FEDERAL RURAL UNIVERSITY OF PERNAMBUCO

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GRADUATE PROGRAM IN FISHERY RESOURCES AND AQUACULTURE

BIOCOMPOUNDS FROM AN ENDOSYMBIOTIC DINOFLAGELLATE: GENESIS, COMPOSITION, BIOLOGICAL ACTIVITIES AND SUSTAINABILITY

Carlos Yure Barbosa de Oliveira

Thesis presented to the Graduate Program in Fishery Resources and Aquaculture of the Federal Rural University of Pernambuco as a requirement to obtain the title of Doctor.

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Thesis considered suitable for obtaining the title of Doctor in Fishery Resources and Aquaculture. Defended and approved on 25/11/2022 by the following Jury:

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I dedicate this work to my family and friends.

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ABSTRACT

Modern baselines that make up the paradigm of sustainable development of aquaculture highlight microalgae as promising organisms for the suitable fish production growth. Microalgae biomass offers opportunities of processes and products not only for aquaculture sector, but also for human nutrition, wastewater treatment, bioenergy, biosensors, and new drugs development. For this latter application, marine dinoflagellates have emerged considerably in recent years due to the chemodiversity of secondary metabolites produced by them. However, a number of challenges are related to dinoflagellate cultivation, for example: sensitivity to shear stresses, nutritional complexity, sensitivity to thermal fluctuations, non-growth under sub-optimal conditions, etc. In view of this, the present thesis was built aimed at contributing to the improvement of marine dinoflagellates cultivation, in particular, for the endosymbiotic dinoflagellate *Durusdinium glynnii*. This thesis is organized into two main sections, the first one consisting of three review articles, and the second organized over three of research articles.

In the first article, global dinoflagellate research output was analyzed based on a scientometric approach using the Scopus database published between 1970 and 2020. The historical data proven that dinoflagellate research is an active research area, highlighting the themes of "harmful algal blooms" and "symbiosis with coral reefs". In analyzing data from the last decade of research, it was possible to identify a potential interest in cultivation and biotechnology of dinoflagellate. In the second article, some of the main genera of dinoflagellates (i.e., *Alexandrium*, *Amphidinium*, *Gymnodinium*, *Karlodinium*, and dinoflagellates of the family Symbiodiniaceae) with potential for cultivation were presented and reviewed. This article applications of dinoflagellates in aquaculture were also pointed out. Finally, in the third article, the potential of microalgae to achieve the important Sustainable Development Goals proposed by the United Nations was reviewed. In this article, the main challenges and the sustainable techniques used in the upstream and downstream processing of the microalgae production chain were presented.

In the fourth article an investigation of the effects of irradiance on growth and biochemical composition of *D. glynnii* was conducted. Under optimal growth conditions, *D. glynnii* accumulated high levels of docosahexaenoic acid (DHA), while the accumulation of the carotenoid peridinin occurred when exposed to high irradiance – proving a photoprotective role of this carotenoid. Additionally, extracts of *D. glynnii* biomass showed potential scavenging free radicals by means of antioxidant activity assays. In the fifth article, nutritional strategies,

based on nitrogen source and concentration, were evaluated as a way to alleviate thermal and light stress – two important parameters for productive scale-up of dinoflagellates. For light stress, the use of sodium nitrate as a nitrogen source was more suitable, while for thermal stress, only urea in high concentrations was able to allow *D. glynnii* cell division. Furthermore, some ecological implications of the findings of this study were demonstrated towards contribute to the knowledge of the phenomena related with the resistance of certain coral reefs to temperature rise. Finally, in the last article, a holistic approach for the production of antibacterial compounds using wastewater from shrimp production was proven. *D. glynnii* showed better growth performance using aquaculture wastewater than using a traditional culture medium, and the biomass produced in the wastewater was able to inhibit two *Vibrio* strains. These findings contribute to the development of circularity in aquaculture.

In conclusion, dinoflagellate research is associated with several important issues for society in terms of social, economic, and public health issues. Recent advances in cultivation and biotechnology of dinoflagellates can help to understand the occurrence of harmful blooms in natural environments, as well as in the development of new sustainable products and processes.

Keywords: antibiotics, antioxidants, docosahexaenoic acid, peridinin, zooxanthellae.

RESUMO

As linhas de base modernas, que compõem o paradigma do desenvolvimento sustentável, da aquicultura destacam as microalgas como organismos promissores para o crescimento adequado da produção de peixes e de camarões. A biomassa de microalgas oferece oportunidades de processos e de produtos não apenas para o setor aquícola, mas também para a nutrição humana, o tratamento de efluentes, a geração de bioenergias, a produção de biossensores e para o desenvolvimento de novos medicamentos. Para esta última aplicação, o interesse por dinoflagelados marinhos emergiu consideravelmente nos últimos anos devido à quimiodiversidade de metabólitos secundários produzidos por estes. No entanto, vários desafios estão relacionados ao cultivo de dinoflagelados, como por exemplo: sensibilidade a tensões de cisalhamento, complexidade nutricional, sensibilidade a flutuações térmicas, não crescimento em condições subótimas, etc. Diante disso, a presente tese foi construída com o objetivo de contribuir para o aprimoramento do cultivo de dinoflagelados marinhos, em especial, para o dinoflagelado endossimbionte *Durusdinium glynnii*. Esta tese está organizada em duas seções principais, sendo a primeira composta por três artigos de revisão, e a segunda composta por três artigos experimentais.

No primeiro artigo, a produção científica global de dinoflagelados foi analisada com base em uma abordagem cientométrica usando o banco de dados da plataforma Scopus no período entre 1970 e 2020. Os dados históricos comprovaram que a pesquisa com dinoflagelados é uma área de pesquisa ativa, destacando–se os temas "floração de algas nocivas" e " simbiose com recifes de coral". Ao analisar os dados da última década de pesquisa, foi possível identificar um potencial interesse no cultivo e biotecnologia de dinoflagelados. No segundo artigo, alguns dos principais dinoflagelados (i.e., *Alexandrium*, *Amphidinium*, *Gymnodinium*, *Karlodinium* e dinoflagelados da família Symbiodiniaceae) com potencial para cultivo foram apresentados e revisados. Neste artigo também foram apontadas aplicações da biomassa de dinoflagelados na aquicultura. Por fim, no terceiro artigo, foi revisado o potencial das microalgas para alcançar os importantes Objetivos de Desenvolvimento Sustentável propostos pelas Nações Unidas. Neste artigo, foram apresentados os principais desafios e as técnicas sustentáveis utilizadas no processamento *upstream* e *downstream* da cadeia produtiva de microalgas.

No quarto artigo foi realizada uma investigação dos efeitos da irradiância no crescimento e na composição bioquímica de *D. glynnii*. Em condições ótimas de crescimento *D. glynnii* acumulou altos níveis de ácido docosaexaenoico, enquanto o acúmulo do carotenoide peridinina ocorreu quando exposto a alta irradiância – comprovando um papel fotoprotetor

desse carotenoide. Adicionalmente, extratos de biomassa de *D. glynnii* mostraram potencial no sequestro de radicais livres por meio de ensaios de atividade antioxidante. No quinto artigo, foram avaliadas estratégias nutricionais, baseadas na fonte e na concentração de nitrogênio, como forma de aliviar o estresse térmico e luminoso – dois parâmetros importantes para o escalonamento produtivo de dinoflagelados. Para o estresse luminoso, o uso de nitrato de sódio como fonte de nitrogênio foi mais adequado, enquanto para estresse térmico, apenas a ureia em altas concentrações foi capaz de permitir a divisão celular de *D. glynnii*. Além disso, algumas implicações ecológicas dos achados deste estudo foram demonstradas no sentido de contribuir para o conhecimento dos fenômenos relacionados com a resistência de certos recifes de coral ao aumento da temperatura. Por fim, no último artigo, foi comprovada uma abordagem holística para a produção de compostos antibacterianos utilizando águas residuais da produção de camarão. *D. glynnii* apresentou melhor desempenho de crescimento usando águas residuais da aquicultura em comparação ao meio de cultura tradicional, e a biomassa produzida nas águas residuais foi capaz de inibir o crescimento de duas cepas bacterianas de *Vibrio*. Essas descobertas contribuem para o desenvolvimento da circularidade na aquicultura.

Em suma, a pesquisa de dinoflagelados está associada a várias questões importantes para a sociedade em aspectos sociais, econômicos e de saúde pública. Avanços recentes no cultivo e na biotecnologia de dinoflagelados podem auxiliar no entendimento da ocorrência de florações nocivas em ambientes naturais, bem como no desenvolvimento de novos produtos bioativos e processos sustentáveis.

Palavras-chave: ácido docosaexaenoico, antibióticos, antioxidantes, peridinina, zooxantelas.

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LIST OF ACRONYMS

μ	Specific growth rate
μ_{max}	Maximum specific growth rate
ABFT	Autotrophic biofloc technology
ABTS	2,2′-azinobis 3-ethyl-benzothiazoline-6-sulphonate
ANOVA	Analysis of variance
APDs	Amphidinols
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
BC–PBR	Bubble column photobioreactors
BSFC	Brake specific fuel consumption
BTE	Brake thermal efficiency
CFU	Colony-forming unit
Chl–*a*	Chlorophyll-*a*
Chl–*c*	Chlorophyll-*c*
COD	Chemical oxygen demand
CS	CiteScore
DHA	Docosahexaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	Irradiance
EA	Elemental analyzer
EAA	Essential amino acids
EFAs	Essential fatty acids
EGT	Exhaust gas temperature
EPA	Eicosapentaenoic acid
ESI	Environmental sustainability index
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FCP	Fucoxanthin-chlorophyll protein
Fe_3O_4@PEI	Nano-Fe_3O_4 coated with polyethyleneimine
GDP	Gross domestic product
GLM	Generalized linear model
HABs	Harmful algal blooms
HCV	Hepatitis C virus
HDI	Human development index
HRR	Heat release rate
HSV	Herpes simplex virus
IC_{50}	Half-maximum inhibitory concentration
IF	Impact factor
IRMS	Isotopic ratio mass spectrometer
ITO	Indium tin oxide
KmTxs	Karlotoxins
LCFA	Long-chain fatty acid
LC–PUFA	Long-chain polyunsaturated fatty acid
LEDs	Light-emitting diodes
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
Nano Fe_2O_3	Iron (III) oxide nanoparticles
NUR	Nitrogen uptake rate
P_s^B	Maximum photosynthetic rate in the absence of photoinhibition

PAR	Photosynthetically active radiation
PCP	Peridinin–chlorophyll–protein
PE	Photosynthesis–irradiance
PM	Particulate matter
POC	Particulate organic carbon
PP	Peak in–cylinder pressure
PS–II	Photosystem II
PSP	Paralytic shellfish poisoning
PSU	Practical salinity unit
PUFA	Polyunsaturated fatty acid
PUR	Phosphorus uptake rate
SCCCs	Super–carbon–chain compounds
SC–CO_2	Supercritical carbon dioxide
SDGs	Sustainable development goals
SFA	Saturated fatty acids
SFE	Supercritical fluids extraction
SJR	SCImago Journal Rank
STX	Saxitoxins
TCBS	Thiosulfate citrate bile salt agar
TL–PSBR	Twin-layer porous substrate bioreactor
TN	Total nitrogen
TP	Total phosphorus
UN	United nations
VPDB	Vienna Pee Dee Belemnite
$\delta^{13}C$	Carbon stable isotope ratio
$\delta^{14}N$	Nitrogen stable isotope ratio

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1. Thesis presentation

Dinoflagellates are perhaps the most diverse and complex group of microalgae in terms of phylogeny, morphology, and nutrition. Dinoflagellates are popularly known for causing phenomena of red tides (in colloquial language) or harmful algal blooms (in the scientific literature). In addition, they are also recognized for their fundamental role in the functioning of coral reefs. In both cases, dinoflagellates can produce a wide range of secondary metabolites, including hormones, toxins, and super–carbon–chain compounds. These compounds have been arousing considerable interest in biotechnology and pharmacology communities, due to potential antibacterial, antifungal, antiviral, and cytotoxicity activities, in addition to having positive preliminary effects against Alzheimer's and in the treatment for abstinence from heroin drug use. On the other hand, understanding the biosynthesis of these metabolites, in laboratory trials using isolated strains, can help to better understand the formation mechanisms of harmful dinoflagellate blooms and also the defense mechanisms against stressors in coral bleaching events. In view of this, the present thesis was built with the aim of contributing to the studies on dinoflagellate biotechnology. This thesis is divided into two main sections: the first section is composed by review articles, and the second one composed by research articles/papers.

Understanding the evolution of scientific dinoflagellates literature was an initial step for the knowledge of the state–of–art of this research field. Thus, the first article reviews global dinoflagellate research output based on a scientometric approach. An evaluation quantitative and qualitative of dinoflagellate documents from Scopus database was carried out. This review article was published in *Publications* (Percentile: 89%, Impact Factor: 3.705; v. 8, p. 50, 2020 DOI: 10.3390/publications8040050) and it was recognized as one of the top 10 most cited articles published in the last two years.

The second article reviews the main dinoflagellate genera with potential (and biotechnological interest) for biomass production. In addition, this review reveals key information on dinoflagellate cultivation and discuss on the major challenges, new insights, and future direction in the promising dinoflagellate production chain. This review article was published in *Borneo Journal of Marine Science and Aquaculture* (Percentile: N/A, Impact Factor: N/A; v. 4, p. 1-5, 2020. DOI: 10.5281/zenodo.4469212).

Microalgae represent a promising sustainable alternative for development of several industries, making its utilization important to modern world. In recent years, microalgae have been identified as suitable organisms to the achievement of important Sustainable Development

Goals (SDGs) set up in 2015 by the United Nations General Assembly. More specifically, microalgae can support eight SDGs: 1 – no poverty, 2 – zero hunger, 3 – good health and well-being, 6 – clean water and sanitization, 7 – affordable and clean energy, 12 – responsible consumption and production, 14 – life below water, and 15 – life on land. The contribution of microalgae biotechnology to achieving these important SDGs was discussed in the third article. This review article was the baseline for the sustainable practices that were employed in the development of the experimental trials conducted in the present thesis. This article was published in *Journal of Environmental Management* (Percentile: 95%, Impact Factor: 8.91; v. 320, p. 115897, 2022).

Opening section 2 of research articles, the fourth article covers an investigation of the effects of irradiance on growth performance, pigments and fatty acids composition, and antioxidant activity of the endosymbiotic dinoflagellate *Durusdinium glynnii*. In this manuscript, *D. glynnii* was cultured under different irradiances (ranging from 100 to 800 µmol photons m^{-2} s^{-1}) provided by light emitting diode (LED) lamps. The use of LEDs lamps results in less energy usage and has a less environmental impact than conventional light sources. We used a strategy of consecutive subcultures (resulting in more than 70 days of cultivation) to fit a robust photoacclimation model for this dinoflagellate. Our study shows that *D. glynnii* can accumulates high levels of docosahexaenoic acid (DHA) and peridinin, an exclusive carotenoid presents in prototrophic dinoflagellates, under certain light conditions. Following antioxidants assays by different methods, it was possible to correlate the production of antioxidant compounds to different metabolites present in the *D. glynnii* biomass. In particular, this article contributes to the achievement of the SDGs 3, 12 and 14, and it was published in *Applied Microbiology and Biotechnology* (Percentile 87%, Impact Factor: 5.560; v. 106, p. 6263-6276, 2022, DOI: 10.1007/s00253-022-12131-6).

