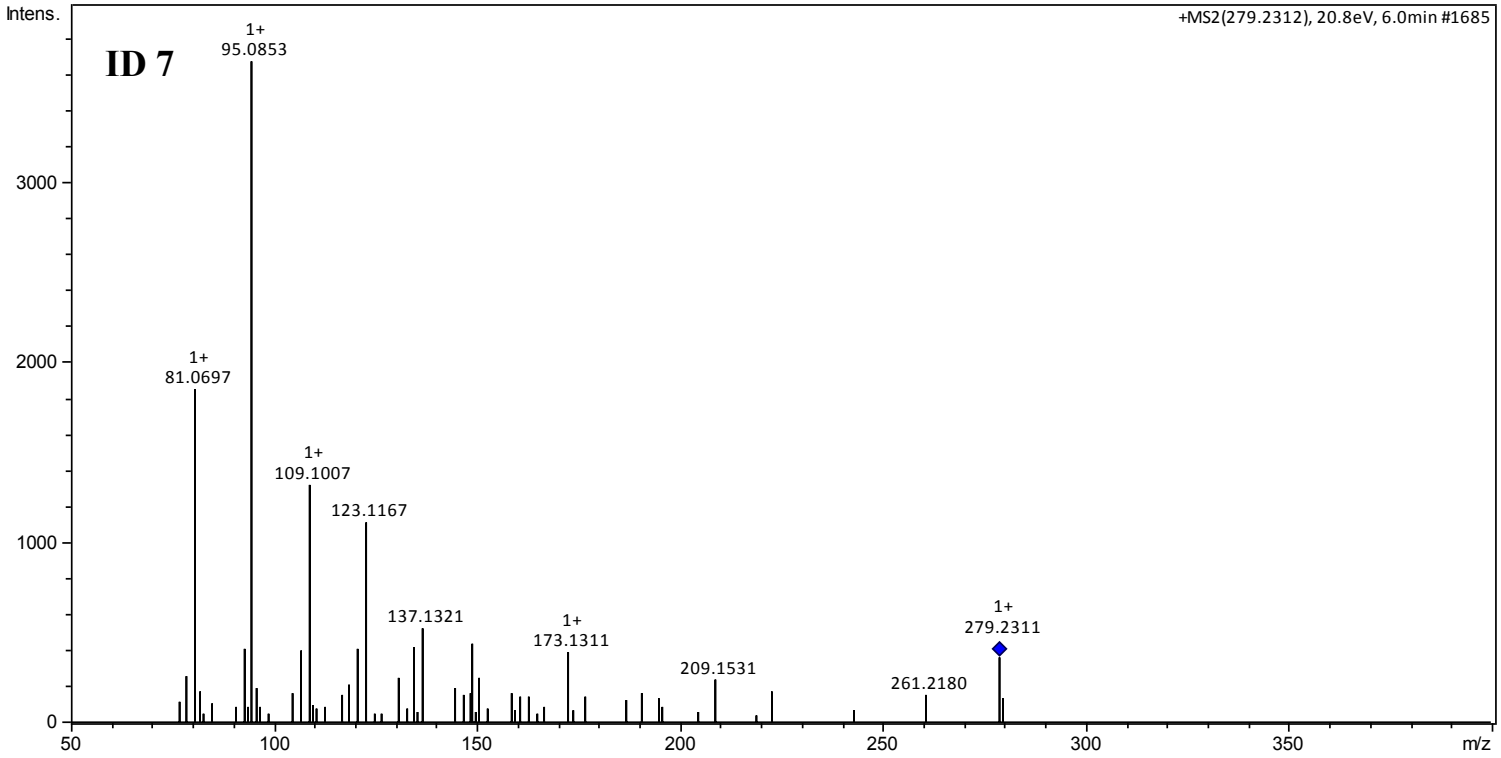


Fig S7 9(S)-HODE ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane.

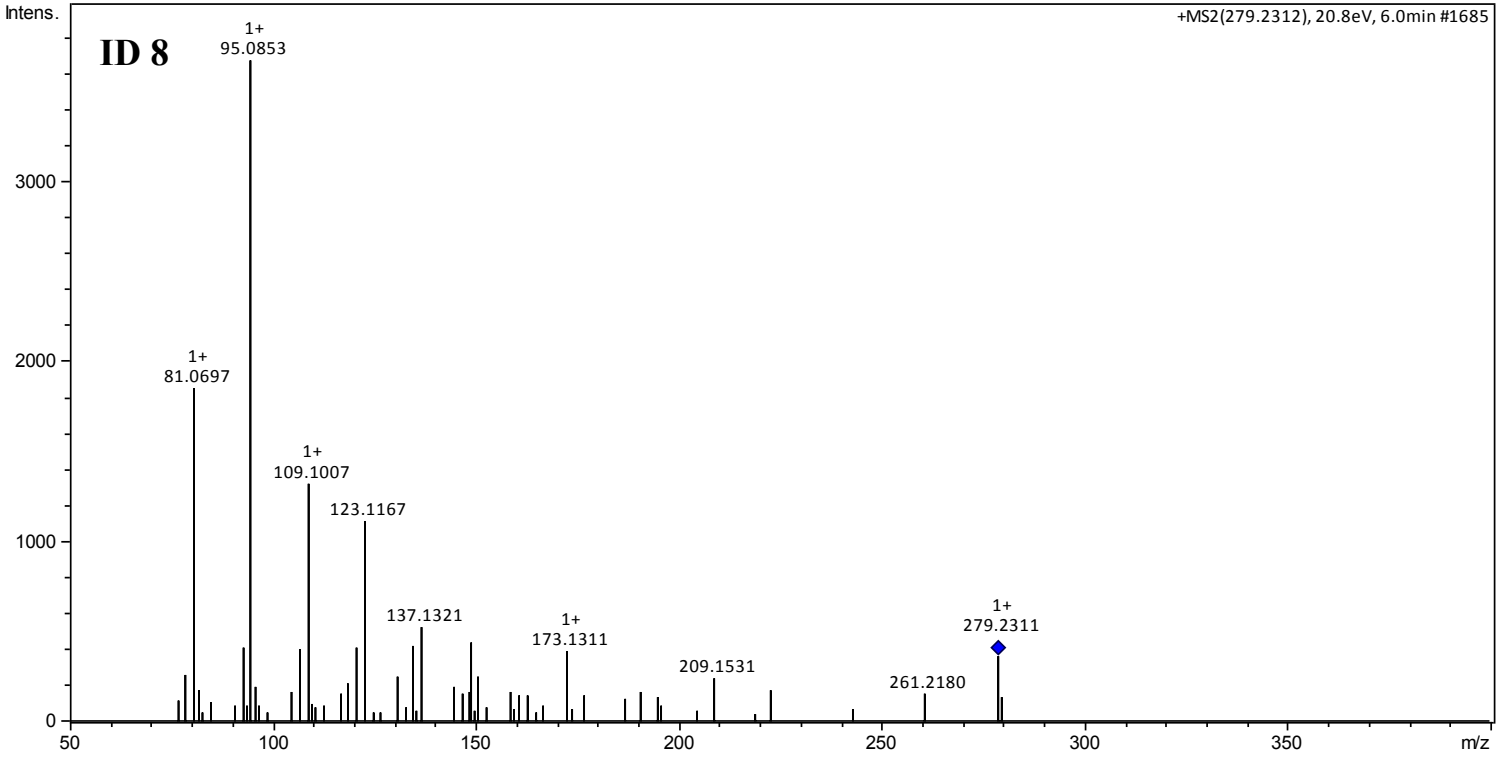


Fig S8 Pinolenic acid ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane.