An investigation on the effects of the source (sodium nitrate and urea) and the concentration (1760 and 440 µM) of nitrogen on the thermal and light resistance of *D. glynnii* was carried out in the fifth article. Both stressors are crucial for the outdoor scalability of microalgae cultivation. The nitrogen isotopic signatures (δ^{13} N) were different between the sources, proven the effectiveness in the usage of the two nitrogen forms. High nitrogen concentrations (i.e., 1760 µM), regardless of source, increased *D. glynnii* growth and chlorophyll–*a* and peridinin levels. During the pre–stress period, the use of urea accelerated the growth of *D. glynnii* compared to cells grown using sodium nitrate. During the luminous stress, the high nitrate condition has increased the cell growth, but changes in pigments composition

was not observed. On the other hand, during thermal stress was observed by a steep and steady decline in cell densities over time, except for high urea condition, where there is cellular division and peridinin accumulation 72 h after the thermal shock. This paper will be submitted to *Coral Reefs* (Percentile 90%, Impact Factor: 4.640).

Based on recent trends in circular bioeconomy business models, and contributing to SDGs 3, 6, 12, and 14, the investigation conducted in the sixth article showed the ability of *D. glynnii* to grow in wastewater from shrimp farming. As biorremediator organisms, dinoflagellates can bioconvert organic residual carbon, nitrogen– and phosphorus–based compounds, and other oligoelements (such as, heavy metals, pharmaceuticals, and other emerging pollutants), into several valuable metabolites. Here, we have proven that extracts from *D. glynnii* biomass cultured in an aquaculture wastewater has antioxidant and anti-*Vibrio* activities. Some *Vibrio* spp. cause massive mortality in shrimp farms every year, and the use of a raw material produced using waste from shrimp farming, can become a valuable and low–cost approach in a holistic process with ultra–low environmental impact. These findings of this research will be submitted to *Aquaculture* (Percentile 92%, Impact Factor: 5.135).

Additionally, another review article covering a multidisciplinary review of one of the most studied microalgae, in terms of number of published articles, in the world – *Tetradesmus obliquus* – was published. Some of the main research hotspot (i.e., light, nitrogen source and concentration, and the use of wastewater as culture medium) in this microalga research was selected to apply these knowledges for the experimental studies using dinoflagellates herein presented. This review was published in *Reviews in Aquaculture* (Percentile 99%, Impact Factor: 10.618, v. 13, p. 1594-1618.) – the leading Fisheries journal (according to Web of Science JCR 2022) – and recently, it was recognized as one of the top 3 most cited publications between 2021 and 2022 from this journal. This review article is presented in Appendix A.

2. Objectives

The main objective of this Thesis was to increase the understanding and deepen the knowledge on cultivation of marine dinoflagellates. To achieve this general objective, the following specific goals were completed:

1. To examine trends and scientific gaps, using bibliometric tools, in worldwide dinoflagellates research.
2. To explore the main genera of dinoflagellates with potential for the production of biomass and biocompounds.
3. To know the main sustainable practices onto microalgal biotechnology that can be applied to products and processes from marine dinoflagellate.
4. To analyze the research hotspots with a microalga model for conducting experiments with marine dinoflagellates.
5. To determine the effects of irradiance on growth, the profile of pigments and fatty acids, and the production of antioxidant compounds in the biomass of the dinoflagellate *D. glynnii*.
6. To explore the effects of nitrogen source and concentration on the increase resistance to thermal and light stressors in *D. glynnii*.
7. To develop a holistic quasi-zero waste model integrating wastewater treatment from aquaculture with the production of antibacterial compounds.

3. Chapter 1 – Review papers

3.1. Article 1: A scientometric overview of global dinoflagellate research

Research in this field is supported by the following journal publication	
Title	A scientometric overview of global dinoflagellate research
Authors	**CYB Oliveira**, CDL Oliveira, MN Müller, EP Santos, DMM Dantas, AO Gálvez
Journal	Publications
Year	2020
Volume	4
Pages	50
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Article

A Scientometric Overview of Global Dinoflagellate Research

Carlos Yure B. Oliveira [1,*], Cicero Diogo L. Oliveira [2], Marius N. Müller [3], Elizabeth P. Santos [1], Danielli M. M. Dantas [1] and Alfredo O. Gálvez [1]

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Abstract: Understanding the evolution of scientific literature is a critical and necessary step for the development and strengthening of a research field. However, an overview of global dinoflagellate research remains unavailable. Herein, global dinoflagellate research output was analyzed based on a scientometric approach using the Scopus data archive. The basic characteristics and worldwide interactions of dinoflagellate research output were analyzed to determine the temporal evolution and new emerging trends. The results confirm that dinoflagellate research output, reflected in the number of publications, is a fast-growing area since the mid-1990s. In total, five research subareas emerged using a bibliometric keywords analysis: (1) "symbiosis with coral reefs", (2) "phylogeny", (3) "palynology", (4) "harmful algal blooms" and (5) "nutrition strategies". Dinoflagellate publications were modeled by fish production (both aquaculture and fisheries) and economic and social indexes. Finally, directions for future research are proposed and discussed. The presented scientometric analysis confirms that dinoflagellate research is an active and important area with focus on mitigating economic impacts, especially in regard to fish production.

Keywords: bibliometric analysis; Dinophyceae; publications; microalgae; Scopus

1. Introduction

Dinoflagellates are protists characterized by two flagella and the pigment peridinin in combination with chlorophyll a, b or c. A large fraction of these microorganisms is mixotrophic, combining photosynthesis with phagotrophy and/or myzocytosis facilitating bloom formation when nutrients are scarce in the euphotic zone of coastal waters [1]. This functional group of microalgae has a primary marine occurrence but is also commonly found in freshwater and estuarine environments. Dinoflagellates are a highly diverse and abundant group of microalgae species and in terms of cell size substantially smaller than diatoms [2].

Claims by botanists, zoologists and micropaleontologists, regarding the taxonomic classification of dinoflagellates, were recurrent until the use of molecular tools which improved the evolutionary understanding and the complex life cycles of dinoflagellates. In fact, the literature on dinoflagellates, especially those published in the last century, is widely diffused, complex and partly contradictory. Nonetheless, dinoflagellates have been recognized for their essential role in the functioning of aquatic ecosystems, especially with regard to: primary productivity [3], symbiosis with reef-building corals [4], harmful algal blooms (HABs) [5] and toxin production and associated cascading trophic effects [6].

Dinoflagellates have been studied in regard to their production of highly diverse secondary metabolites. These compounds are, in general, not vital for the cell's survival and reproduction, and include a variety of hormones and allelochemicals. The toxins are a group of allelochemicals that can have harmful effects on higher organisms, such as fish, birds and mammals [7]. Historical records of toxins produced by dinoflagellates include (1) the Captain George Vancouver's crew poisoning after eating contaminated shellfish in 1793 [8], (2) human poisoning caused by the consumption of mussels in 1927 (California, United States), which has been related to the presence of *Alexandrium catenella* [9], and (3) the first proven cases of paralytic shellfish poisoning (PSP) in humans were recorded in 1976 [10]. Before 1970, toxic dinoflagellate blooms were recorded in Europe, North America and Japan and as of 1990, toxic species were observed in the Southern Hemisphere in conjunction with a general global increase in the distribution of toxins due to transportation of many HAB species via ship ballast waters [8,11]. Since then, HABs have caused serious public health problems and negatively impacted fishing and aquaculture industries, including the recent Godzilla Red Tide event in Chile—the largest recorded fish farm mortalities [11–14]. Additionally, dinoflagellates may cause problems in freshwater environments. Particularly *Ceratium* spp. have been reported in several freshwater environments in South America since the mid-2000s, and, although it is not a toxic genus, the postbloom accumulation of *Ceratium* spp. biomass may cause low oxygen environments due to an increased bacterial activity impacting survival rates of fish and crustacean species [15,16].

Bibliometric or scientometric analyses have become fundamental tools for analyzing current trends within the scientific literature and provide guidelines and motivations for future research in specific fields or areas. Recently, bibliometric analyses have been reported on eutrophication [17], diatom research [18], microcystins in China [19], photosynthesis [20] and microalgae research [21]. However, although these reports indirectly addressed dinoflagellate research, curiously this area has not been specifically emphasized—with the exception of Barbosa Noga and Ferreira Gomes [22] summarizing Brazilian dinoflagellate studies.

Exclusively quantitative bibliometric research is not necessarily the best approach to assess and discuss global scientific productivity [23], however, when combined with qualitative data, it can generate valuable indices for recognizing current status and future prospects within a given research field/area. Herein, dinoflagellate research was quantitatively and qualitatively analyzed to provide an improved understanding of the global research situation and emerging trends. The basic characteristics, development of publications, worldwide distribution, mainstream journals, keywords and genera of dinoflagellates research were analyzed in detail. Diverging research trends and subareas were identified to raise awareness on possible gaps in scientific cooperation.

2. Methods

2.1. Data Collection

The information of scientific publications was based on the Elsevier Scopus database (obtained on 31 January 2020). A detailed search was carried out using [TITLE-ABS-KEY (dinoflagellate)] as search query. This search resulted in 17,871 publications after limiting the search timescale from 1970 to 2019. Although the term "dinoflagellates" has presented the same number of publications as "dinoflagellate", it should be noted that if a different term is used, such as "Dinophyceae" (10,475 publications), variations may occur. The obtained results were processed by author keywords with identical meanings and by discarding keywords not related to phycology such as "article", "priority journal" and "non-human". Particularly the term "non-human" appears in the sixth place (2995 publications) among the most frequent keywords, however, when analyzing in detail the publications [TITLE-ABS-KEY (dinoflagellate) AND LIMIT-TO (EXACTKEYWORD, "Nonhuman")], the results did not contain any publications with the term "non-human" and, conclusively, this keyword was discarded.

2.2. Bibliometric Analysis

The publications obtained were organized and processed using the OpenRefine software. This software tool allows for the eliminating of duplicate records or grouping of different representations of the same reality [21,24].

The characteristics of bibliometric analyzed literature include both qualitative information and quantitative data. Herein, the elements investigated were: the document type and the language, the number of publications per year, the distribution of publications by research institutions and country, the keywords, the sources and research networks. In the case of research networks, a community can be defined as a set of nodes that are more densely connected with each other than with the rest of the network. Community detection was carried out using the VOSviewer software (version 1.6.14). This software enables the creation of charts categorized by countries or keywords what is represented by a node. The connections between two nodes represent the collaboration between the two keywords (or countries) in a research file.

2.3. Mapping and Modeling Scientific Production

Population and territorial extension data were obtained from the Worldometer website (http://www.worldometers.info/world-population/population-by-country/) and were used for normalization of scientific production per inhabitants and per territorial extension. A world map was colored according to number of publications of each country to compare the spatial distribution of published dinoflagellate publications.

In order to verify the influence of economic, environmental and social data on scientific production of dinoflagellates, we fitted generalized linear model (GLM), with a Gaussian error distribution and an identity link function for continuous data. The GLM was made using the 'lmer' function from the package 'lme4' [25]. Gross Domestic Product (GDP) and Human Development Index (HDI) were downloaded from The World Bank Database (https://data.worldbank.org/). Environmental Sustainability Index (ESI) was downloaded from the Yale University (https://epi.yale.edu/). This index summarizes 32 performance indicators and 11 pollutant emission categories in a single score for environmental health and ecosystem vitality. Additionally, agricultural production data were evaluated according to the global fish production by aquaculture (Aqua) and by capture (Capt) and to the fertilizer consumption (Fert) downloaded from the Fisheries Division of the Food and Agriculture Organization of the United Nations (http://www.fao.org/fishery/topic/16140) and from The World Bank Database, respectively. Data were extracted from 1970 to 2019 (depending of data availability) to calculate the according averages to be used in the model.

All analyses were performed in the RStudio software v 1.2.5 (Boston, MA, USA).

3. Results

3.1. Basic Characteristics of the Dinoflagellate Literature

The 17,871 publications, resulting from the initial search performed on the Elsevier Scopus platform, were composed of 16,258 articles (91%), 619 reviews (3.46%), 480 conference papers (2.68%), 214 book papers (1.19%) and 300 others (1.67%). Publications were published in English (17,071 ≅ 95.52%), Chinese (205 ≅ 1.15%), Spanish and French (174 ≅ 0.97%), Japanese (116 ≅ 0.65%) and other languages (211 ≅ 1.18%).

3.2. Temporal Development of Publications

The annual numbers of publications between 1970 and 2019 are presented in Figure 1. In 1970, 30 documents on dinoflagellates were published, and until the early 1980s a low oscillation in the number of publications could be observed (ranging from 26 to 90). From the quantitative perspective, a crucial moment for dinoflagellates research was registered in 1991 with a remarkable increase in the number of publications. In addition, in 2003 the number of publications exceeded 500 for the

first time. In mid-2014 the number of publications diverged from the fitted exponential trend. As a result, in 2017, only 862 publications were achieved, a lower number than projected (1000 publications). Furthermore, dinoflagellate publications of the last decade corresponded to 43.96% (7856 publications) of all publications since 1970. Among these publications, articles (92.11%) and the English language (95.69%) presented similar percentages when compared to the last 50 years. A linear regression model was fitted to the data of the last decade and is showed in Figure S1. Although the linear model of the last decade (R^2 = 0.75) was less expressive than the exponential one presented in Figure 1 (R^2 = 0.96), it projected that in 2025, 1000 publications on dinoflagellates would be achieved (8 years later when compared to the exponential model).

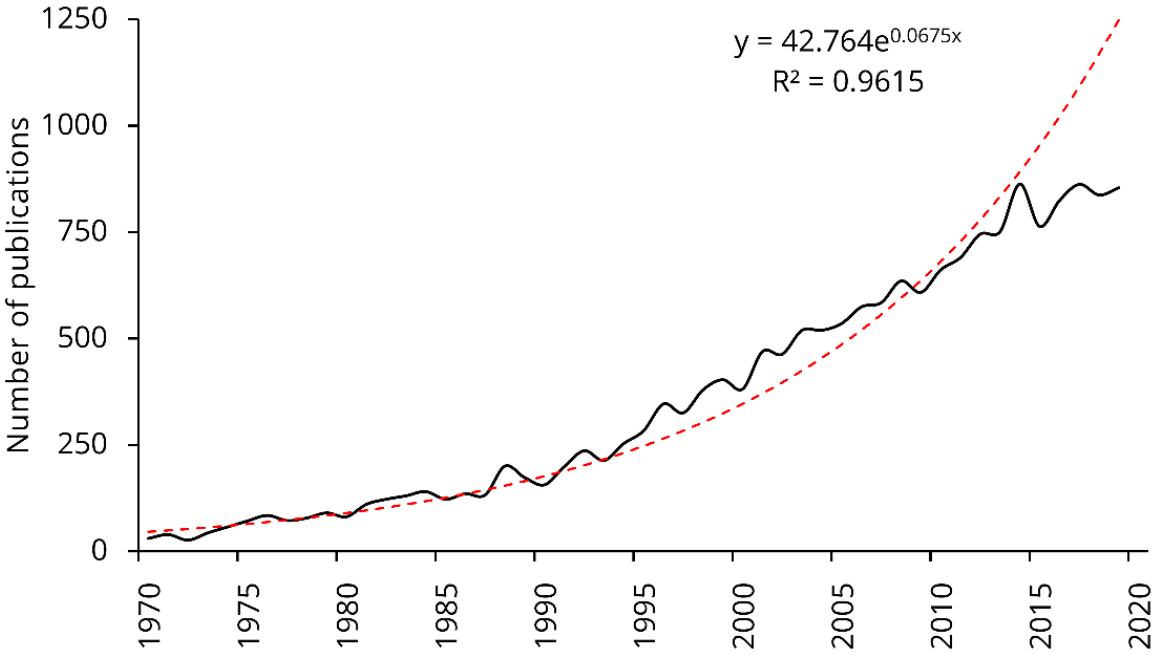

Figure 1. Trend in the number of publications from 1970 to 2019.

3.3. Global Distribution of Publications

A world map of the scientific production (Figure 2) indicates research on dinoflagellates has been conducted on all continents. The countries that published the most publications were the United States, Japan and the United Kingdom. In general, specific European countries published more publications than countries of America, Asia, Oceania and Africa. This ranking order changed when the scientific production was normalized to the population data and resulted in a new ranking with New Zealand, Norway and Australia in the top three positions. China and India, with populations of over one billion people, occupied the last positions in this normalized ranking. Using the normalization per land area data, Netherlands, the United Kingdom and Denmark were in the top three positions. Only the United Kingdom appeared multiple times in the top three positions of all applied rankings (Table 1). In relation to the countries that presented the most publications on dinoflagellates over the last decade, the United States remained at the same position, followed by China, Germany and France. The most dominant continent in terms of the number of publications was Europe with Germany, France, the United Kingdom and Spain among the top 10 countries (Table S1).

The top 20 research centers are listed in Table 2 and headed by: Center National de la Recherche Scientifique (CNRS), Alfred–Wegener-Institute and the Chinese Academy of Sciences. Although the United States leaded the ranking of publications by countries, only one North American institution (Woods Hole Oceanographic Institution) did appear in the top 20 institutions. This ranking was led by European and Asian institutions (three French, two German, two Spanish, one Dutch, one Danish, three Japanese, three Chinese and one Russian).

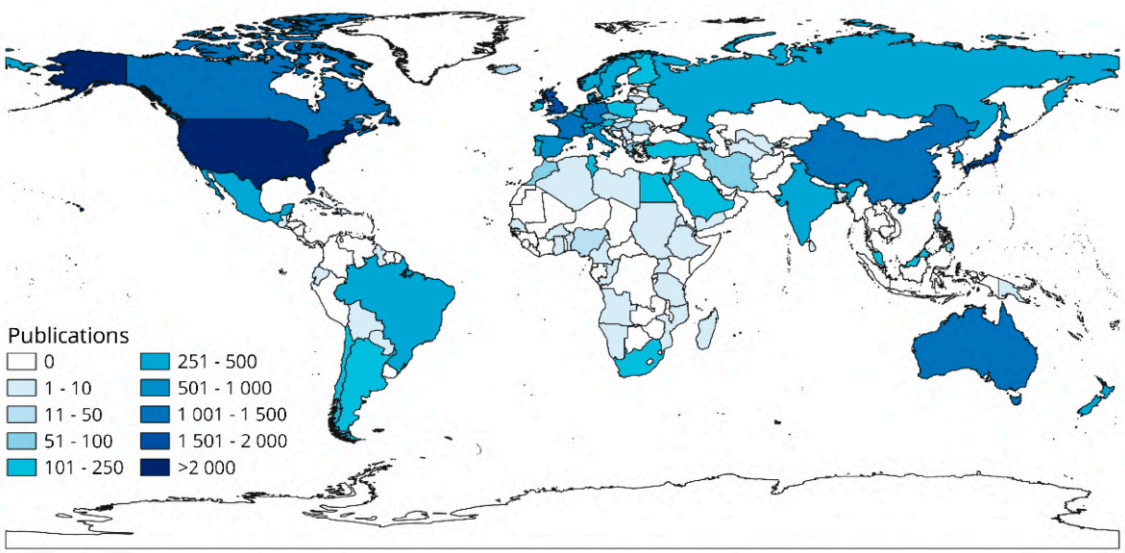

Figure 2. World map according to the number of documents.

Table 1. Publications (n) distribution by countries.

Country	M Habitants	Dimension (M km^2)	*n*	*n*/M Habitants	*n*/10^3 km^2
United States	331.00	9,147,420	4992	15.08	0.638
Japan	126.48	364,555	1590	12.57	4.265
United Kingdom	67.89	241,930	1541	22.70	6.354
Germany	83.78	348,560	1470	17.54	4.121
France	65.27	547,557	1393	21.34	2.526
China	1439.32	9,388,211	1256	0.87	0.131
Canada	37.74	9,093,510	1225	32.46	0.123
Australia	25.50	7,682,300	1110	43.53	0.144
Spain	46.75	498,800	958	20.49	1.898
Italy	60.46	294,140	619	10.24	2.054
South Korea	51.27	97,230	564	11.00	5.696
Denmark	5.79	42,430	260	44.89	6.036
New Zealand	4.82	263,310	437	90.62	1.615
Netherlands	17.13	33,720	423	24.69	10.186
Norway	5.42	365,268	419	77.29	1.088
Sweden	10.10	410,340	399	39.51	0.886
India	1380.00	2,973,190	352	0.25	0.107
Russian Federation	145.93	16,376,870	336	2.30	0.002
Mexico	128.93	1,943,950	336	2.61	0.171

Figure 3 shows the affinity of the collaboration of countries with >250 publications on dinoflagellates. The 20 countries, plotted in this analysis, were distributed across four communities: the first formed by Asian countries, the United States and Canada; the second formed by European countries, Brazil and Mexico; the third formed by European countries, India and the Russian Federation; and the fourth formed by Australia and New Zealand. The United States and the United Kingdom demonstrated connections with all four communities. More frequent connections (represented by the thickness of the line) could be observed between the United States and Australia, and the United Kingdom and Germany.

Table 2. Top 20 research centers.

Affiliation	Country	*n*
CNRS Centre National de la Recherche Scientifique	France	494
Alfred-Wegener-Institut Helmholtz-Zentrum für Polar-und	Germany	423
Chinese Academy of Sciences	China	378
Woods Hole Oceanographic Institution	United States	338
IFREMER Institut Francais de Recherche pour I'Exploitation de la Mer	France	331
Sorbonne Universite	France	286
Københavns Universitet	Denmark	285
Hokkaido University	Japan	250
University of Bremen	Germany	238
CSIC—Instituto de Ciencias del Mar ICM	Spain	233
The University of British Columbia	Canada	222
University of Tokyo	Japan	203
Scripps Institution of Oceanography	Canada	201
Instituto Espanol de Oceanografia	Spain	202
Utrecht University	Netherlands	197
Ministry of Education China	China	194
Russian Academy of Sciences	Russian Federation	193
University of Queensland	Australia	194
Tohoku University	Japan	193
Ocean University of China	China	188

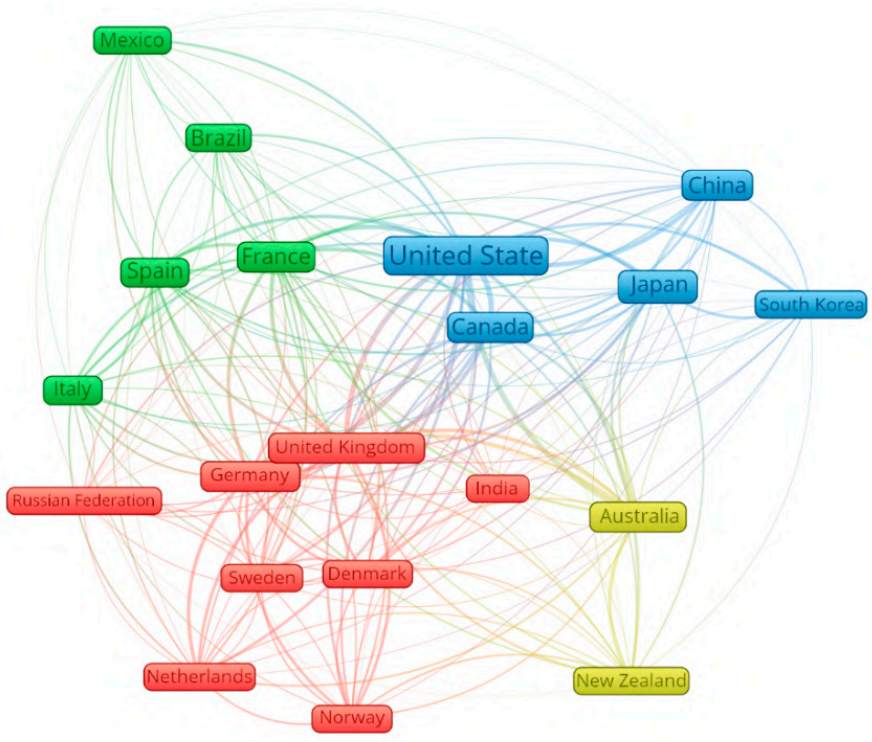

Figure 3. Visualization map of countries communities regarding dinoflagellate research.

3.4. Sources and Citations

The total number of publications, the JCR Impact Factor and the SJR CiteScore of the top 20 journals are listed in Table 3. The highest number of publications on dinoflagellates was found in the journal *Harmful Algae*, which was associated with the highest CiteScore index. On the other hand, *Marine Pollution Bulletin* had the highest Impact Factor of this ranking. Among the high impact phycology journals (IF ≥ 2), *Algal Research* (IF = 4.008), *Journal of Applied Phycology* (IF = 3.016), *European Journal of Phycology* (IF = 2.756) and *Algae* (IF = 2.914) did not appear in this ranking.

In addition, *PLoS ONE* was the only multidisciplinary journal listed in this ranking. In the last decade of publications, *Harmful Algae* continued to lead the number of publications. Furthermore, the journal *Marine Drugs* (IF = 4.073), which did not appear in the previous ranking, emerged on the ninth position in relation to the number of publications and it achieved the highest impact factor index (Table S2).

Table 3. Top 20 journals with published dinoflagellate research with the associated Impact Factor, CiteScore and number of documents (*n*).

Journal	Impact Factor (2019)	CiteScore (2019)	*n*
Harmful Algae	3.707	8.8	749
Journal of Phycology	2.328	4.6	614
Marine Ecology Progress Series	2.326	4.2	509
Journal of Plankton Research	2.146	3.9	401
Marine Biology	2.050	4.3	322
Review of Palaeobotany and Palynology	1.425	3.1	251
Toxicon	2.201	4.1	259
PLoS ONE	2.740	5.2	257
Journal of Environmental Marine Biology and Ecology	2.247	4.6	237
Limnology and Oceanography	3.778	7.5	234
Palynology	1.330	2.0	236
Hydrobiologia	2.385	4.7	195
Aquatic Microbial Ecology	1.841	3.2	195
Phycologia	2.276	3.7	188
Estuarine Coastal and Shelf Science	2.333	4.5	186
Marine Pollution Bulletin	4.049	6.7	181
Palaeogeography Palaeoclimatology Palaeoecology	2.833	5.1	181
Deep Sea Research. Part II Tropical Studies in Oceanography	2.697	6.6	167
Journal of Eukaryotic Microbiology	2.143	4.6	148
Marine Micropaleontology	2.207	3.7	145

The top 20 most cited publications and their main information are listed in Table 4. This ranking was headed by the journal *Limnology and Oceanography*, with four publications, followed by *Nature* and *Science*, with three and two publications, respectively. The publication with the highest total number of citations "Hoegh-Guldberg et al., 2007" received also the highest number of citations per year. Although *Harmful Algae* was the journal with the highest number of published publications, it did not appear in this specific list. Exclusively, the journals *Limnology and Oceanography*, *Phycologia* and *Journal of Phycology* appeared in both rankings.

Table 4. Top 20 mostly cited publications on dinoflagellate research.

Title	Authors	Year	Journal	Cited by	Citations per Year
Coral reefs under rapid climate change and ocean acidification	Hoegh-Guldberg, O. et al.	2007	*Science*	3029	252.42
A general method for isolation of high molecular weight DNA from eukaryotes	Blin, N. et al.	1976	*Nucleic Acids Res.*	2284	53.12
A review of harmful algal blooms and their apparent global increase	Hallegraeff, G.M.	1993	*Phycologia*	1694	65.15
Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton	Menden-Deuer, S., Lessard, E.J.	2000	*Limnol. Oceanogr.*	1418	74.63
Valuable products from biotechnology of microalgae	Pulz, O., Gross, W.	2004	*Appl. Microbiol. Biotechnol.*	1087	72.47
Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment	Camargo, J.A., Alonso, Á.	2006	*Environ. Int.*	945	72.69
The evolution of modern eukaryotic phytoplankton	Falkowski, P.G. et al.	2004	*Science*	855	57.00
The effects of harmful algal blooms on aquatic organisms	Landsberg, J.H.	2002	*Rev. Fish. Sci. Aquac.*	793	46.65

Table 4. *Cont.*

Title	Authors	Year	Journal	Cited by	Citations per Year
Microalgal biomarkers: A review of recent research developments	Volkman, J.K. et al.	1998	*Org. Geochem.*	793	37.76
Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea	Smayda, T.J.	1997	*Limnol. Oceanogr.*	778	35.36
Iron-limited diatom growth and Si:N uptake ratios in a coastal upwelling regime	Hutchins, D.A., Bruland, K.W.	1998	*Nature*	754	35.90
Nuisance phytoplankton blooms in coastal, estuarine, and inland waters	Paerl, H.W.	1988	*Limnol. Oceanogr.*	693	22.35
Ocean acidification causes bleaching and productivity loss in coral reef builders	Anthony, K.R.N. et al.	2008	*P. Natl. Acad. Sci. USA*	690	62.73
The phagotrophic origin of eukaryotes and phylogenetic classification on protozoa	Cavalier-Smith, T.	2002	*Int. J. Syst. Evol. Micr.*	680	40.00
The role of microorganisms in coral health, disease and evolution	Rosenberg, E. et al.	2007	*Nat. Rev. Microbiol.*	678	56.50
Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity	Moon-Van Der Staay, S.Y. et al.	2001	*Nature*	672	37.33
Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton	López-García, P. et al.	2001	*Nature*	629	34.94
Flexibility and Specificity in Coral-Algal Symbiosis: Diversity, Ecology, and Biogeography of *Symbiodinium*	Baker, A.C.	2003	*Annu. Rev. Ecol. Evol. Syst.*	620	38.75
Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing	Schmidt, T.M. et al.	1991	*J. Bacteriol.*	566	20.21
Identification of group—and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence Analysis of a fragment of the LSU rRNA gene	Scholin, C.A. et al.	1994	*J. Phycol.*	558	22.32

3.5. Keywords Analysis

Keyword co-occurrence and their affinity are indicated in Figure 4. In this analysis, 41 keywords were plotted and distributed into five communities. The first community (red color) revolved around the keywords "harmful algal blooms", "red tide" and "toxins", probably influenced by research related to the increase in harmful algal blooms involving the taxa "*Alexandrium*" and "*Karenia brevis*". The second community (green color) revolved around the keywords "phytoplankton", "mixotrophy" and "grazing", probably influenced by enforcements to understanding dinoflagellate interactions with others organisms (e.g., bacteria, diatoms and cyanobacteria) and their nutrition strategies. The third community (yellow color) revolved around the keywords, "taxonomy", "ultrastructure" and "phylogeny", probably influenced by evolutionary relationships and taxonomic refinements and corrections within this group. The fourth community (purple color) revolved around paleontological studies that used the keywords "dinoflagellate cysts", "biostratigraphy" and "palynology". Finally, the fifth community (blue color) revolved around the keywords "*Symbiodinium*", "Coral bleaching" and "photosynthesis", influenced by coral–endosymbiont interactions and the effects of climate change on coral ecosystems. An additional keywords analysis of the last decade data, using author keywords that appeared at least 50 times, was carried out and the results were represented as a cloudword (Figure S2). As in Figure 4, the same keywords could be observed with the emergence of some new keywords: "*Marine drugs*", "*Chemistry*" and "*Microalga*".