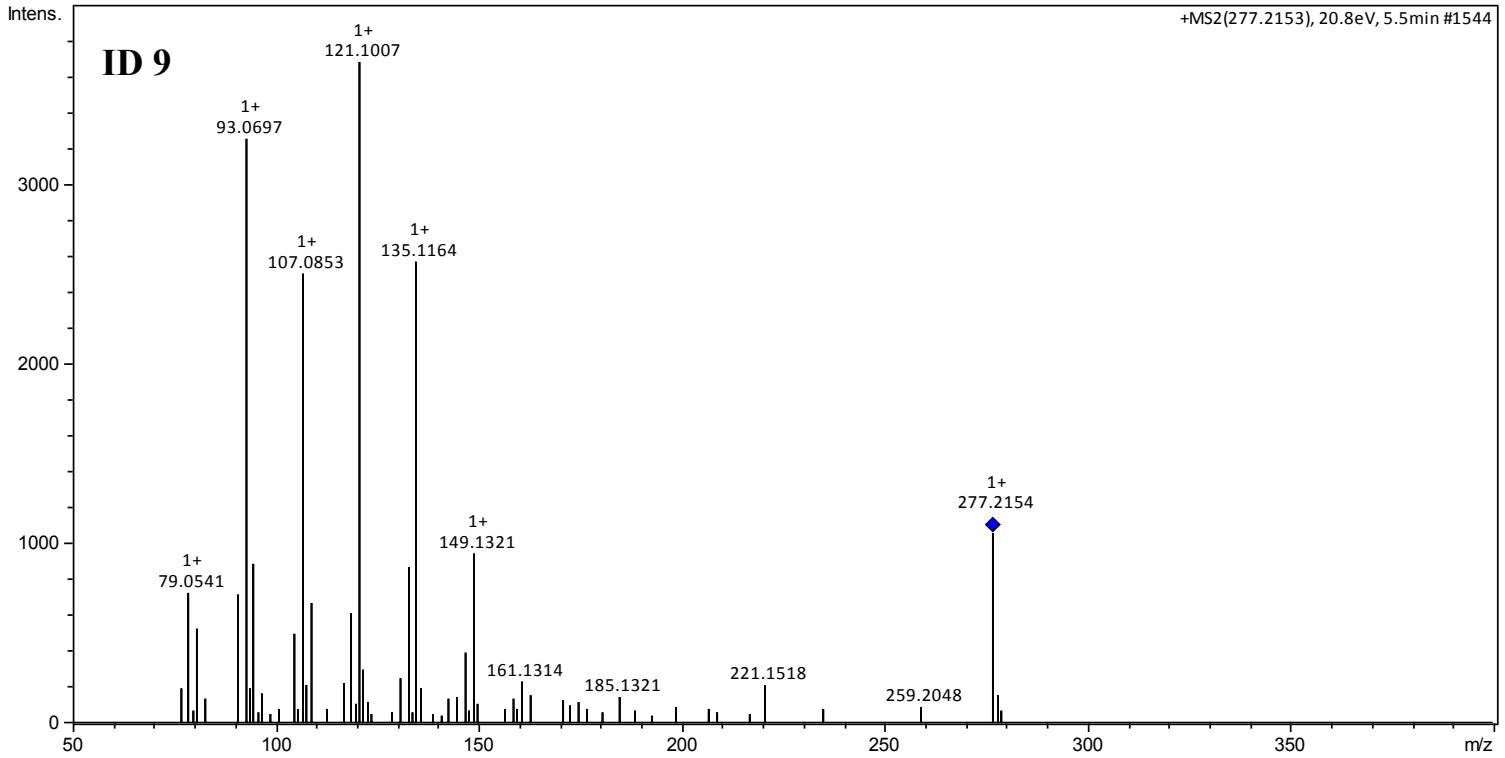


Fig S9 Stearidonic acid ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted dichloromethane.