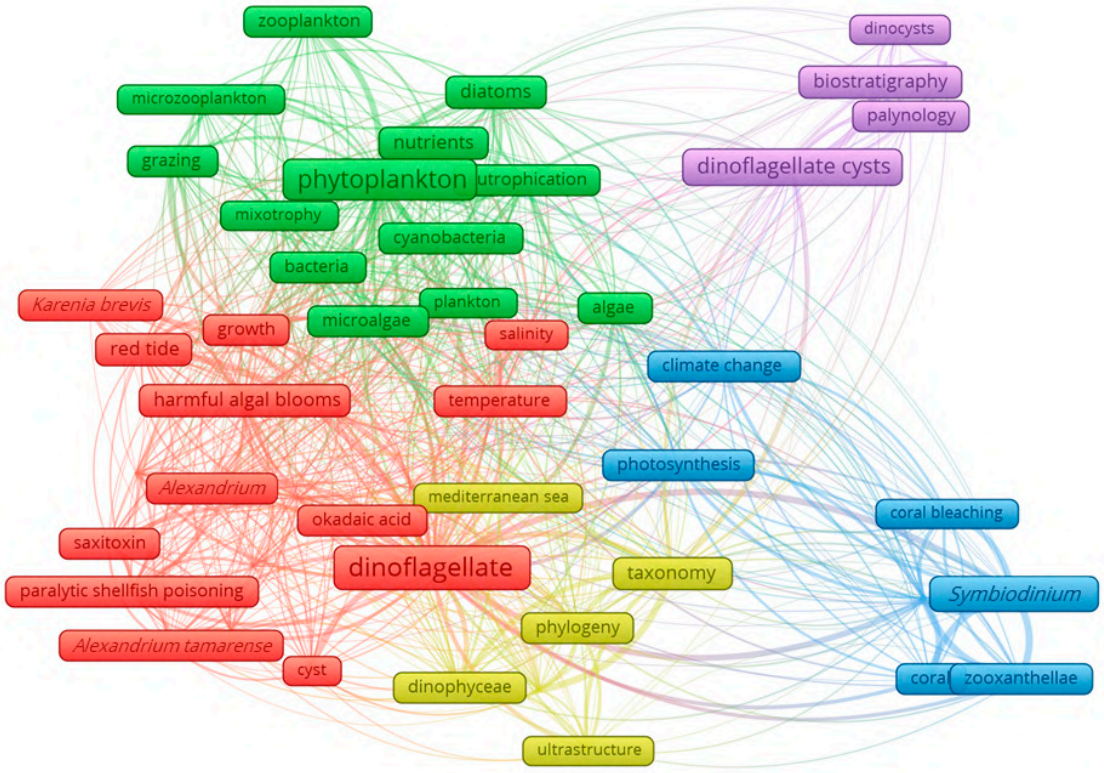

Figure 4. Keywords communities and their associations regarding dinoflagellate research.

Figure 5 shows the top ten genera of dinoflagellates that appeared in the title, abstract or keywords of the published publications. *Alexandrium* leaded this ranking, followed by *Prorocentrum*, *Symbiodinium* and *Gymnodinium*—these appeared in at least a thousand publications. *Ceratium*, *Protoperidinium* and *Noctiluca*, located at the last positions of this ranking, were associated to almost six times fewer published publications than *Alexandrium*.

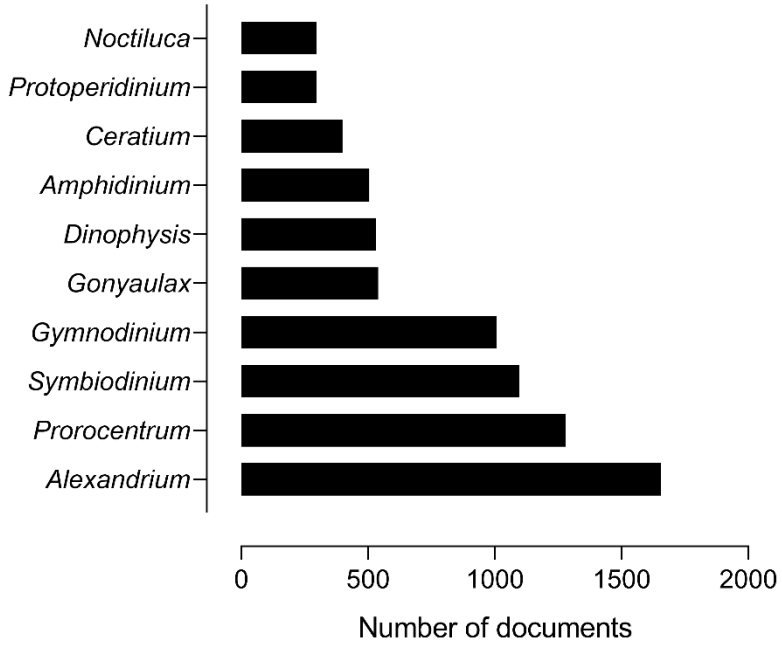

Figure 5. Representation of the number of publications related to specific genera of dinoflagellates.

3.6. Modeling Scientific Production

Economic (GDP), environmental (EPI) and social (HDI) indexes indicated significant influence on the scientific production of dinoflagellate literature. Fish production (both aquaculture and capture) was also the outcome of the model, however, while the capture had a positive relationship with dinoflagellate publications, the aquaculture had a negative effect. The fertilizer consumption did not indicate a significant influence (Figure 6).

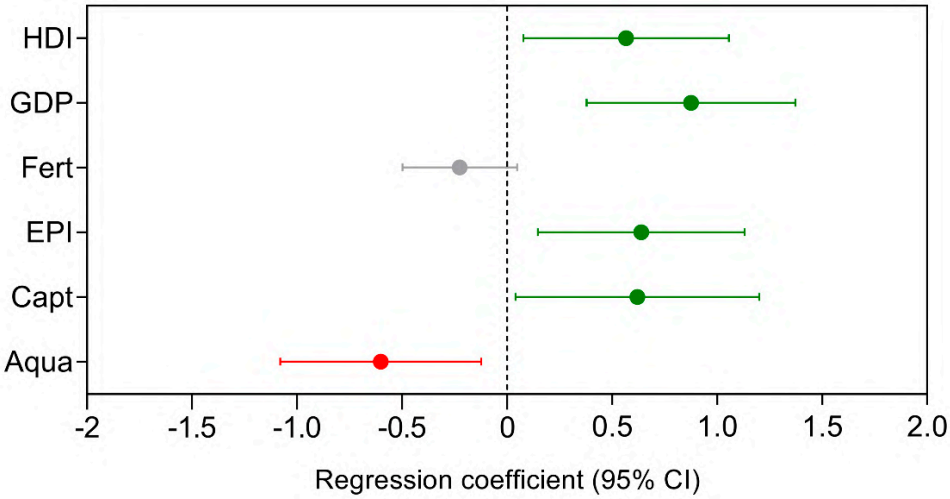

Figure 6. Coefficient estimates (± 95% confidence intervals) indicating the magnitude and direction economic, environmental and social variables tested for a model of scientific production of dinoflagellates. Gray markers represent predictors without significant influence, red and green represent negative and positive effects, respectively.

4. Discussion

First of all, it is necessary to mention that the number of publications on dinoflagellates is certainly greater than those reported in this study. This is related to many local, regional or national journals that are not indexed in large and international databases (such as Elsevier Scopus). Many publications on dinoflagellates in Latin America, for example, have not been published in indexed journals (e.g., [26–28]). However, recent research efforts have been initiated to showcase Latin American research conducted on harmful algal, including dinoflagellates [29]. Some possible motives of publishing in local journals include: (1) meeting the requirements of funding agencies, (2) easier publishing in the native language and/or (3) contributing to the evolution of local journals [30].

Interestingly, 1000 publications should have been achieved in 2017 according to the exponential regression model, which, however, did not occur (Figure 1). The divergence between the number of publications and the exponential trend hints towards a resource limitation, such as for example human resources and/or financial funding limitation. It is likely that this divergence may have been induced by the financial crisis of 2008. The implemented interventions by public authorities to alleviate the consequences of the financial crises resulted in multiple financial cuts for scientific research of several countries [31] with a presumably time delayed effect on dinoflagellate research output. In summary, a linear regression model expressed well the growth of the number of research papers on phytoplankton between 1991 and 2013 [32]. Two linear trends were observed within the worldwide microalgae research, the first was from 1970 to 2005 and the second from 2005 to the present [21]. Therefore, although linear models are often used successfully for phytoplankton studies, in the present study an exponential model was more suitable and it allowed illustrating the political and economic evidence that negatively affected dinoflagellate research.

The global scientific production on dinoflagellates can be related to social, economic and environmental aspects. The United States has long been the most productive and influential research

country in the world, and when analyzing data from 1970 to 2019, the USA still appears as the top country that publishes the most publications in several bibliometric studies [21,33,34]. However, China overtook the United States in 2018 and became the quantitative largest producer of scientific articles in the world. The United States still invests the highest amount of financial resources on research and development (around USD 500 billion), while China stood second (around USD 400 billion) in 2015 with increased funding trends on research and development over the recent years. The spent funding of the United States, on the other hand, remained on the same level in relation to previous years [35]. Although the United States spearheaded the two analyses of publications by country (Figure 2 and Table S1), a substantial increase in the number of publications by China was observed and it rose from the sixth to the second place, when analyzing both the last 50 and 10 years, respectively.

The Elsevier Scopus classifies the CNRS and the Chinese Academy of Sciences as research centers, as well as the Alfred–Wegener-Institut, Woods Hole Oceanographic, University of Tokyo. However, it should be mentioned that CNRS is the French National Centre for Scientific Research and it provides a significant amount of funding for basic research in France. In a similar way, the Chinese Academy of Sciences is an umbrella body of research centers. Therefore, they cannot be directly compared to regional research institutions. Furthermore, although the United States leads the ranking in the number of publications on dinoflagellates, only one American institution appears in the top 20 research centers. This observation may be related to American research being distributed over several research centers with diverse funding resources and not being centralized as for example in case of the French CNRS and the Chinese Academy of Sciences.

Others phytoplankton groups (mainly, diatoms and cyanobacteria) were identified within dinoflagellate research, (Figure 3). This can be explained by the assumed dominant role of diatoms for primary production and the carbon cycle in the oceans. Diatoms have been considered model organisms for oceanic phytoplankton research which has been reevaluated over the past few decades [36–38]. On the other hand, cyanobacteria are the largest group of prokaryotic organisms known for their potential toxicity in marine, freshwater and eutrophic environments and therefore closely related to HAB research [39,40]. It is likely that dinoflagellate research on understanding physiological dynamics, bloom causes and consequences, and the biosynthesis of secondary metabolites has been studied together with other HAB species.

Figures 4 and 5 highlight the *Symbiodinium* genus probably due to two issues: (1) the great effort in the last decade to reorganize the diversity of this genus into a revised hierarchical structure and (2) the close relation to coral reef research. The nine clades recognized in the literature [41] have recently been reorganized into six new genera (in addition to *Symbiodinium*) belonging to the Symbiodiniaceae family [42]. In addition, the most active countries in the fifth community of keywords (blue color) (Figure 4) are countries in Oceania which may be related to the location of the Great Barrier Reef and of other tropical coral reefs, and the reoccurrence of coral reef bleaching events in these regions [43–45]. The evolutionary success of reef corals over time has been strongly linked to the mutualistic relationship with endosymbiont dinoflagellates. The benefits of this relationship include the supply and exchange of inorganic nutrients (carbon, nitrogen, phosphorus, etc.) that are converted into carbohydrates, amino acids and other secondary metabolites under photoautotrophic pathways. Although dinoflagellates from the Symbiodiniaceae family are almost always associated to a symbiotic lifestyle, they can also be found in free-living mode [46]. The mutualistic relationship can lead to biased conclusions because many publications on coral reefs may occasionally contain in their keywords terms such as "*Symbiodinium*" or "Zooxanthellae"—as, for example, in the case of the most cited publication reported in our investigation. Figure 4 also shows the palynology as another active subarea in dinoflagellate research. A number of dinoflagellate species produce resting cysts that have the potential (1) to become fossilized in sediments or (2) to be transported via ships' ballast waters [47,48]. The transportation of sediments and water containing dinoflagellate cysts has led to a global dispersion of bloom-forming dinoflagellate species. Efforts in this research subarea have been

related to fossil dinoflagellate cysts which are a useful tool for reconstructing past environmental and oceanographic conditions [48,49].

Figure 5 demonstrates that the number of publications related to nontoxic dinoflagellate genera (*Ceratium* and *Noctiluca*) was lower than toxic dinoflagellate genera, which indicates a greater research interest on toxin-producing species. *Alexandrium* genus, that led the ranking of dinoflagellate genera, is the major harmful dinoflagellate bloom genus with respect to diversity, impact potential and cascading ecosystem consequences [11]. This suggests that interest in studying a certain dinoflagellate genus (or species) increases as new evidence of a toxic potential is reported. Moreover, it should be taken into account that some dinoflagellate species were taxonomically reclassified over time (for example, *Karlodinium veneficum* and its basionym *Gymnodinium veneficum* and *Ceratium* genus that recently was subdivided into *Ceratium* and *Tripos* for freshwater and marine species, respectively) [50,51].

Regarding the GLM, positive relationship between fish catch and dinoflagellate publications may be related to HAB issues and their impacts on the fishery industry—which is mainly composed by marine fishing (~87.5% of total fish catch) [52–54]. On the other hand, a negative relationship between aquaculture production and dinoflagellate publications was observed maybe due to aquaculture production includes most freshwater (51 million tons) than marine (30.8 million tons) production [55]. Understanding the main related factors leading to harmful dinoflagellates blooms is a crucial step for appropriately managing aquaculture and agriculture activities. The escalation on food production increased the amount of nitrogen compounds released in receiving waters [56,57]. Moreover, regions that reported an increase in the agricultural activities (terrestrial and aquatic) experienced, almost in the same proportion, the increasing frequency and environmental impact of HABs [56]. Although the nitrogen role on dinoflagellate blooms have not been fully elucidated, this algae group have the highest urease activity per cell than any other phytoplankton group [58], and in some cases, the urea uptake can increase the toxicity of dinoflagellate [59]. Thus, understanding the causes and controlling factors of dinoflagellate blooms can contribute to reducing the associated impacts on fishing activity [14].

The Recent trends analysis (Supplementary Materials) indicates the emergence of a new dinoflagellate research subarea: cultivations and biotechnology. This is related to the appearance of new keywords over the past decade, such as "*Marine drugs*", "*Chemistry*" and "*Microalga*"—a common term in publications that refer to cultivation and biotechnology of phytoplankton (Figure S2). These results, when related to the rise of the journal *Marine Drugs*, may possibly be associated to the interest in cultivating dinoflagellates to produce potential raw material for new drugs formulation with biological activities.

5. Future Directions

The results presented in the previous sections give evidence to two emerging future hotspots in dinoflagellate research: (1) taxonomy and classification, (2) harmful dinoflagellate blooms and (3) cultivation and biotechnology. Figure 7 demonstrates a flowchart of the main emerging subareas for dinoflagellate research. Research using "omics" (i.e., genomics, transcriptomics, proteomics and metabolomics) approach is collated on the top of the flowchart and can help in the identification of species, the detection of nutritional strategies, the interaction of symbiotic relationship with bacteria and cnidarias, and the biosynthesis of biomolecules—especially regarding secondary metabolites. These issues can contribute to the knowledge of the metabolic pathways and mechanisms involved during bloom formation. At the bottom of the flowchart, attention was given to the cultivation of dinoflagellates, and it made, according to the procedures, for optimizing the production of biomass from other cultivated microalgae species.

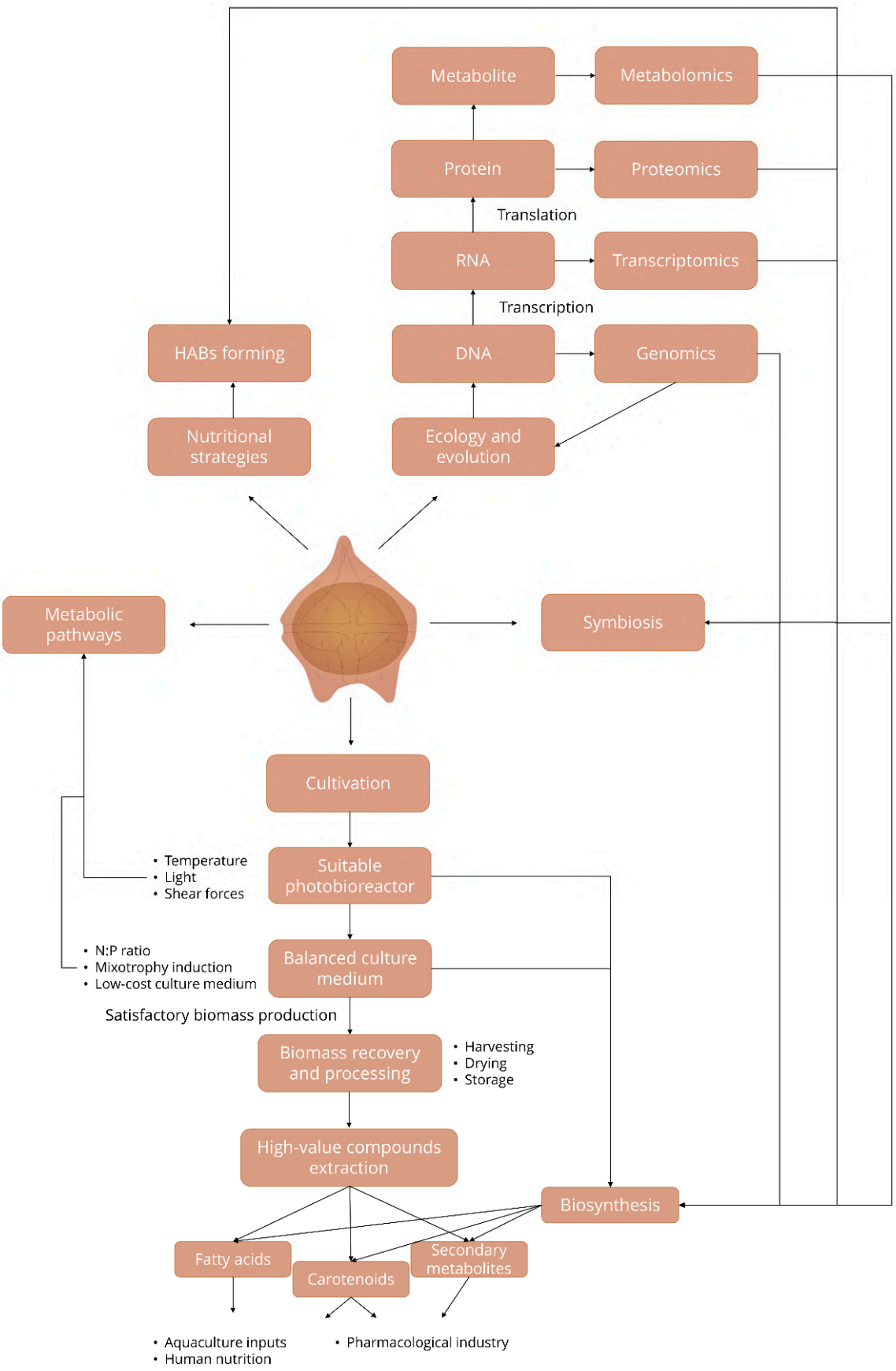

Figure 7. Perspectives for future dinoflagellate research.

The development and use of robust platforms for data sharing and knowledge transfer/dissemination will certainly contribute and facilitate a fast achievement of new solutions regarding taxonomic and metabolic classifications. Several existing databases (e.g., Dictionary of Natural Products, AntiBase, MassBank and Global Natural Products Social Molecular Networking) can be used to share raw data that can contribute to the research advancement on biomolecules from dinoflagellate biomass [60,61].

The main products obtained from cultivating dinoflagellate biomass include: fatty acids that have sustainable applications for animal and human food, and for biodiesel production [62–64], the peridinin apocarotenoid and toxins that have strong antioxidant properties and helps to prevent the formation of tumors [65–67], and also the amino acids and polysaccharides that can also be used in biorefinery models.

The lack of suitable methods for culturing certain dinoflagellates limits potential in vitro and in vivo tests and the commercialization of new drugs. The main difficulty in cultivating dinoflagellates is the sensitivity to the shear forces [68]. The success in the production of dinoflagellate biomass, as well as an improvement in the biosynthesis of secondary metabolites, will be an important step to be achieved and represents a highly promising industry for the next decades [69].

6. Conclusions

This scientometric overview demonstrated a constant increase in the number of publications on dinoflagellates from 1970 to 2019. Most of the top publishing countries were recognized for an important marine fish production economy, with a clear interest in mitigating the impacts of harmful algal blooms on capture production and the associated economy. In addition, the United States and the United Kingdom are highly intertwined within a global research network. The bibliometric analysis of dinoflagellate-related publications indicated that there are more publications in developed regions compared with undeveloped regions. Furthermore, a clear research trend towards toxin producing dinoflagellate genera is evident compared to nontoxin-producing genera.

Although a high number of publications have been reported in this study, it is clear that dinoflagellate research will remain active and growing regarding (1) taxonomy and classification issues, (2) harmful dinoflagellate blooms and (3) cultivation and biotechnological use of dinoflagellate biomass. To address these gaps, international cooperation to make higher quality research can focus in future works based on published data (for example, meta-analysis-based approaches) to clarify taxonomic issues. Moreover, studies on the life-cycle assessment of dinoflagellate production must be considered in future works.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-6775/8/4/50/s1, Figure S1: Number of publications by years at the last decade in the dinoflagellates research, Figure S2: Cloudword representation of main author keywords (min. 250 times) on dinoflagellate research at the last decade, Table S1: Top 10 countries in number of publications on dinoflagellates research, Table S2: Top 10 journals in the number of publications in the last decade. Most information about these journals can be visualized in Table 3.

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3.2. Article 2: A mini review on challenges and opportunities in dinoflagellates cultivation

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A mini review on challenges and opportunities in dinoflagellates cultivation

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Abstract

Dinoflagellates are photosynthetic protists commonly distributed in marine and freshwater environments and can be found in symbiotic associations. They are a significant primary producer and play a fundamental role in the functioning of aquatic ecosystems – especially for coral reefs. Dinoflagellates can produce a wide variety of secondary metabolites, and their toxins can affect fish, birds and mammals. In recent years these toxins have been found to have potential cytotoxic, anticancer, antibiotics, antifungals activities. This mini review covers the main genera of dinoflagellates, and challenges and advances in their cultivation in addition to prospects for development of dinoflagellates-based products.

Keywords: Microalgae, Biomass, Secondary metabolites, Toxins

Background

Interest in microalgae has increased considerably in recent decades, mainly due to demand for sustainable biomass and bioprocesses, such as aquaculture, where microalgae play essential roles as live food for molluscs, and larvae of crustaceans and fish (Muller-Fuega, 2000; Garrido-Cardenas et al., 2018). Besides other applications, these photosynthetic microorganisms have also aroused interest in wastewater treatment and production of high commercial value molecules (eg., fatty acids, carotenoids and amino acids) and biofuels (Daroch et al., 2013; Salama et al., 2017; Oliveira et al., 2020a). According to Garrido-Cardenas et al. (2018), even with various species of microalgae isolated, global production of and research on microalgae are limited to a small number of taxa, such as *Arthrospira* of Spirulina group, that are intended mainly for human food or as a dietary supplement (Pan-Utai et al., 2018); *Chlorella* spp. for being a potential producer of β-1,3-glucan, an active immunostimulator with antioxidant capacity (Carballo et al., 2019); *Dunaliella salina*, as a source of β-carotene (Ben-Amotz, 2004) and; *Haematococcus pluvialis*, for astaxanthin extraction (Panis & Carreon, 2016).

Dinoflagellates are a eukaryotic group of microalgae common in marine, estuarine and freshwater environments. Besides the species diversity (around 6,000 species), dinoflagellates have various structural shapes (amoeboid, coccoid, palmelloids, etc.), habitats (planktonic, benthic and epicontinental) and nutritional modes (photoautotrophic, heterotrophic, mixotrophic and phagotrophy). They play a significant role as primary producers and contribute to the functioning of aquatic ecosystems, especially coral reefs. The ecological activities of coral reefs heavily depend on the symbiosis between reef-building corals and zooxanthellae (reviewed in Jephcott et al., 2016 and Suggett et al., 2017).

In addition, dinoflagellates also receive interest in research because some of their species produce toxins and they also cause harmful algal blooms (HABs) (Gravinese et al., 2018). Despite their great diversity, about 90 species have been reported as potential toxin producers (Burkholder et al., 2008; González-Rodríguez et al., 2010; Speight & Henderson, 2010; Saldarriaga & Taylor, 2017).

Toxins from dinoflagellates can affect human and ecosystem health and, for a long time, this was the main reason for interest in their studies. However, in recent years dinoflagellate toxins have been found to have potential pharmaceutical applications (i.e. cytotoxic, anticancer, antibiotics, antifungals activities). In this context, this mini review reveals key information about dinoflagellates cultivation. The discussion also takes into account the major challenges, new insights and potential of the biomass production of dinoflagellates.

Dinoflagellate Genera

Specific dinoflagellate genera have been studied as a source of bioactive molecules (secondary metabolites): *Alexandrium*, *Amphidinium*, *Gymnodinium*, *Karlodinium* and *Symbiodinium* (Wang & Hsieh, 2002; Parker et al., 2002; Band-Schmidt et al., 2014; Benstein et al., 2014; Lage et al., 2014; Molina-Miras et al., 2018; Langenbach & Melkonian, 2019).

2.1 *Alexandrium*

The genus *Alexandrium* is one of the major harmful algal bloom genera. Three different families of toxins were reported in this genus: saxitoxins (STX), goniodomins and spirolides; but they have not been fully characterized (Balech, 1989; Touzet et al., 2008; reviewed in Anderson et al., 2012). *Alexandrium* spp. are considered opportunistic in relation to nutrition - different species have been found in both nutrient-

rich (Spatharis et al., 2007) and nutrient-poor waters (Collos et al., 2014). In addition, bacteria and microalgae (dinoflagellate, *Amphidinium carterae*) have been observed to contain food vacuoles (reviewed in Jeong et al., 2010). Moreover, growth under the mixotrophic nutritional mode has also been reported for *Alexandrium catenella* (Legrand & Carlsson, 1998).

2.2 *Amphidinium*

Amphidinium spp. are toxic dinoflagellates found in coastal waters and tempered tropical estuaries (Steidinger & Jangen, 1996). It is known for HABs that may produce mainly ichthyotoxins (Huang et al., 2009) and hemolytic substances (Echigoya et al., 2005). Abundance of peridinin (an apocarotenoid), located in the photosynthetic complex of most dinoflagellates, has been extensively studied in *Aphidinium carterae* (Hofmann et al., 1996); this apocarotenoid possesses strong antioxidant properties and can act against the tumors (Nishino, 1998; Barros et al., 2001). Recently, amphidinols (APDs), secondary metabolites produced by this genus, have aroused a growing interest by presenting potential antifungal, antibacterial, antioxidant and antitumor agents (Satake et al., 2017; Iwamoto et al., 2017; Martínez et al., 2019). Although the structure of APDs is well-documented (Satake et al., 2017), several factors related to the biosynthesis of these molecules are still not well understood.

2.3 *Gymnodinium*

Gymnodinium catenatum is the only dinoflagellate species of this genus that produces paralytic shellfish poisoning (PSP) and its greatest relevance is due to the fact that it can affect human health with neurological and gastrointestinal disorders, usually as a result of the consumption of contaminated shellfish (Band-Schmidt et al., 2008; Martínez et al., 2016). This species is widespread in temperate and tropical waters in many regions of the world (Hallegraeff et al., 2012) and the toxin profile may vary according to environmental factors (Negri et al., 2001; Oliveira-Proença et al., 2001; Holmes et al., 2002; Oh et al., 2010). As for the other dinoflagellates, studies on *G. catenatum* have mainly involved the ecophysiological approach for understanding the influence of environmental factors on the production of toxins.

2.4 *Karlodinium*

In the genus *Karlodinium*, a cosmopolitan species of temperate regions that has been more thoroughly studied is *Karlodinium veneficum* (García-Camacho et al., 2007; Gallardo-Rodríguez et al., 2012; López-Rosales et al. 2015). *K. veneficum* is a producer of karlotoxins (KmTxs) and it can feed by ingesting diatoms and copepods (Bachvaroff et al., 2009; Waters et al., 2010; Place et al., 2012). The KmTxs can be easily isolated, and like APDs, it has hemolytic and ichthyotoxic activity. KmTxs are also more likely to function as anti-grazing and allelopathic. Investigations have shown that *K. veneficum* is able to reconfigure its cellular metabolic machinery and regulate dynamic protein expressions to cope with the stress caused by excess light. This is an interesting strategy for intensive cultivation to produce biomass (Cui et al., 2017). For this reason, *K. veneficum* proves to be a promising species for production of biomolecules.

2.5 *Symbiodinium* (family Symbiodiniaceae)

Symbiodinium spp. were recognized by arbitrary letters (e.g., A, B, C) that became referred to as "clades". Recently, in short, the genus *Symbiodinium,* based on genetics and ecology data, was split into seven new genera belonging to family Symbiodiniaceae, (LaJeunesse et al. 2018). Regardless of taxonomic classification, they are commonly approached for their endosymbiotic association with coral reefs (but they can also be associated with some species of anemones, jellyfish, sponges and others) (reviewed in Stat et al., 2006; Krueger & Gates, 2010). For these associations, most studies have sought to investigate the effect of environmental parameters on endosymbiosis with coral reefs and to clarify the main causes involved in coral bleaching events (McIlroy et al., 2016; Grégoire et al., 2017; Bernasconi et al., 2019). However, peridinin and toxins contents have also aroused, albeit simple, interest in cultivation aimed at the biotechnological applications of *Symbiodinium* spp. biomass (Benstein et al., 2014; Langenbach & Melkonian, 2019; Tsirigoti et al., 2020).

Biomass Production

Difficulties in reaching high biomass concentrations in cultures of dinoflagellates limit the commercial applications. This is mainly due to the sensitivity of many dinoflagellates to shear forces. Recently, the application of twin-layer porous substrate bioreactor (TL-PSBR) has been investigated in the laboratory. However, although projections are commonly made for large TL-PSBR (g m-2), operation of this bioreactor on an industrial scale is still doubtful (Langenbach & Melkonian, 2019). In addition to the TL-PSBR, bubble column photobioreactors (BC-PBR) have been used successfully for the biomass production of dinoflagellates (López-Rosales et al. 2016, 2017). The BC-PBR also controls shear stress, ensuring healthy growth of dinoflagellate cells. Moreover, the BC-PBR is likely to be more productive than the TL-PSBR because they have a larger photosynthetically active area than the biofilm of TL-PSBR. The improvement of photobioreactors for the intensive cultivation of dinoflagellates is still a basic process necessary for the development of this production chain.

Potential Aplications in Aquaculture and Future Directions

Due to the production of allelopathic compounds and the ability to grow under mixotrophic nutritional mode the dinoflagellates have a great potential to treat wastewaters. The microalgae, because of their use in wastewater treatment, have attracted increasing attention; they can

convert inorganic compounds into polyunsaturated fatty acids (PUFA), carotenoids, amino acids and others biomolecules, in addition to the secondary metabolites (Zeller et al., 2013; Oliveira et al. 2020b). This potential has not been sufficiently explored (Molina-Miras et al. 2020). In the case of PUFA, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), exhibit biological activities and are considered in the treatment of heart disease, cancer, type 1 diabetes and others (reviewed in Mendes et al., 2009). Hitherto, fish oil is the most widely used product of this category in the market even with some negative characteristics (e.g. distracting odor, allergic reactions, high refinery costs etc.). In addition, this use amounts to unsustainable exploitation of wild prey fish in aquaculture of fish and shrimp feed (Naylor et al., 2000). Based on this potential, a simplified model for production of dinoflagellate biomass using aquaculture wastewater is shown in **Figure 1**.