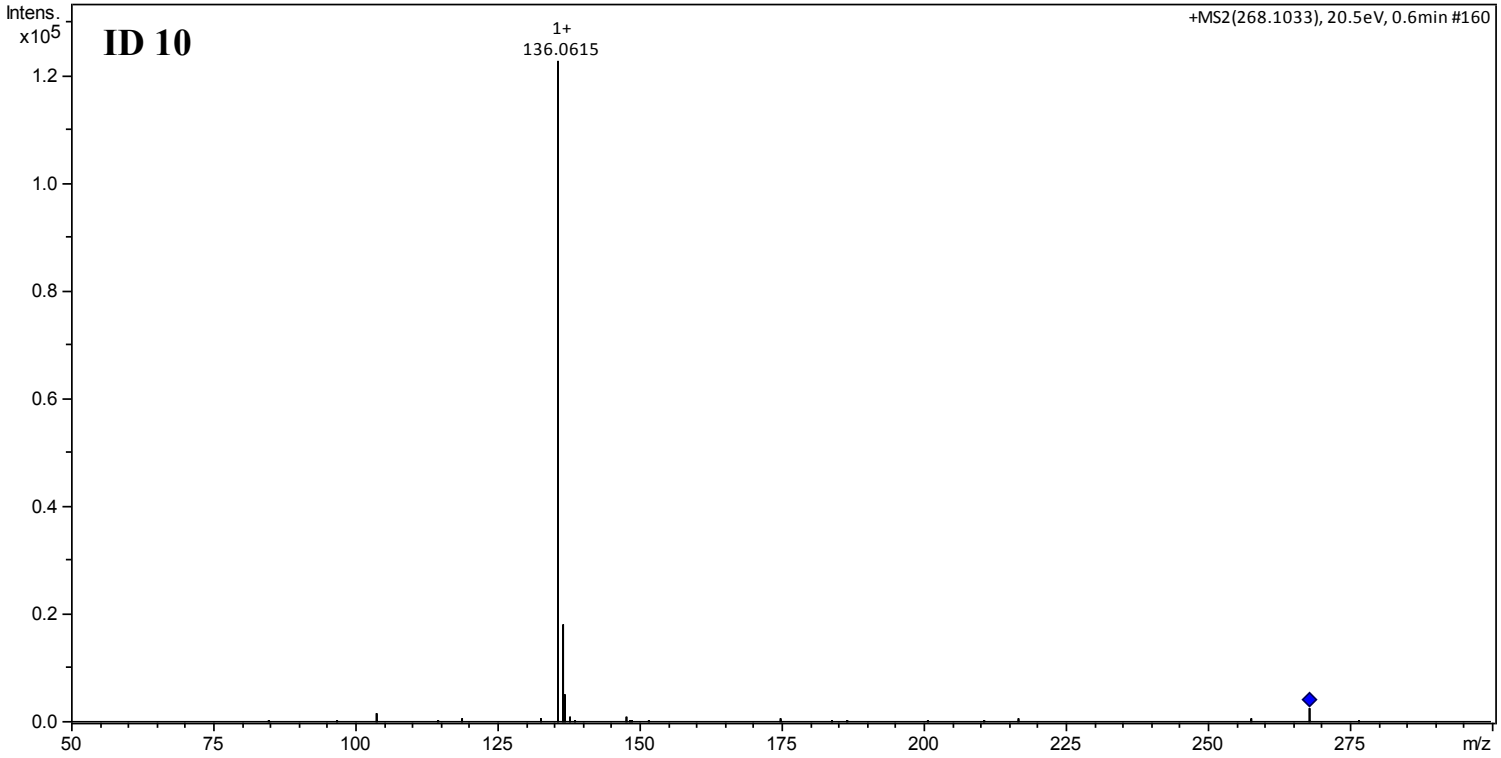


Fig S10 Adenosine ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

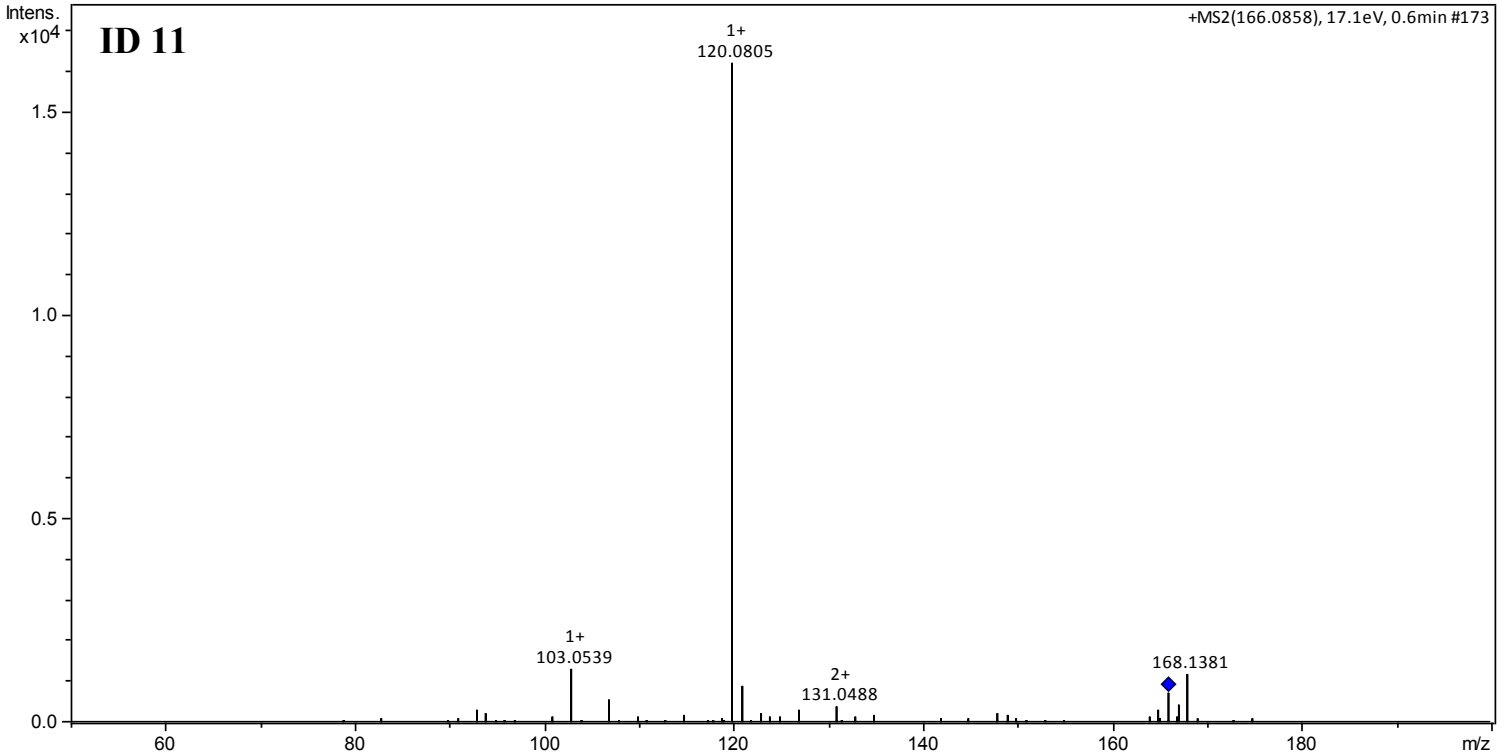


Fig S11 L-Phenylalanine ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

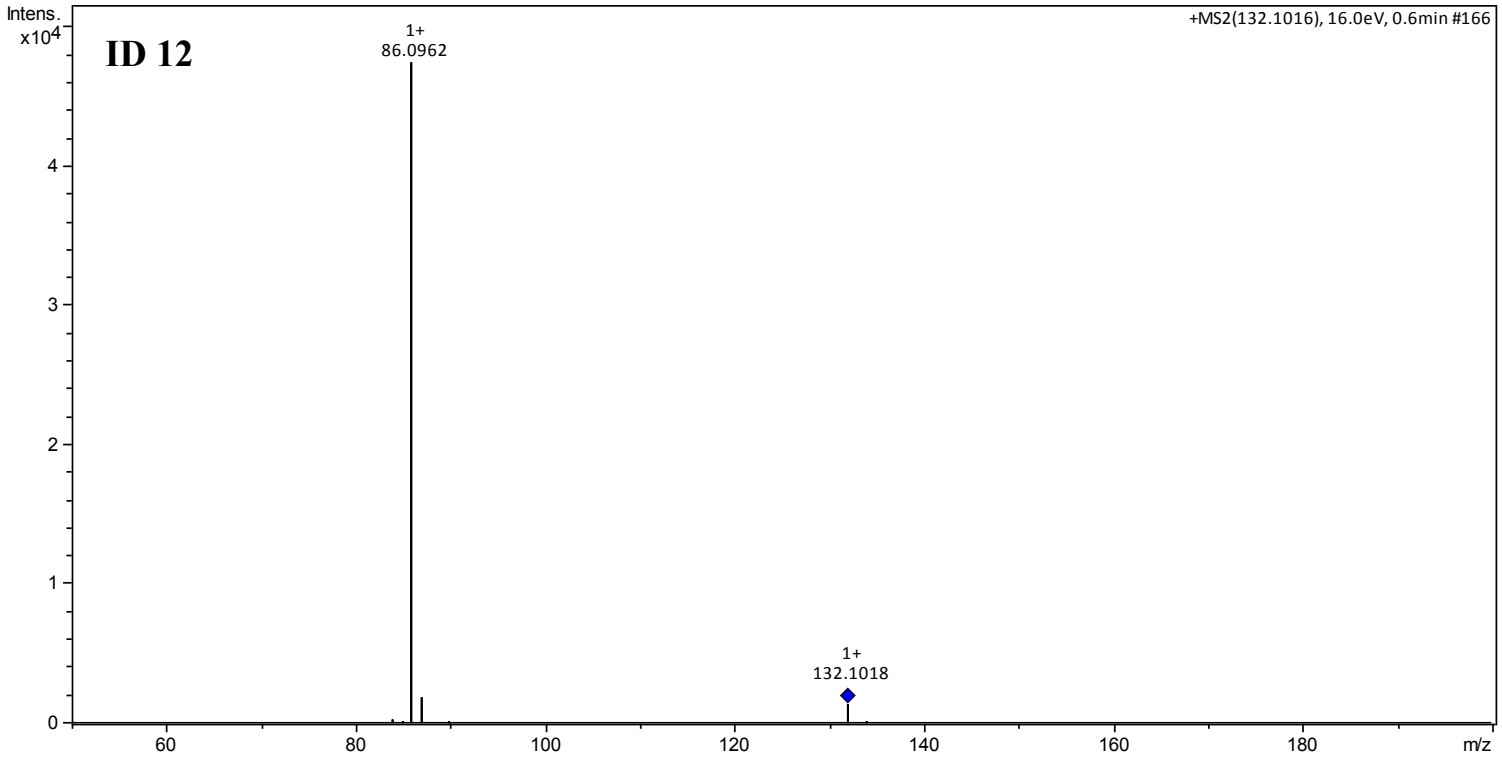


Fig S12 L-Leucine ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

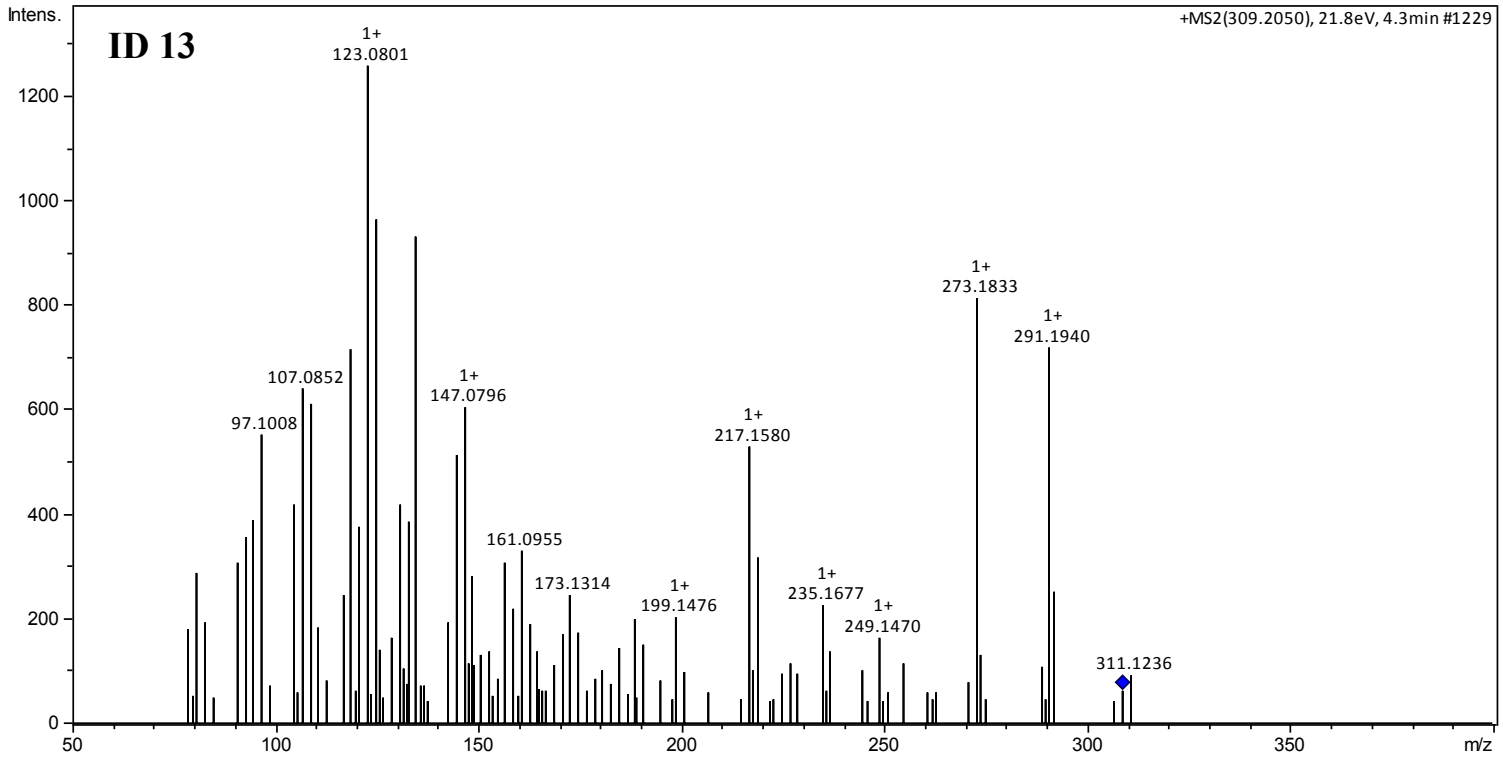


Fig S13 2,3-Dinor Prostaglandin E1 ion spectra obtained by UPLC-MS of microalgae biomass extracted methanol.

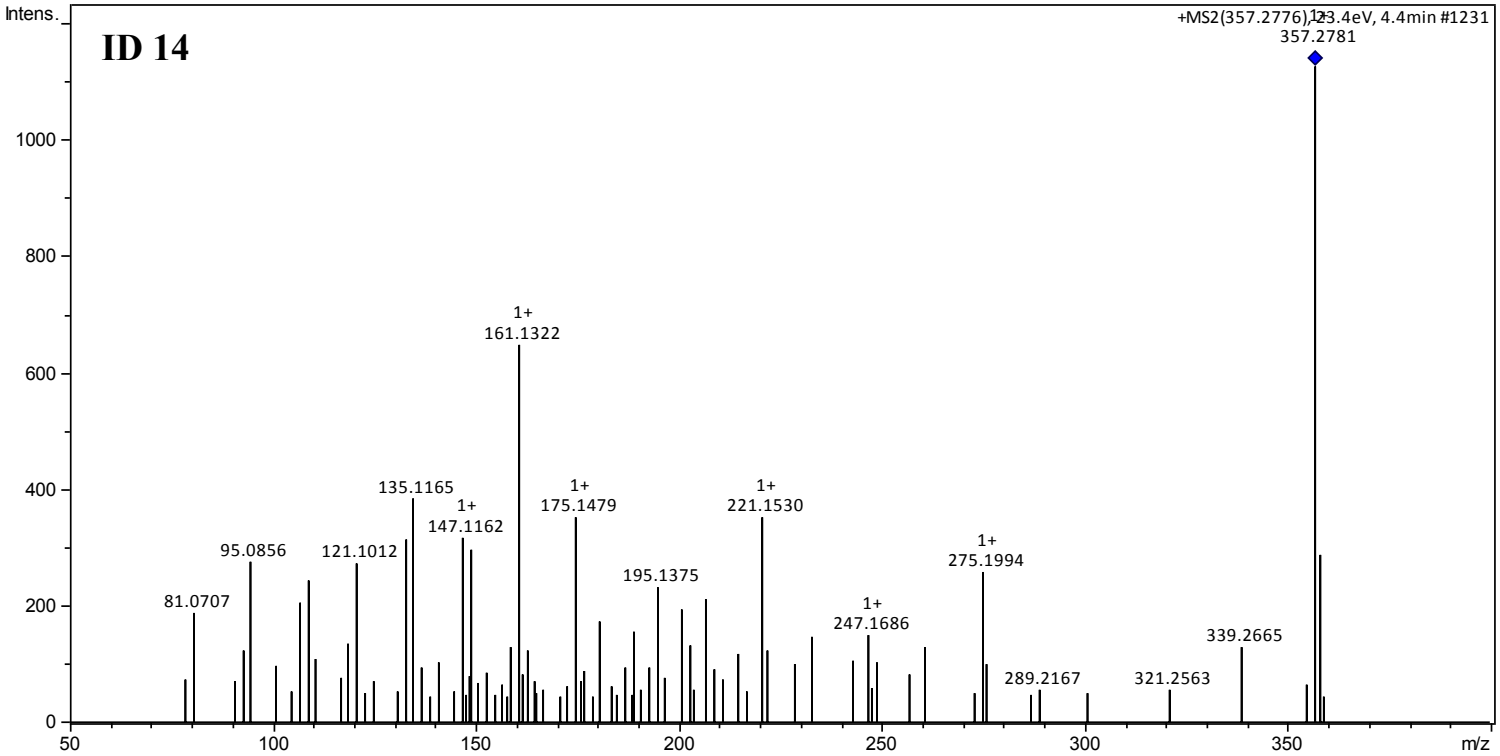


Fig S14 3b-Hydroxy-5-cholenoic acid ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