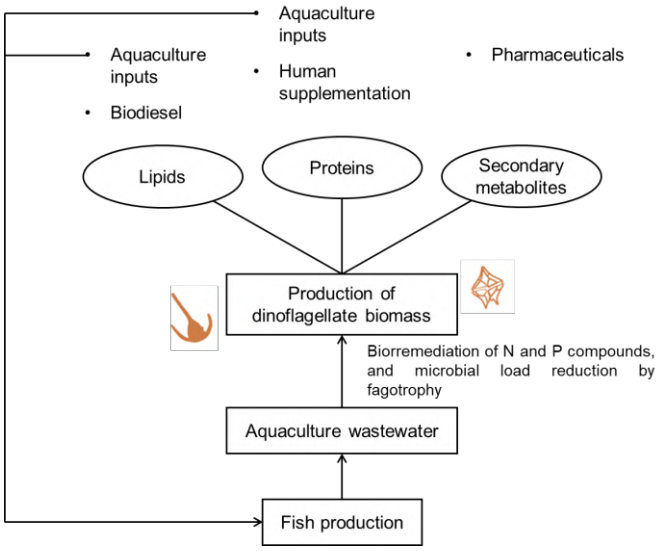

Figure 1. Simplified integrated model for the production of dinoflagellate biomass using aquaculture wastewater.

Recent interest in the cultivation of dinoflagellates has already resulted in substantial improvements and technological advances in the production processes. Limitation on commercial application of pigments and secondary metabolites produced by dinoflagellates is due to the lack of a reliable natural source of these macromolecules, since industrial-scale cultivation of dinoflagellates still faces barriers. Addressing some of these constraints will be a significant step towards the large-scale development of new inputs and drugs.

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3.3. Article 3: An overview on microalgae as renewable resources for meeting sustainable development goals

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Review

An overview on microalgae as renewable resources for meeting sustainable development goals

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ABSTRACT

The increased demands and dependence on depleted oil reserves, accompanied by global warming and climate change have driven the world to explore and develop new strategies for global sustainable development. Among sustainable biomass sources, microalgae represent a promising alternative to fossil fuel and can contribute to the achievement of important Sustainable Development Goals (SDGs). This article has reviewed the various applications of microalgal biomass that includes (i) the use in aquaculture and its sustainability; (ii) commercial value and emerging extraction strategies of carotenoids; (iii) biofuels from microalgae and their application in internal combustion engines; (iv) the use and reuse of water in microalgae cultivation; and (v) microalgae biotechnology as a key factor to assist SDGs. The future prospects and challenges on the microalgae circular bio economy, issues with regard to the scale-up and water demand in microalgae cultivation are also highlighted.

1. Background

Microalgae biomass offers opportunities for sustainable development of several industries, making its utilization important to drive global sustainable development. Microalgae can be found almost in any aquatic body that contains inorganic nutrients (such as carbon, nitrogen, phosphorus, and others oligoelements) and light (for performing oxygenic photosynthesis) although they can also grow heterotrophically using organic substrates (Chisti, 2008). Microalgal biomass can be converted into biodiesel, bioethanol, and biogas via processes such as liquefaction, pyrolysis, transesterification, fermentation, and anaerobic digestion (Abomohra et al., 2016; Muhammad et al., 2021; Oliveira et al., 2021a). In the food and pharmaceutical industries, microalgae are a proven source of essential amino acids and long–chain polyunsaturated fatty acids with antimicrobial, anti–cancer and antioxidants activities (Dantas et al., 2019; Lauritano et al., 2016).

Although the microalgae production chain is considered highly sustainable, many hurdles related to the high demand for water and high loads of organic waste, including residual microalgal biomass, still remain under–explored (Bui et al., 2015; Ramos-Suárez and Carreras, 2014; Serrà et al., 2020a). The current scenario of the large–scale production of microalgae biomass is predominantly based on extensive practices, *i.e.*, it uses old, inexpensive, and low–productivity systems that require large volumes of water (Colling Klein et al., 2018; Yadav et al., 2019). Furthermore, some microalgae market, such as *Haematococcus pluvialis* and *Dunaliella salina*, for example, that have a specific market directed to the extraction of high–value compounds, with components that do not exceed 10% of the biomass weight (astaxanthin and β–carotene, respectively), producing organic residues from microalgal biomass. In this way, providing further solutions to the remaining/leftover biomass is therefore considered a key step for long–term development of microalgae industry.

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Microalgae are also an ecofriendly and sustainable wastewater treatment option. Most wastewater contains macronutrients, *i.e.*, carbon, nitrogen and phosphorus, required for microalgal metabolism and growth, and therefore the growth of microalgae on wastewater for water treatment as well as energy production and/or other useful resources has encouraged research for development of circular economies (Hussain et al., 2021; Jaiswal et al., 2020). Effective wastewater treatment using microalgae, while producing a valuable biomass and improving the water quality levels, can also reduce coastal eutrophication and its negative impacts on fishery and aquaculture, tourism, and public health (Mardones et al., 2021; Oliveira et al., 2020).

This review attempts to deal with the knowledge gap for improving the sustainability of microalgae cultivation for feed, food, and bioenergy production through exploring water reuse strategies, and also the circular economy concept. Furthermore, microalgae biotechnology as a factor to assist United Nations Sustainable Development Goals is also presented. It is noteworthy that overcoming these current challenges related to the microalgae production will be beneficial not only for environment healthy but also for offset microalgal production costs.

2. Microalgal biomass applications

2.1. Aquaculture

Microalgae production is highly intended for aquaculture demand. Large volumes of microalgae cultures are produced daily for all growth stages of bivalve molluscs (clams, mussels, oysters, and scallops) and abalone, crustaceans (shrimp and prawns), fish, and for zooplankton as live food (Ashour et al., 2019; Muller-Feuga, 2000). Over the last decades, several hundred new species of microalgae have been reported, containing different biochemical compositions and biomolecules, but less than twenty microalgae species have been widespread use in aquaculture. Diatoms are the most prominent group of microalgae used in marine aquaculture due to its high abundance in marine and estuarine environments, and therefore serving as good choice for feeding larvae and adults of different fish species (Brown, 2002; Brown et al., 1997). The diatom *Conticribra* (*Thalassiosira*) *weissflogii* is a suitable source of proteins and carbohydrates for marine bivalve molluscs and crustacean larvae, while *Chaetoceros muelleri* stands out due to its fatty acids profile, cell size, and thin cell wall, which facilitates the digestion of intracellular compounds by shrimp larvae (Brown, 2002). Commonly mixed diets containing two or more microalgae species have been a successful strategy for different life stages molluscs and fish. For example, the use of mixed diets containing *Isochrysis galbana* and *Phaeodactylum tricornutum* improved the survival and growth rate of *Anomalocardia brasiliana* when compared to single microalga diets (de Oliveira et al., 2016). On the other hand, green algae such as Scenedesmaceae (i.e., *Scenedesmus*, *Desmodesmus* and *Tetradesmus* genera) and *Chlorella*–type species are more utilized in freshwater aquaculture, due to not only their cell size and biochemical composition, but also due to the cosmopolitan nature and simple isolation from environment and easy cultivation (Marques et al., 2019; Muller-Feuga, 2000). Moreover, other specific microalgae that synthetize significant amounts of biocompounds, such as astaxanthin from *Haematococcus pluvialis* used in salmonid industry, also deserve to be highlighted (Benemann, 1992). The main microalgae species intended for aquaculture purposed are highlighted in Table 1.

Historically, the type of production system used to produce microalgae in aquaculture hatcheries are simply designed photobioreactors, such as plastic bags, which may hinder the availability of light along the water column. Due to the low technology, the estimated cost for the production of microalgal biomass in this type of system is high i.e. USD 50–400 kg^{-1} (Oostlander et al., 2020). Although the use of mass microalgae systems is necessary to improve the sustainability of live foods for aquaculture, few efforts have been evolved in reducing the demand for water, which is already too high.

Microalgae can be grown using various aquaculture wastewater. The use of aquaculture wastewater to reduce the water demand and produce a valuable biomass is indeed a highly sustainable approach. Some studies have reported the successful cultivation of both freshwater and marine microalgae using wastewater from different aquaculture systems (Andreotti et al., 2020; de Oliveira et al., 2020; Liu et al., 2019). Nevertheless, some microalgal exudates have the potential to inhibit beneficial bacteria in some systems, such as biofloc technology, making this route of water reuse unfeasible (Natrah et al., 2014). Despite that, the effects of adding microalgae to aquaculture systems that have a well–established microbiota, such as biofloc and synbiotic systems, has not been fully explored.

Another emerging topic, which has a very promising market, is the usage of algal concentrates instead of using fresh microalgae cultures (Militz et al., 2018, 2021). Microalgae concentrates can replace facilities for massive cultivation of microalgae to feed larvae of fish and crustaceans that depend on microalgae for their nutrition. However, the benefits and harms of using microalgae concentrates have not yet been fully explored due to the scarcity of information and data available in the specific literature.

Table 1
Main microalgae species used in aquaculture and their biocompounds.

Species	Water environment	Utility	Interest/ biocompounds
Isochrysis galbana	Marine	Bivalve molluscs, Crustacea larvae, Zooplankton	PUFAs, Fucoxanthin
Pavlova lutheri	Marine	Bivalve molluscs	PUFAs
Chaetoceros spp.	Marine	Bivalve molluscs, Crustacea larvae, Zooplankton	PUFAs
Thalassiosira/ Conticribra spp.	Marine	Bivalve molluscs and Crustacea larvae	EAA and Carbohydrates
Navicula spp.	Marine	Crustacea larvae	PUFAs
Nannochloropsis spp.	Marine	Zooplankton and finfish	PUFAs
Chlorella spp.	Freshwater	Zooplankton and finfish	PUFAs, EAA
Scenedesmus spp.	Freshwater	Zooplankton and finfish	PUFAs and EAA
Ankistrodesmus sp.	Freshwater	Zooplankton and finfish	PUFAs and EAA
Arthrospira platensis (Spirulina)	Freshwater	Protein input	EAA and Phycobiliproteins
Haematococcus pluvialis	Freshwater	Coloring purpose (pigments)	Astaxanthin

PUFAs = polyunsaturated fatty acids; EAA = essential amino acids.

2.2. Carotenoid's market

Carotenoids are lipophilic compounds that constitute a class of terpenoid pigments derived from a 40–carbon polyene chain (Gong and Bassi, 2016; Guedes et al., 2011). They are responsible for the yellow, orange, brown, and red coloration of algae, plants, and animals (like flamingos, crustaceans and fishes) (Cezare-Gomes et al., 2019; Negro and Garrido-Fernández, 2000; Saha et al., 2020).

2.2.1. Astaxanthin

Astaxanthin, a red colored carotenoid, is a powerful antioxidant that has drawn interest from many industries. Because of the presence of hydroxyl (–OH) and ketone (–CO) functional groups, the structure of astaxanthin is considered polar and susceptible to oxidation, thereby providing antioxidant properties (Mota et al., 2021; Rammuni et al., 2019). Due to these properties, this compound is widely explored in many areas, such as human health (pharmaceuticals, nutraceuticals and dietary supplements), cosmetics and nutrition (food, animal feed, pigments for food and beverages).

Total market value of astaxanthin is expected to reach US$ 800 million by 2022, with a compound annual growth rate of 8%. Although most of the commercially exploited astaxanthin is produced by synthetic routes, there is a growing demand for products derived from natural and sustainable sources, such as microalgae (Fábryová et al., 2020).

Therefore, microalgae may play a fundamental role in this scenario, as many of them can synthesize astaxanthin as a secondary metabolite. *Chlorella vulgaris, Chlorella zofingiensis, Chlorococcum wimmeri, Dunaliella salina, Botryococcus braunii* and *Scenedesmus obliquus* are some species that have the potential to produce this carotenoid (Markou and Nerantzis, 2013; Rammuni et al., 2019). However, *Haematococcus pluvialis* is considered the best source of natural astaxanthin, and even under unfavorable conditions, i.e., nutrient deficiency, high light intensity or high temperature, the microalga cells are able to synthesize up to 7% of dry weight of astaxanthin, and 90% of its total carotenoids content (Marinho et al., 2022; Shah et al., 2016). In this microalga, astaxanthin is found mainly in the form of esters comprising of astaxanthin monoesters (70%) and astaxanthin diesters (25%). Thus, only 5% of the total astaxanthin in *H. pluvialis* corresponds to free–astaxanthin (non–esterified form) (Lorenz and Cysewski, 2000). Commercially, astaxanthin is mainly offered as an oleoresin, in which only 10–15% (w/w) corresponds to total astaxanthin, being the majority composed by acylglycerols and other minor carotenoids (Shah et al., 2016). In fact, esterified astaxanthin may be responsible for biological properties attributed to the free astaxanthin form, once these oleoresins are considered to be composed by the latter, overlooking the fact that this pigment is present in only low amounts (Holtin et al., 2009; Régnier et al., 2015; Richard et al., 2008). In a study conducted by Hosseini et al. (2020), the biomass of microalga *H. pluvialis* was mainly composed of carbohydrates, followed by proteins and lipids, and minor content of ash and moisture. According to these authors, the residual biomass consisted primarily of carbohydrates (~49%), which included a high percentage (~36%) of starch (α–glucan) and 13% of structural carbohydrates (glucan, mannan, and other carbohydrates), which corroborates with findings of Haque et al. (2017). The high carbohydrate content of the residual biomass highlights the potential for using it as a carbon source for subsequent bioethanol production, which not only proves that integration processes but also supports a sustainability improvement.

There are many ways of recycling the residual microalgae biomass, and anaerobic digestion is one of the most common practices. By stabilizing the waste in the absence of air, the anaerobic digestion process transforms the microalgal biomass into CO_2, CH_4 and also, a nutrient rich digestate, which can be used for production of biogas, biofertilizers, and animal feed (Onorato and Rösch, 2020).

2.2.2. Lutein

Lutein, a xanthophyll and yellow colored pigment, is an oxygenated carotenoid found primarily in plants, but also known to be produced by certain microalgae species, which, in fact, are able to achieve a higher lutein production rate than that in plants (Guedes et al., 2011; Lin et al., 2015). The global market of this compound is increasing considerably and it is predicted that USD 357.7 million will be achieved by 2022.

There are several microalgae species already identified as potential lutein producers, such as *Chlorella sorokiniana* (Chen et al., 2017), *Parachlorella* sp. (Heo et al., 2018), *Chlamydomonas* sp. (Zhao et al., 2019), *Desmodesmus* sp. (Xie et al., 2014), *Tetradesmus obliquus* (Ho et al., 2015), and *Chlorella vulgaris* (Gong and Bassi, 2017). It is worth noting that some of these species might be able to produce up to 5 g of free lutein per kg biomass (Ochoa Becerra et al., 2020). The antioxidant (Ávila et al., 2016) and anti–inflammatory (Buscemi et al., 2018) properties, and its protective role in age–related macular degeneration (Eisenhauer et al., 2017) and diabetic retinopathy (Neelam et al., 2017) has made lutein very popular as a food and feed additive (*e.g.*, to modify the color of egg yolk in hen farming) (Lin et al., 2015; Mussagy et al., 2019). Besides this, researchers have reported other beneficial health effects of lutein such as the promotion of infant brain development and anti–atherogenic, antihypertensive, antiulcer, and anticancer activities (Fitzpatrick and Dhawan, 2014; Kim and Park, 2016).

In a study conducted by Nobre et al. (2013), the microalga *Nannochloropsis* sp., which is able to synthetize lutein, was cultivated in polyethylene bags of 10 L capacity to produce biomass feedstock for the production of fatty acids for biodiesel, biohydrogen, and pigments with high added–value. The residual biomass, after extraction of carotenoids, used as a substrate in a dark fermentation process in combination with *Enterobacter aerogenes*, could successfully produce green hydrogen. Huang et al. (2020) demonstrated the feasibility of ethanol production from pigment–extracted residual biomass of *Chlamydomonas* sp. The authors stated that the biomass residue was composed mainly of carbohydrates (65–67% starch, 8–10% cellulose). In addition, *Chlamydomonas* strain appears to be a microalgae candidate for integrated production of carbohydrate (64.3%, 438 mg L^{-1} d^{-1}) and lutein production (5 mg g^{-1}, 3.5 mg L^{-1} d^{-1}).

2.2.3. β–carotene

β–carotene, an orange pigment, is a chemical compound produced by several microalgae, such as *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Muriellopsis* and *Haematococcus* spp. (Gateau et al., 2016; Pourkarimi et al., 2020). It is precursor of vitamin A and have applications as food coloring agent and additive to cosmetics. Owing to the antioxidant properties, β–carotene is extensively used in food and animal feed. Besides, β–carotene increases immunity (Vílchez et al., 2011; Yaakob et al., 2014), inhibits and prevent several types of cancer and tumors, and also shows effectiveness in controlling cholesterol levels and reducing the risk of cardiovascular diseases (Gateau et al., 2016; Rammuni et al., 2019).

Under certain conditions of light and salt, *Dunaliella salina* can accumulate β–carotene up to 14% of its dry weight, while other microalgae such as *Chlorella zofingiensis* and *Arthrospira platensis* may reach 0.1–2.0% of dry biomass weight (Raposo et al., 2015). Moreover, *D. salina* can also synthesize high amounts of triglycerides (Minhas et al., 2016), with the fatty acid content reaching up to 30–60% of its dry weight (Shah et al., 2016; Ye et al., 2008). Hence, the lipids extracted from the residual biomass, after carotenoid recovery can be employed for co–production of biodiesel through transesterification. Also, the carbohydrate content in this microalga can be enhanced up to 50–60% of dry weight, thereby making it viable to produce bioethanol as a by–product in biorefineries (Doan et al., 2012).

Likewise, Francavilla et al. (2015) conducted a study where in residual mass of *D. tertiolecta*, post extraction of β–carotene and other high added–value products, was suitable for bio–oil and char production through fast pyrolysis. It is worth noting that the char produced was rich in nitrogen and other macro–elements, highlighting its use in agriculture as biofertilizer. Similarly, in a new method developed by Damergi et al. (2017), glycerin was recovered as a coproduct of β–carotene extraction from the filtered residue of the saponification of chlorophylls.

There are many opportunities for future research, including explorations of less studied carotenoids, such as peridinin (found in phototrophic dinoflagellates) and myxol (found in freshwater cyanobacteria), and their applications (Novoveská et al., 2019; Oliveira et al., 2020). On the other hand, a number of studies have been pointed fucoxanthin as a promising carotenoid for large–scale production. Thus, it is expected that the fucoxanthin market will be consolidated in the coming years.

2.3. Bioenergy

In recent years, microalgae feedstock based third–generation biofuels are in the spotlight as new source of bioenergy and fuel production. This can be attributed to the sustainable aspects of microalgae cultivation compared to traditional generation biofuels from plants, which had high production cost, large land usage, higher water requirement with low conversion rates. The derivative products from microalgae biomass are diverse and range from liquid to gaseous fuels with various

applications mainly in the field of internal combustion engines. The next section will briefly describe the multiple possibilities of bioenergy from microalgae biomass and its application in different internal combustion engines.

2.3.1. Classification of bioenergy based on microalgae biomass processing

Microalgae as a feedstock is an excellent source of biomass to derive various forms of bioenergy. Microalgal biomass can be converted into multiple forms of renewable biofuels such as biodiesel, bio–oil, biogas, bioethanol and bio–hydrogen through transesterification, pyrolysis, liquefaction, fermentation and gasification, respectively (Günay et al., 2019). The conversion methodology to convert algal biomass into these bio–energies can be classified as thermochemical, chemical and biochemical conversion as illustrated in Fig. 1. The most commonly exploited derivative of microalgae biomass is the synthesis of biodiesel and bio–oil. Biodiesel is comprised of a mixture of monoalkyl esters which are long–chain fatty acid methyl esters obtained from the lipid part of the algal biomass. Intracellular lipids can be extracted from the biomass using certain solvent extraction methods such as Bligh and Dyer, Soxhlet method, Folch method, pressurized liquid extraction and supercritical carbon dioxide (SC–CO_2) extraction (Ryckebosch et al., 2014). The oil acquired from the lipids is converted into biodiesel using multiple chemical reactions between triglycerides and alcohols through direct transesterification. Acid or alkali catalysts are used to mediate the reactions using an external heat source where the methyl esters separate from layer glycerol.

Various solvents and their ratios used for lipid extraction and transesterification of algal oil extracted from different microalgae species are listed in Table 2. Also, the lipid quantity and their respective yield rates alongside with several microalgae species are highlighted. *Chaetoceros muelleri*, *Nannochloropsis* spp., *Botryococcus braunii*, and *Spirogyra* sp. have the highest lipid quantities and yield rates (Arias--Peñaranda et al., 2013; Duan and Shi, 2014; Jiménez Callejón et al., 2014; Nagle and Lemke, 1990; Yuvarani et al., 2017). Other notable microalgal species that showed high content in fatty acid methyl esters are *Dunaliella salina* and *Schizochytrium limacinum*. As per American Society for Testing and Materials (ASTM) standards, the physicochemical properties of most microalgal biodiesel fuels can be directly applied in the existing designs of diesel engines with minor adjustments (Ryckebosch et al., 2014). Bio–oil is another microalgal biomass derivative, which can be synthesized by treating the microalgae biomass to pyrolysis. The bio–oil produced after pyrolysis meets the ASTM standards for transportation sectors (Castello et al., 2018). Several parameters such as ash content, pyrolysis temperature, water, biomass composition and vapor residence time are taken into account to quantify the algal bio–oil productivity. Production of bioethanol from microalgae biomass is made possible by fermentation of the biomass with engineered bacterium that produces chemical changes from organic (carbohydrates or starch) substrates through the action of some enzymes. Microalgae species such as *Chlorella vulgaris*, *Spirogyra* and *Chlorococcum* contain about 37% of starch by dry weight which is ideal for bioethanol or other higher–chained alcohol production like butanol (Behera et al., 2015). Microalgae derived biodiesel and bio–oil can be homogenized with conventional diesel fuel at optimal conditions and utilized as fuels in compression ignition engines without any additional components or modifications. Equally, microalgae–based bioethanol or other higher alcohols can theoretically be used as a fuel additive with diesel in compression ignition engines or directly in a spark–ignition engine with conventional gasoline fuel. However, there are not many studies incorporating alcohols derived from microalgae biomass in internal combustion engines, which should be explored more by researchers. The next section deals with the applications of these biofuels in internal combustion engines with their respective performance, combustion and emission characteristics for different blend ratios.

2.3.2. Application of microalgae biofuels in internal combustion engines

The physicochemical properties of most biofuels such as kinematic viscosity, cetane number, density, lubricity, cloud point, flash point,

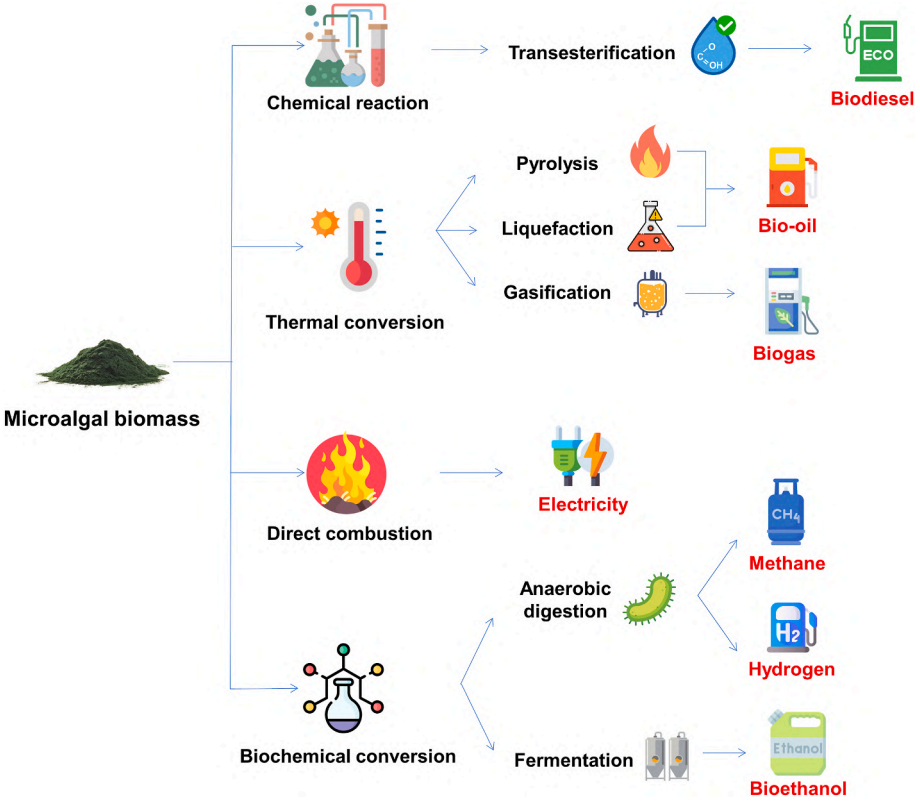

Fig. 1. Classification of microalgal biofuels based on the processing of its biomass.

Table 2
Extraction solvents of multiple microalgae species with their lipid content for biodiesel and bio-oil production.

Microalga	Method of extraction	Transesterification solvents and catalysts	Lipid content (%)	Yield rate (% dry weight)	Nature of fuel			Ref.
					Biodiesel	Bio-oil	Alcohol	
Ankistrodesmus falcatus	Bligh and Dyer's extraction (1:1)	–	4–12	10–13	✓	✓	✓	El-Sheekh and Hamouda (2016)
Botryococcus braunii	Solvent extraction	Solution of Nano calcium oxide in methanol	83–87	75–80	✓	✓	×	Prasad et al. (2015)
Chaetoceros muelleri	Hexane:2-propanol- 2:3 extraction solvents	Sodium hydroxide-methanol and hydrochloric acid-methanol	46–90	10–16	✓	✓	×	Nagle and Lemke (1990)
Dunaliella salina	Soxhlet apparatus (n-hexane)	Saponification (ethanoic potassium hydroxide (20%) + hydrochloric acid)	60–70	37–45	✓	✓	×	El-Ayouty et al. (2015)
Nannochloropsis gaditana	Saponification oil separation (potassium hydroxide + methanol)	Chloroform/methanol (1:1) and hexane + isopropanol	35–60	27–58	✓	✓	×	Ryckebosch et al. (2014)
Nannochloropsis oculata	Photo-catalysis and methanolysis	Methanol + 1% sulfuric acid	50–53	31–68	✓	✓	×	Arias-Peñaranda et al. (2013)
Nanocholoropsis sp.	Saponification/centrifugal (hexane solvent)	Methanol + Sulfuric acid	50–56	20–25	✓	✓	×	Jiménez Callejón et al. (2014)
Neochloris oleoabundans	Ethanol butanol acetone extraction/Bligh and Dyer's extraction	For 1 L of algae oil, 200 mL of methanol + sodium hydroxide at 60–80 °C	13–15	2–6	✓	✓	✓	(Du et al., 2016, 2018)
Phormidium valderianum	Supercritical CO_2 extraction	Alcoholysis using petroleum ether	4–6	5–10	✓	✓	✓	Chatterjee and Bhattacharjee (2014)
Scenedesmus incrassatulus	Bligh and Dyer's extraction (1:2:0.8)	Oil: methanol-1:6	20–25	17–21	✓	✓	✓	Arias-Peñaranda et al. (2013)
	Bligh and Dyer's extraction (1:2:0.8)	Enzymatic transesterification (novozym oil: methanol- 1:6)	23–24	19–21	✓	✓	×	
Schizochytrium limacinum	Bligh and Dyer's extraction	methanol, sulfuric acid, and chloroform heated at 90 °C for 40 min	63–37	36–39	✓	✓	✓	Johnson and Wen (2009)
Spirogyra sp.	Soxhlet method (chloroform: methanol-2:1)	2% sulfuric acid dissolved in methanol	10–15	–	✓	✓	×	Kumar et al. (2011)
	Soxhlet extraction (hexane: oil-1:2)	–	–	55–80	✓	✓	×	Konga et al. (2017)

calorific value, lower heating value, octane number and latent heat of vaporization are fairly similar to conventional diesel or gasoline fuel. Therefore, to further enhance the fuel quality of conventional fuels, homogenizing these biofuels with conventional fuels is a key step to initiate the process (Kumar et al., 2011). Microalgae–based biofuels are homogenized using sophisticated ultra sonicators at different compositions such as 80% diesel +20% biofuel (B20), 70% diesel + 30% biofuel (B30), 60% diesel + 40% biofuel (B40) to avoid phase separation. These fuel blends can be operated in compression ignition engines at various operating conditions. The effectivity of microalgal fuel blends can be quantified based on the corresponding engine output parameters such as brake thermal efficiency (BTE), brake specific fuel consumption (BSFC), peak in–cylinder pressure (PP), heat release rate (HRR), exhaust gas temperature (EGT), oxides of nitrogen (NO_X), carbon monoxide (CO), carbon dioxide (CO_2), hydrocarbon (HC), particulate matter (PM) and smoke emissions. All these parameters collectively, fall under performance characteristics (BTE, BSFC), combustion characteristics (PP, HRR, EGT) and emission characteristics (NO_X, CO, PM, CO_2, smoke) (Nautiyal et al., 2020).

Microalgal biodiesel and bio–oil are predominantly used in compression ignition engines compared to other derivates of the biomass. Multiple studies which have incorporated different ratios of microalgal biodiesel and bio–oil with conventional diesel fuel to power a compression ignition engine are illustrated in Table 3. The blend mixtures are categorized alongside the respective microalgae biomass in addition to their effects on engine output characteristics. The studies presented in Table 3 have shown that biodiesel derived from the microalgae *Spirulina* sp., *Chlorella protothecoides, Chlorella vulgaris*, *Scenedesmus* sp., *Botryococcus braunii* and *Navicula* sp. displayed an increase in brake specific fuel consumption (BSFC) when their compositions are higher in the homogenous blend. This can be attributed to the lower heating value and higher density of microalgal biodiesel compared to conventional diesel. Similarly, brake thermal efficiency (BTE) of the microalgal biodiesel blends is lower when considering higher mixing ratios, and vice versa. This is due to the inherent oxygen content of the biodiesel blend at lower ratios which facilitates complete combustion and improves BTE (Rajak et al., 2020a, 2020b, 2020c; Rajak and Verma, 2018; Subramaniam et al., 2020). Combustion parameters, such as peak in–cylinder pressure (PP), have shown a slight reduction at lower loads and an increase in higher loads. This is due to the superior air-fuel mixing at higher loads which increases the in-cylinder temperature in PP. This is also boosted by the lower cetane number of microalgal biodiesel blends, which in turn, creates a longer ignition delay, causing more fuel mass to be combusted. At lower loads, the higher viscosity of the microalgal biodiesel blends dramatically hampers the combustion characteristics (Al-lwayzy and Yusaf, 2017; Krishna Kolli et al., 2019; Uludamar, 2018).