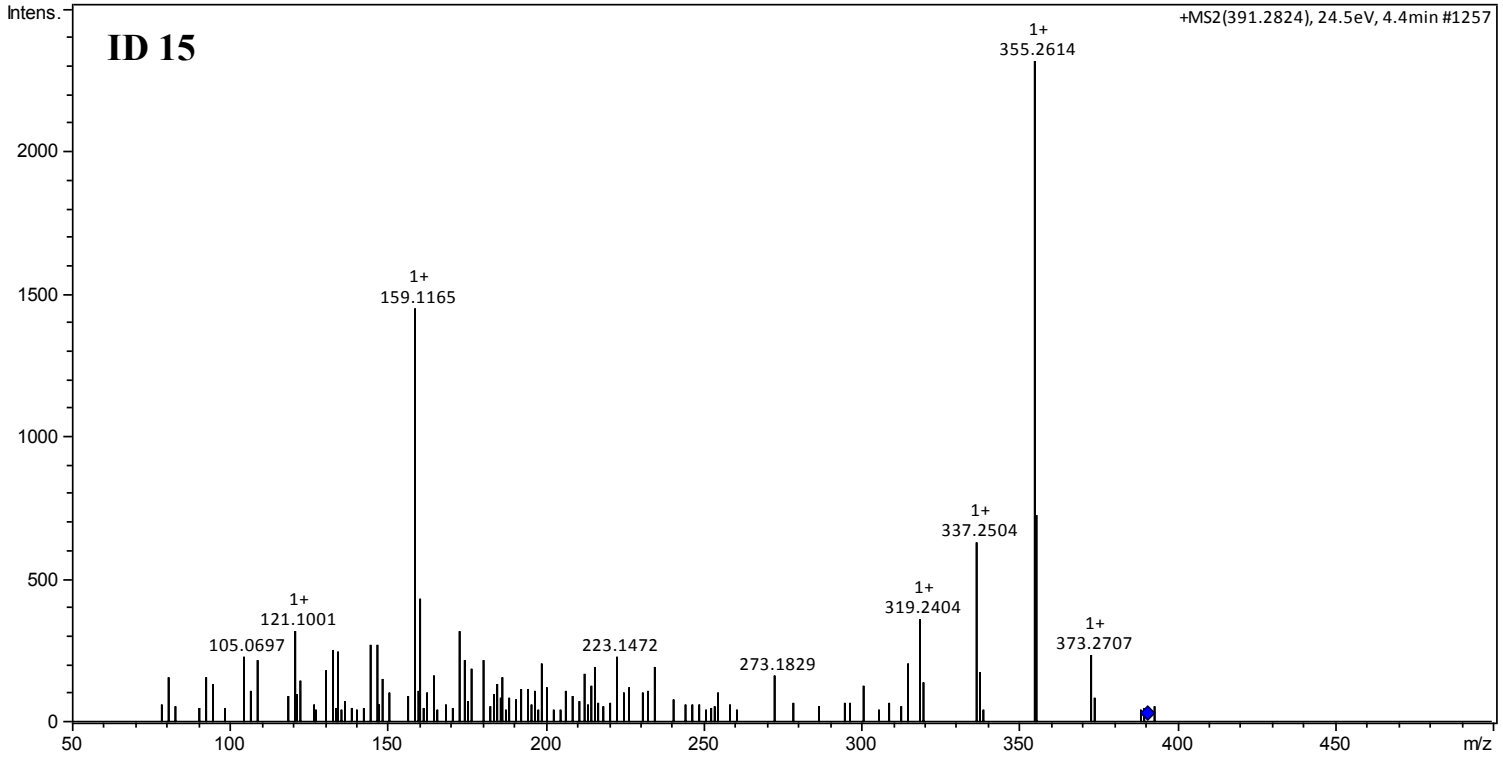


Fig S15 Nutriacholic acid ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

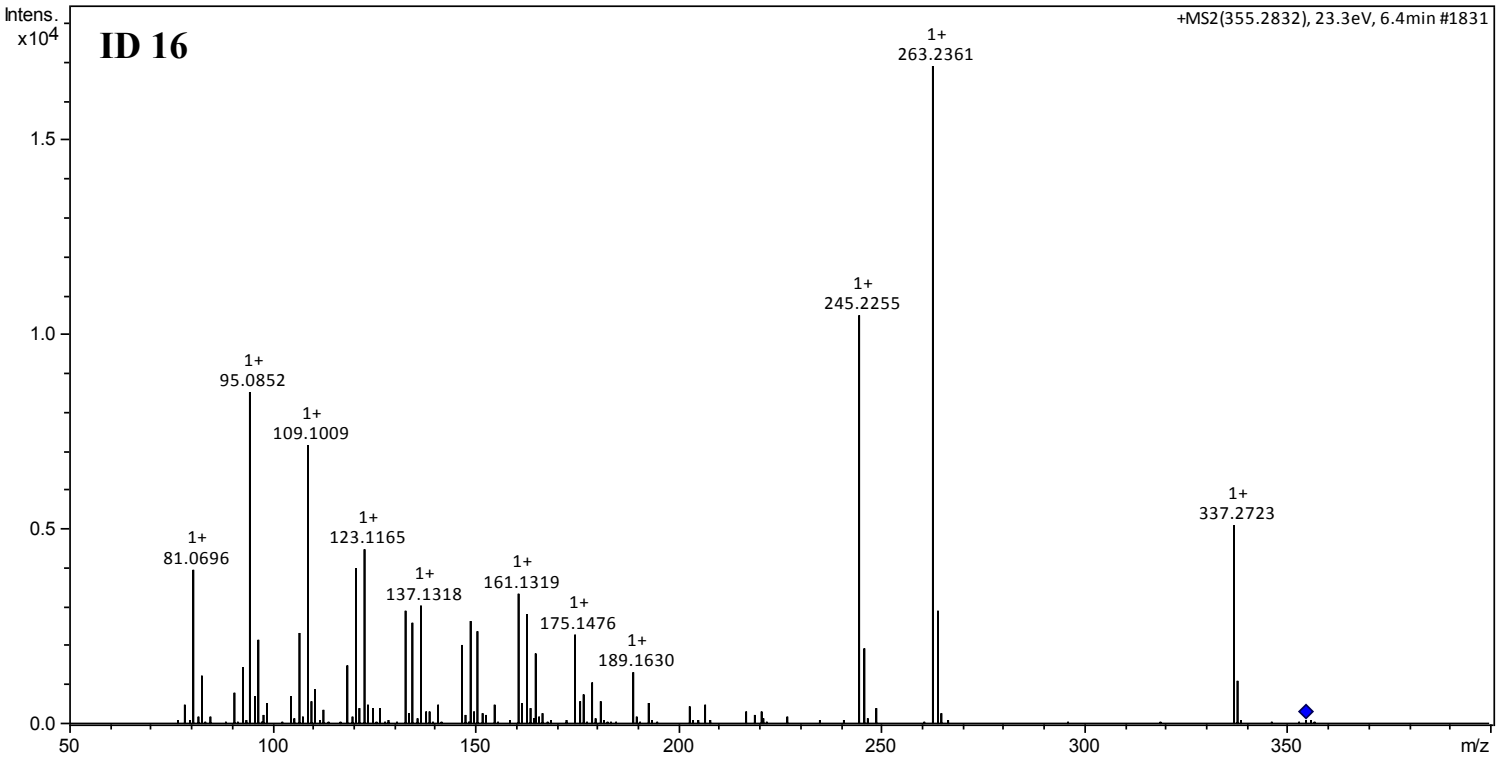


Fig S16 2-Linoleoyl glycerol ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

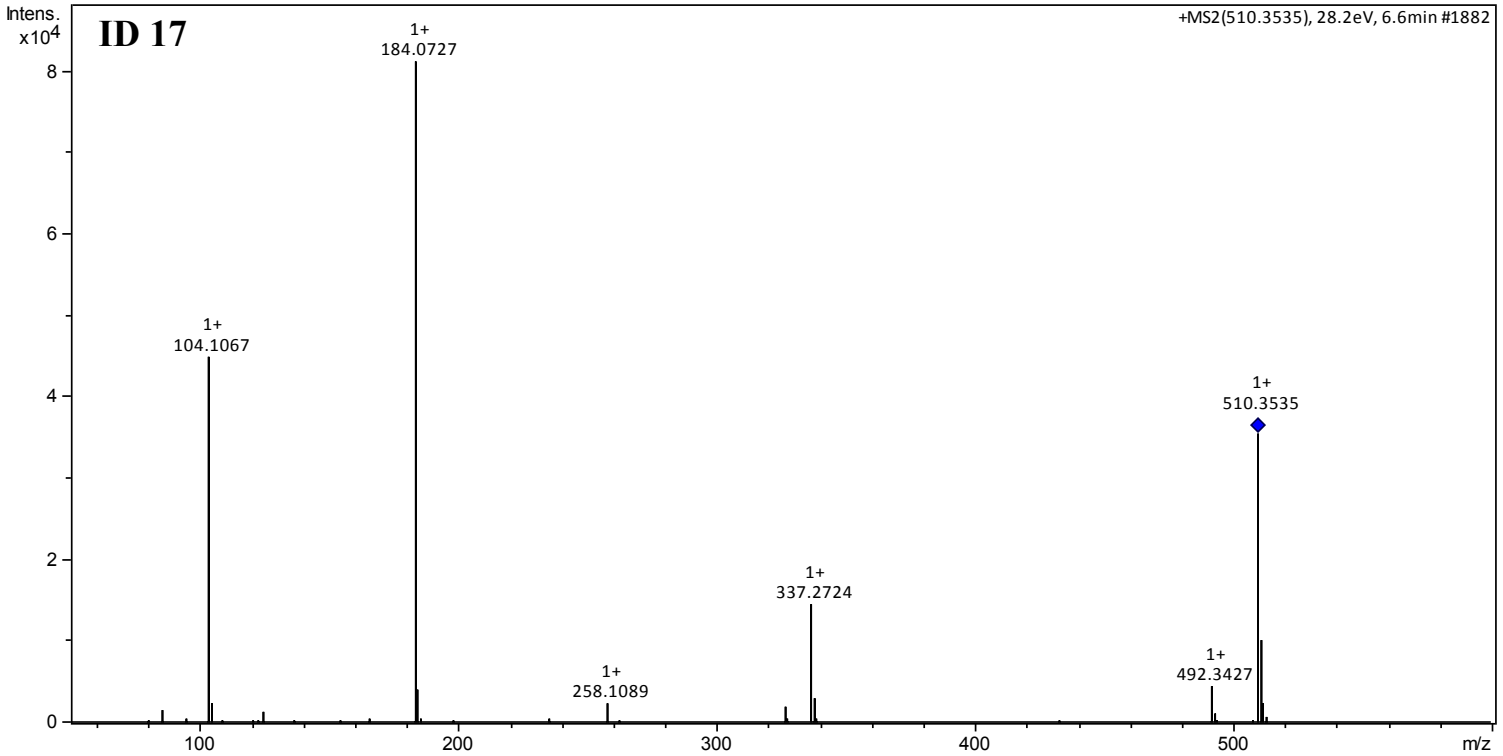


Fig S17 1-Heptadecanoyl-sn-glycero-3-phosphocholine ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

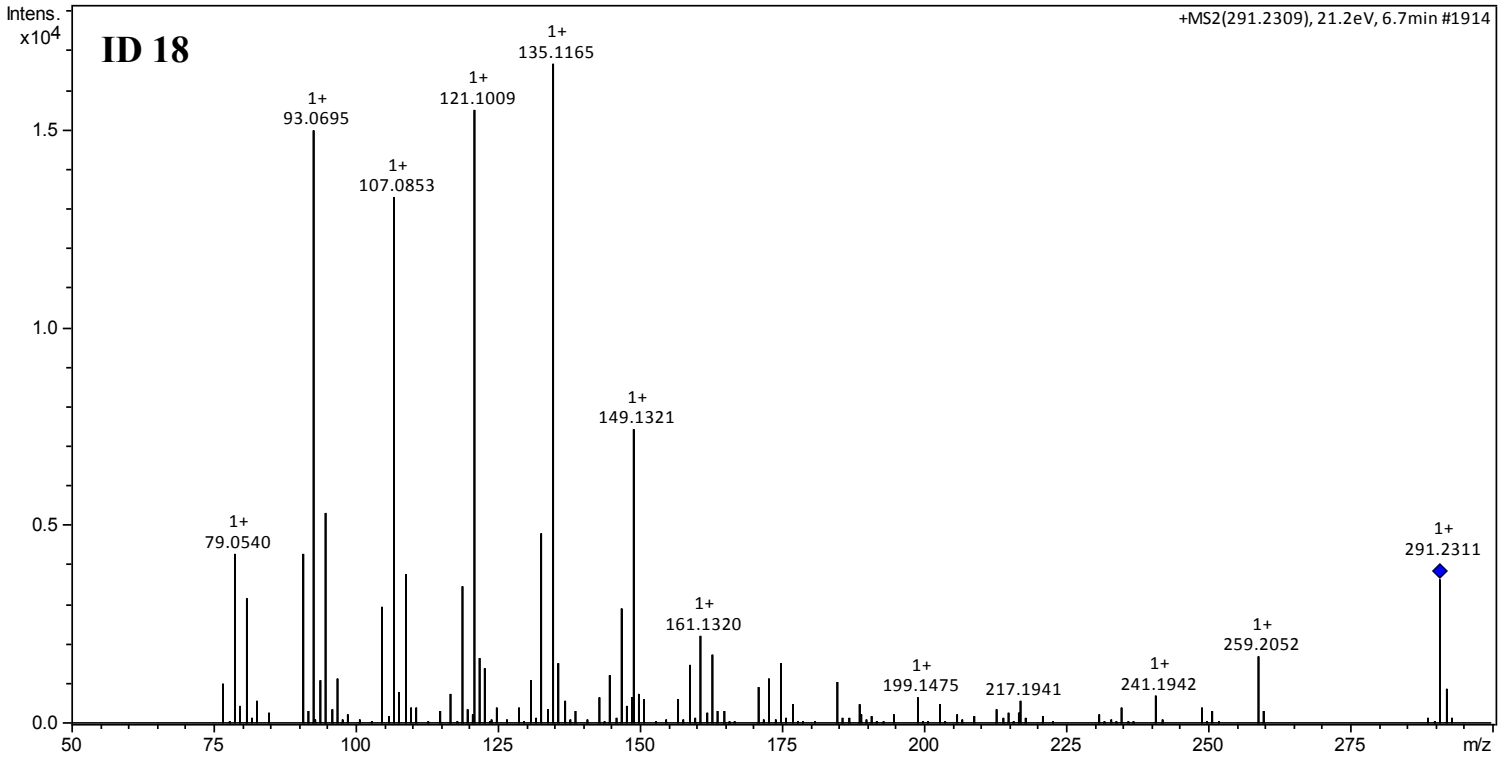


Fig S18 11beta-Hydroxy-5alpha-androstan-17-one ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

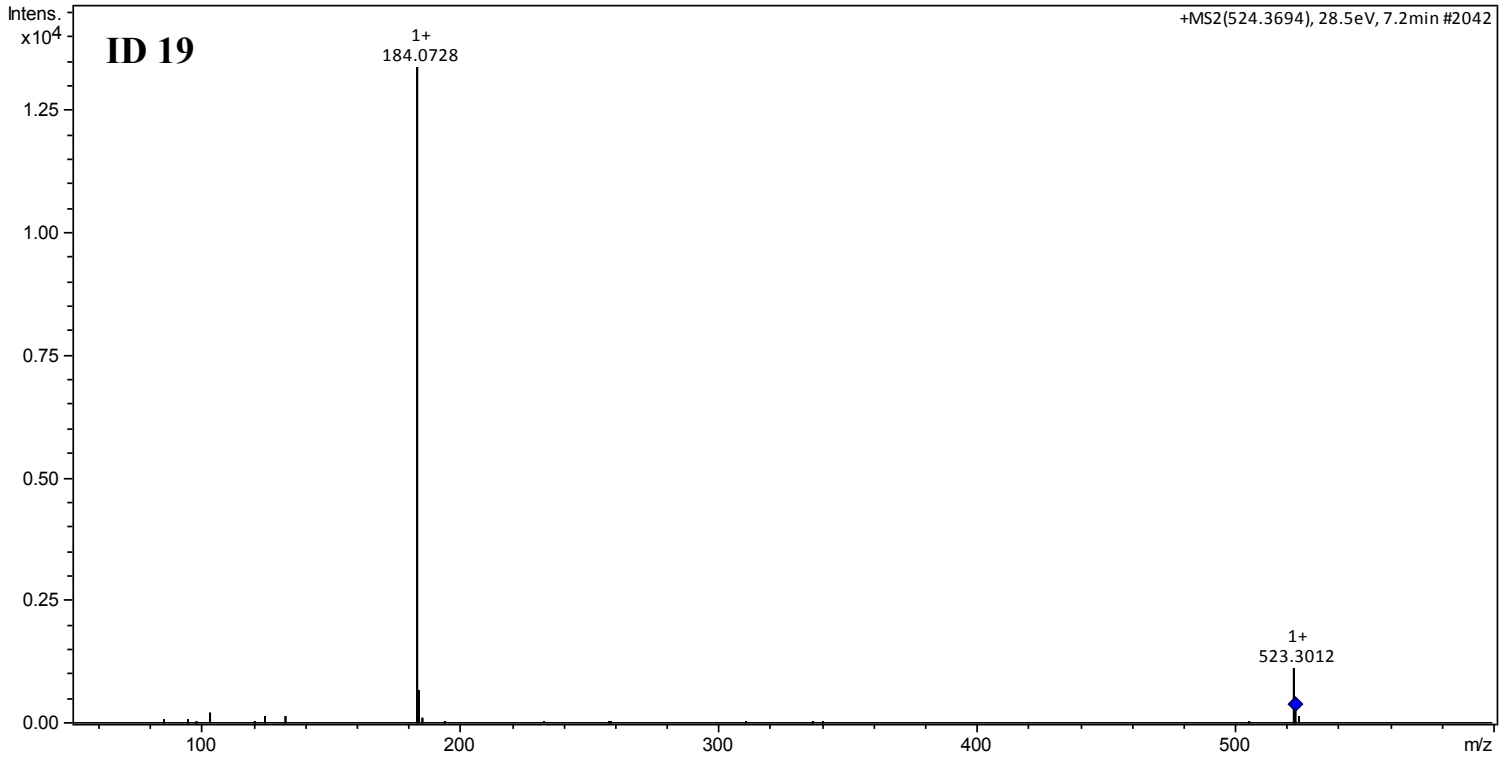


Fig S19 PAF (C16) ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

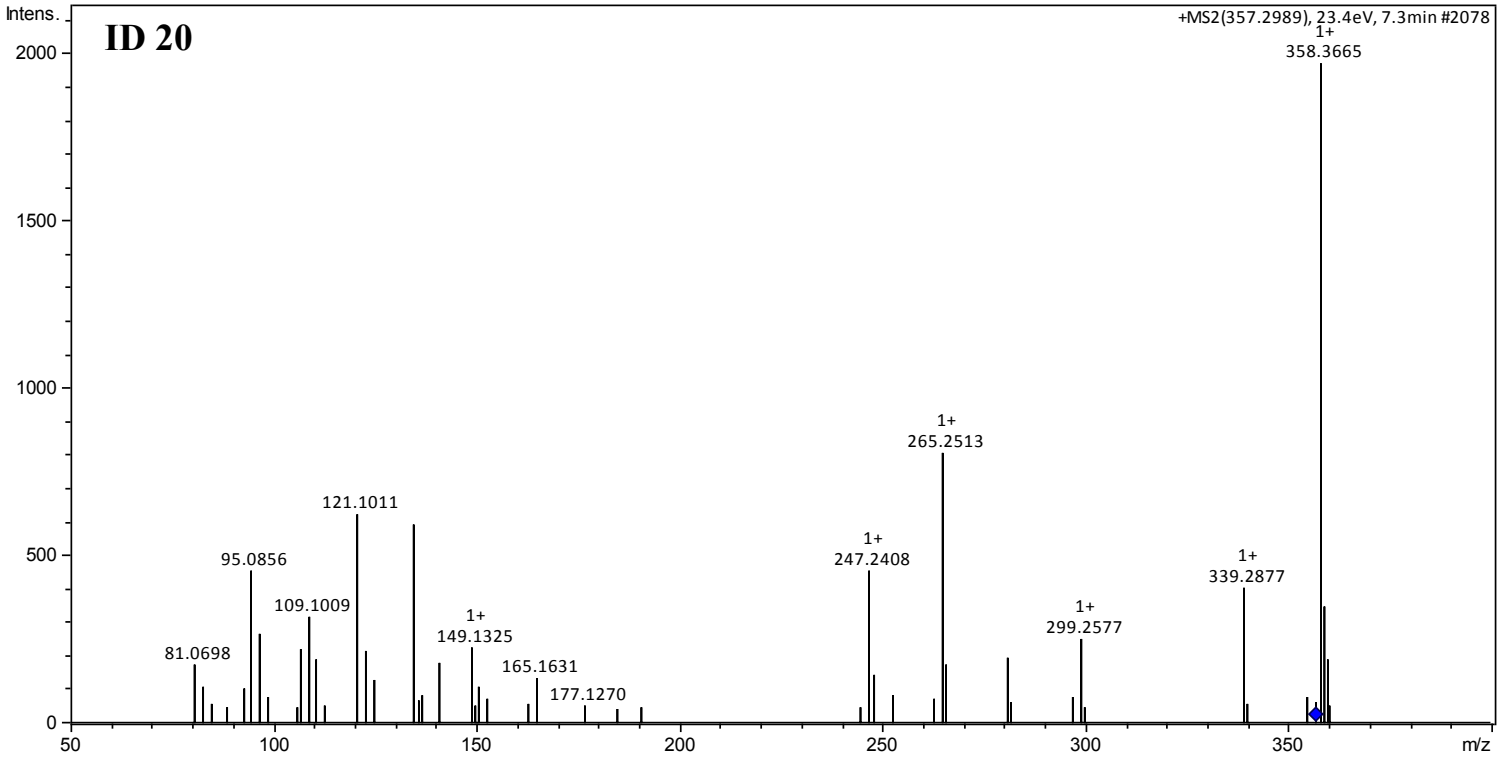


Fig S20 Glyceryl monooleate ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

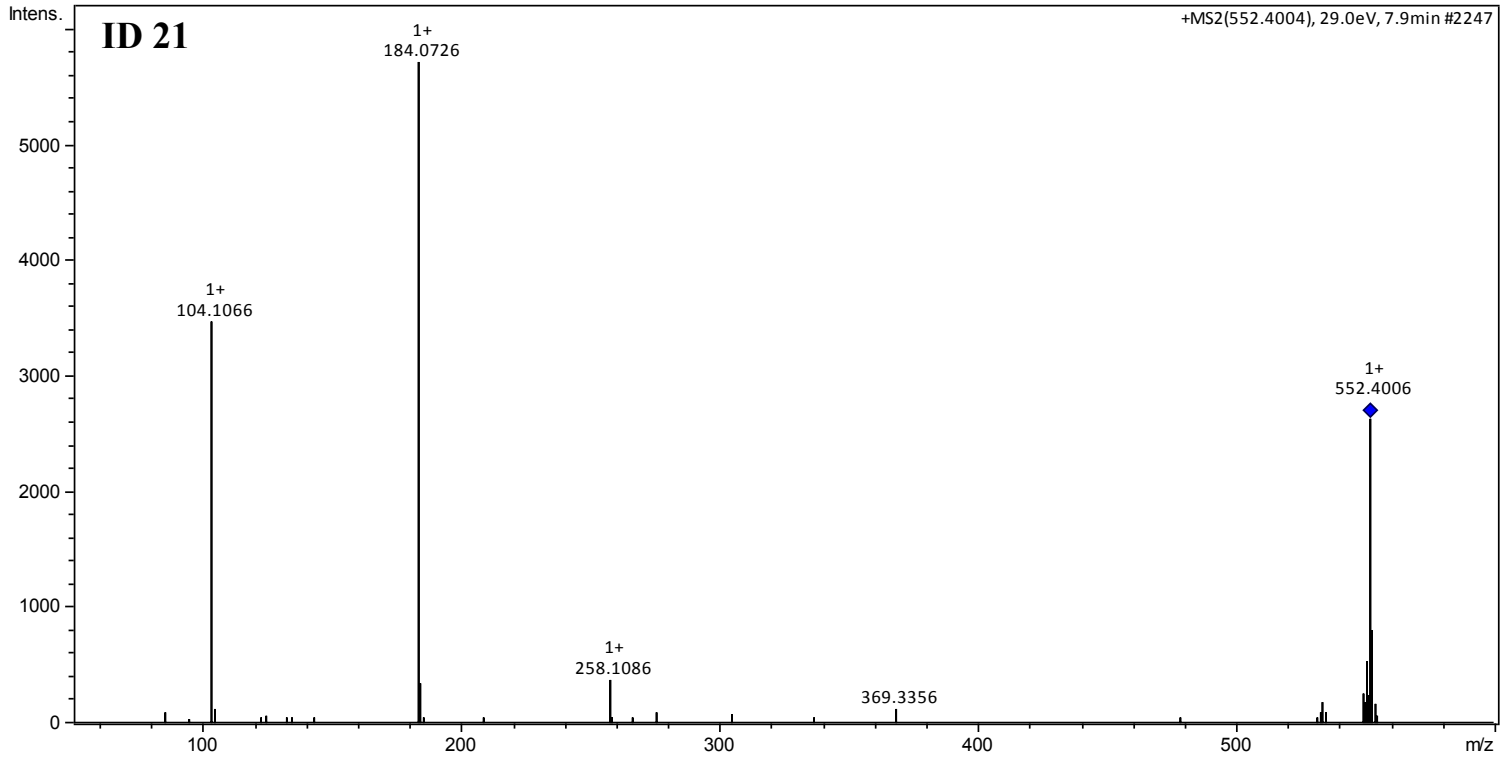


Fig S21 1-hexadecyl-2-butyryl-sn-glycero-3-phosphocholine ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

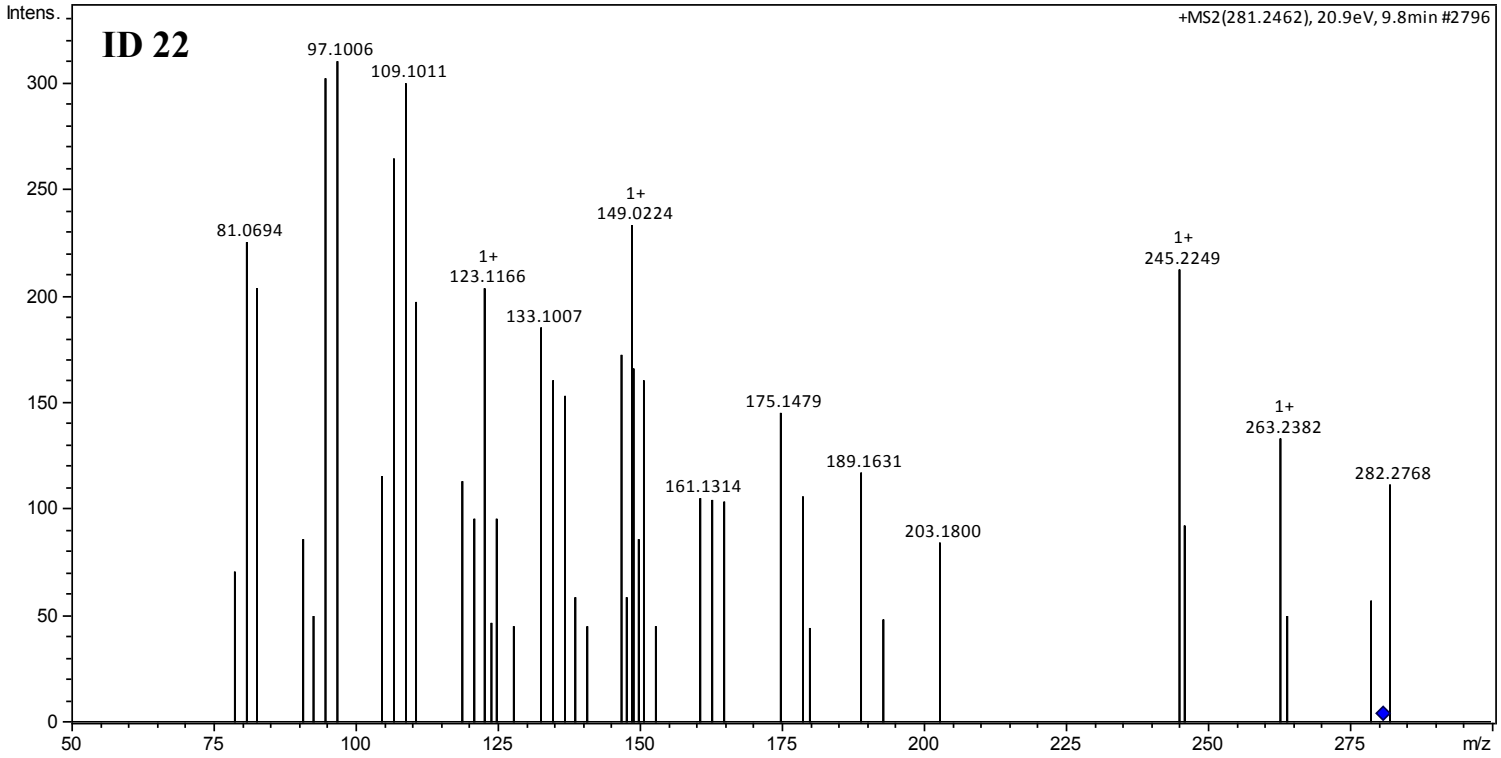


Fig S22 Linoleic acid ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.

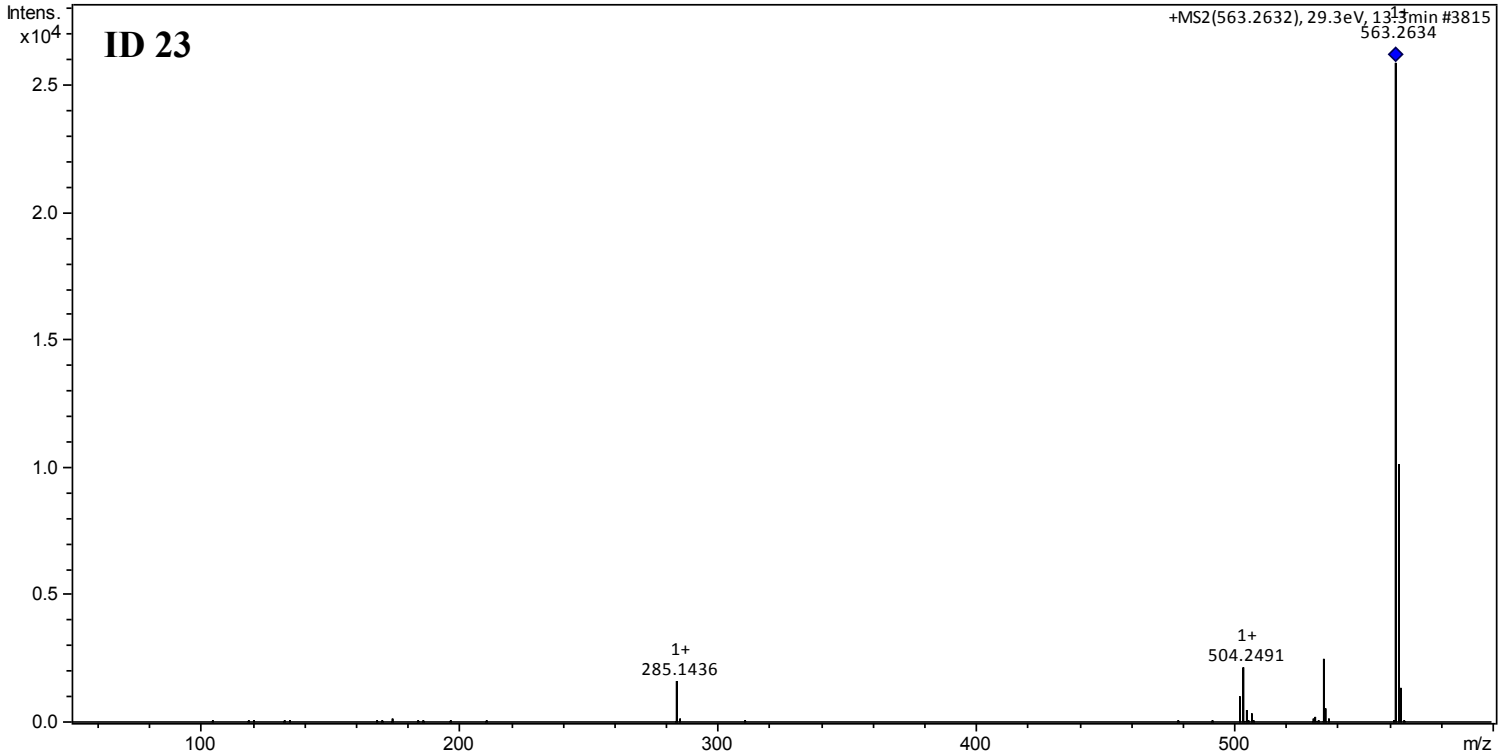


Fig S23 Protoporphyrin IX ion spectra obtained by UHPLC-ESI(+)-MS/MS of microalgae biomass extracted methanol.