In terms of emission characteristics, including CO emissions, fossil diesel is generally lower for the biodiesel derivatives of the above–stated microalgae species due to, in most cases, complete combustion. Also, the excess oxygen (O_2) levels in the microalgal biodiesel oxidize the CO into CO_2 emissions. Likewise, hydrocarbon (HC) emissions that are formed during air–fuel mixture, dramatically reduces for almost all microalgae species. Nitrogen oxide (NO_X) emissions and particulate matter (PM) concentrations have an inverse relationship, trying to reduce one may increase the other. Almost all studies stated in Table 3 shows an increase in NO_X emissions due to the longer residence time of exhaust gases, higher in–cylinder temperature and PP, which is boosted by the highly oxygenated nature of microalgal biodiesel (Arunprasad and Elango, 2020; Hossain et al., 2019; Karthikeyan et al., 2020; Karthikeyan and Prathima, 2017; Tayari and Abedi, 2019). Dramatic reductions in smoke and PM emissions as a NO_X trade–off for all microalgae species are also specified in Table 3.

A generalized view of the effects of multiple microalgae species and

Table 3
Effect of microalgae biodiesel and bio-oil at different compositions in compression ignition engines and the outputs.

Microalga	Fuel nature and blend composition	Combustion characteristics	Performance characteristics	Emission characteristics	Ref.
Botryococcus braunii	Biodiesel B20	–	BTE ↑ for TiO_2–SiO_2 nano additive doping to biodiesel	↓ CO, ↓ HC, ↑ CO_2, ↑ NO_x	Karthikeyan and Prathima (2017)
Chlorella protothecoides	Biodiesel B20	↓Combustion duration	5% ↑ BTE, 3% ↓ BSFC for B100 and B20	22% ↓ HC, 6.5% ↑ NO_x, ↓ smoke emissions for B20 blend	Yasar and Altun (2018)
	Biodiesel B20, B50 and B100	6.1% ↓ EGT	5.7% ↑ BTE, 10% ↑ BSFC for B100	28% ↓ CO, 16% ↓ CO_2, 7.4% ↓ NO_x, 12% ↓ smoke emissions for B100	Al-lwayzy and Yusaf (2017)
	Biodiesel B85	–	6% ↑ BTE, 8.5% ↓ BSFC for EGR with ceramic coated engine	47% ↓ CO, 51% ↓ HC, 18.5% ↓ NO_x, 46% ↓ PM for EGR with ceramic coated engine	Krishna Kolli et al. (2019)
	Biodiesel B20	–	–	10% ↓ CO, ↓ CO_2, 9% ↑ NO_x, on adding hydroxy and hydrogen to B20 blend	Uludamar (2018)
Chlorella vulgaris	Biodiesel B10, B15 and B20	–	BTE ↑ for B20 blend	20% ↓ CO, 31% ↓ HC, 38% ↑ NO_x for B20 blend	Patel et al. (2014)
	Biodiesel B40 and B50	–	BTE ↓, BSFC ↑ with increasing blend percentage	↓ CO, ↓ HC, 1.5% ↓ CO_2	Mathimani et al. (2017)
	Biodiesel B20 with hydrogen	–	6.6% ↑ BSFC B20 blend with hydrogen gas	↓ CO, ↓ HC, ↓ CO_2, ↑ NO_x	Tayari and Abedi (2019)
	Bio-oil and Biodiesel B100	↑ PP	BTE ↓, BSFC ↑	↓ CO, ↓ HC, ↓ smoke, ↓ NO_x	Satputaley et al. (2018)
Navicula sp.	Bio-oil B20	–	BTE ↑, BSFC ↓	↓ CO, ↓ HC, ↓ smoke emissions	Arunprasad and Elango (2020)
Scenedesmus sp.	Biodiesel B25 and B50	↑ PP, ↑ HRR	0.3% ↓ BTE, 3.8% ↑BSFC for B25 blend	23% ↓ CO, 15% ↑ NO_x, 95% ↓ PM	Hossain et al. (2019)
Spirulina spp.	Biodiesel B20, B40 and B100	–	3% ↓ BTE, 3.3% ↑ for B20 blend	NO_x ↓, Smoke emissions ↓ for ternary blends of B20, B40 & B100	Rajak et al. (2020a)
	Biodiesel B20, B40, B60, and B80	↓ PP	3% ↓ BTE, 3.2% ↑ for B20 blend	CO_2↑, NO_x, 19% ↓, 42% ↓ smoke emissions, 41% ↓ PM for B100	Rajak et al. (2020b)
	5, 10, 15 and 20% hydrogen with biodiesel	↑ PP	BTE ↑, BSFC ↓ with 15% and 18% hydrogen addition	36% ↓ CO_2, NO_x↑, 23% ↓ smoke emissions, 51% ↓ PM	Rajak et al. (2020c)
	Biodiesel B20 and B100	↑ PP, ↑ HRR	1.2% ↓ BTE, 3.2% ↑ BSFC for B20 blend	CO_2↑, 6.2% ↓ NO_x, 2.6% ↓ smoke emissions, 12.4% ↓ PM for B20 and B100	Rajak and Verma (2018)
	Biodiesel B0, B20, B40, B60, B80 and B100	1.7% ↓ EGT	BTE ↓, BSFC ↑ with increasing blend percentage	4% ↓ CO, 3% ↓ CO_2, 16% ↓ HC, 7% ↓ NO_x, 12% ↓ smoke emissions for B20 blend	Rajak et al. (2020c)
	Biodiesel B100	1.6% ↓ EGT	2.7% ↓ BTE, 6.4% ↑BSFC for B100	5% ↓ CO_2, 6% ↓ NO_x, 3% ↓ PM for B20 and B100	Rajak et al. (2019)

SFC – Brake specific fuel consumption; BTE – Brake thermal efficiency; EGR – Exhaust gas recirculation; EGT – Exhaust gas temperature; HC – Hydrocarbon; HRR – Heat release rate; NOx – Nitrogen oxides; PM – Particulate matter; PP – Peak in-cylinder pressure.

their biodiesel derivatives with their corresponding engine outputs characteristics while operating in a compression ignition engine is described in Fig. 2. Studies which have incorporated *Arthrospira platensis* (Spirulina) and *Chlorella vulgaris* biodiesel have shown promising engine output results compared to other microalgae species. Moreover, fuel additives such as ethers and nano additives such as metal oxide are added to microalgae biodiesel to enhance their cetane index or ignition improvement by altering the physicochemical properties of the

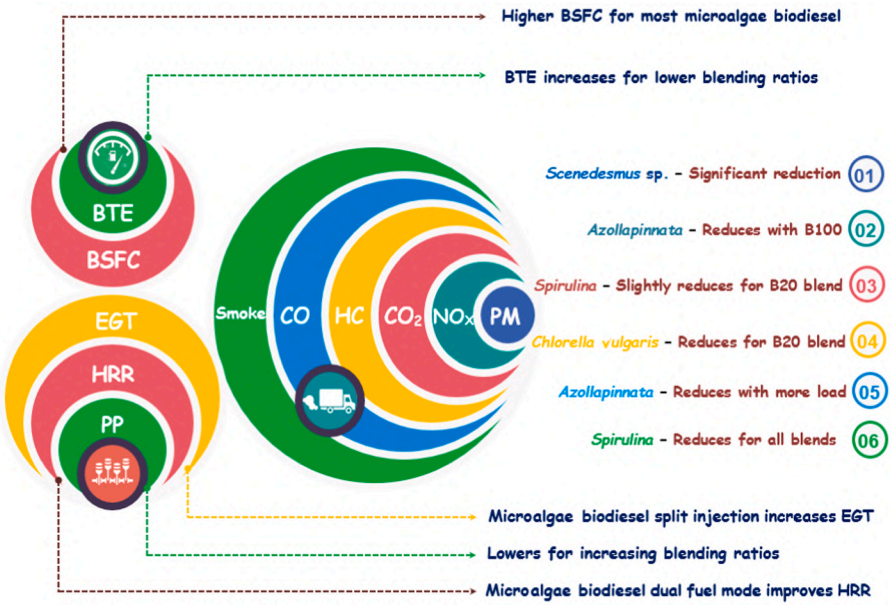

Fig. 2. General norms of microalgal biodiesel on engine output parameters.

homogenous blend. It has been reported that ethers improve the performance and emission characteristics of a microalgae biodiesel blend (Satputaley et al., 2018).On the other hand, metal oxide nano additives improve the volatility and reduce the viscosity of the blend, which in turn leads to improved air–fuel mixing formation, superior atomization and vaporization during injection of fuel (Satputaley et al., 2018). Furthermore, this superior atomization enhances the spray penetration characteristics and ultimately delivers optimal engine output characteristics. Although there is a lack of research microalgae biofuels in spark–ignition engines, it is theoretically possible to draw an inference. Bioethanol and other higher alcohols such as bio–butanol have similar physicochemical properties as conventional gasoline. Hence, at optimal proportions, these bio–alcohols can be homogenized with gasoline to power a spark–ignition engine. Therefore, so far, microalgae biodiesel can adequately replace conventional diesel fuel by producing comparable engine output characteristics with superior engine efficiency in compression ignition engines.

3. Water demand for microalgae cultivation

The high–water demand for aquaculture is one of the environmental concerns for development of environmentally sustainable aquaculture. Microalgae can be cultivated using freshwater, brackish and saline waters. It is important to highlight that freshwater resources will still remain a requirement in the life–cycle processes with uses that may include pond makeup water to maintain target salinities (because of the water evaporation), flushing salt accumulation in ponds, equipment cleaning, and biomass processing (Venteris et al., 2013; Xu et al., 2020). Thus, reducing freshwater demand using feasible and sustainability routes in large–scale microalgae biomass production is of extreme urgency. An interesting study conducted by Xu et al. (2020) reported guidelines for the implementation of microalgae biofuel systems with reduced water demand in the United States. The authors identified that is possible to scale algae biofuel production to 20.8 billion liters of algae–diesel annually without significant water–stress impact in the United States, based on three site–selection criteria: (1) biomass productivity, (2) water–use efficiency and (3) water–stress impact.

Most microalgae cultivation systems were developed thinking in terms of production volume (Nwoba et al., 2019). But, today, the use of large amounts of water as well as high loads of nutrients, labor and extensive land must be considered. However, the land where microalgae production is sited is not so relevant, because microalgae can be cultivated in non–arable land and hence does not compete with traditional agriculture (Chisti, 2007). The cultivation system employed also depends on the species to be cultivated, for example, *Arthrospira platensis*, and other filamentous algae are more appropriated to be cultured in raceways ponds due to the sensitivity to higher shear forces present in closed photobioreactors (Grobbelaar, 2012). Details of different production systems used for microalgae large–scale biomass production are shown in Table 4.

Some of the main microalgae producing countries include China, Germany, Spain, and Italy. In these high economic countries, cultivation occurs mostly in closed photobioreactors (Araújo et al., 2021). However, the use of closed photobioreactors in low–income countries is not an accessible reality as these intensive systems require high investments and management skills, thus hindering the development of microalgae cultures in such countries (Merlo et al., 2021). Therefore, substantial advances in the development and improvement of low–cost systems for intensive microalgae cultivation are needed to contribute to the growth of this activity in low–income countries.

3.1. Water reuse strategies

A number of studies have explored microalgae growth in reused water, and several substances that can stimulate or inhibit microalgal growth and metabolism have been reported (Andrade et al., 2019; Fret et al., 2020; Molina-Miras et al., 2020; Molino et al., 2020). In a comparative study between a *Jatropha curcas* L. plant and microalgae, the water requirement for biodiesel production was 5,787 and 31,361 m^3 per ton of biodiesel, respectively (Zhang et al., 2014). Although microalgae do not compete for water and soil used in agriculture, the total volume requirements remain high. Therefore, reusing the cultivation water as well as recycling wastewater as culture medium, are emerging sustainable solutions for making biodiesel production from microalgae (Lu et al., 2020; Zhang et al., 2020).

3.1.1. Growth stimulation

Chemicals compounds and organisms used for microalgal harvesting can stimulate a microalgae culture. Some flocculants, such as ferric and aluminum salts, and chitosan, increasing the growth rate of microalgae (Delrue et al., 2015). Flocculants can also remove potential growth inhibitors, like ferric chloride–based flocculants that can form bond with some inhibitory macromolecules are removed during flocculation (Lu et al., 2020). Furthermore, microalgae and bacteria exudates, such as polyamines, peptides and glycopeptides synthetized by both microalgae and bacteria can have stimulatory effects (Grabski and Tukaj, 2008; Sabia et al., 2015). Interestingly, the cultivation of a strain of *Spirulina* sp. in reused culture medium for four consecutive cycles led to an increased carbohydrate content, which would be an interesting strategy for the production of biohydrogen and/or bioethanol, contributing to the microalgal circular economy (Andrade et al., 2019).

3.1.2. Growth inhibition

There are several substances that may cause harmful effects on microalgae metabolism when reutilizing water from the spent medium. Such substances involving growth inhibition include cell wall debris, excessive bacteria, high load of dissolved organic matter, salinity and harvesting process (Lu et al., 2020). Some species, such as *Nannochloropsis* spp., split their cell walls during cell division, and glycoproteins and polysaccharides of the cell wall of this type of algal species serve as substrate for inducing the rapid proliferation of bacteria (Rodolfi et al., 2003). In general, when utilizing reused water for microalgae cultivation, bacteria, due to smaller size, remain in the water after microalgal cells are harvested. Thus, some expensive methods, such as ultrafiltration or UV treatment are required to enable water reuse, as bacteria can easily dominate a microalgae culture system due to its accelerated growth and metabolism (Deschênes, 2016). Consequently, the accumulation of organic matter may also hinder the

Table 4
Productive characteristics for different type of system used in microalgae cultivation.

Production system	Implementation cost	Skilled labor	Energy demand	Biomass reached (kg m^{-3})	Water use (m^3 biomass ton^{-1})
Open systems					
Open pond	Very low	No	No	0.05–0.5	20,000–2,000
Aerated tank	Very low	No	Low	0.02–0.8	50,000–1,250
Raceway ponds	Low	No	Low	0.5–1.5	2,000–666.67
Thin-layer	Very high	Yes	High	10–50	100–20
Closed systems					
Photobioreactor	High	Yes	High	2–5	500–200
Fermenter	Very high	Yes	High	5–20	200–50

penetration of light, thus reducing the ability of microalgae to efficiently perform photosynthesis (González-Camejo et al., 2020).

Reused water contains nutrients, which may reduce nutrient addition costs (Lu et al., 2020). However, monitoring the oligoelements concentrations in the reused water is an expensive and maybe an unviable process. Also, accumulation of specific elements (such as magnesium) can also cause cell aggregation (Vandamme et al., 2012). Accumulation of ions in algal cultures can increase the salinity but it can be measured over the time of microalgae cultivation. Chemicals used for harvesting microalgal cells can also be toxic to microalgae. Strong photocatalytic activity of nanocomposites can kill microalgae cells in presence of light (Serrà et al., 2020b). NaOH–residual in reused medium inhibited the photosynthetic activity of *Dunaliella salina* (Pirwitz et al., 2015). Thus, further research on microalgal cells harvesting using green/bio–flocculants may be a promising topic for the improvement of water reuse techniques after flocculation–based harvesting.

3.2. Wastewater as culture medium

Sever drinking water shortage around the globe has urged leading researchers to look for strategies for reutilizing different types of effluents for microalgae cultivation (Oliveira et al., 2021b). Due to their metabolic versatility, microalgae are promising organisms for treating a variety of wastewater types using photoautotrophic, heterotrophic or mixotrophic routes (Wollmann et al., 2019). In recent years, microalgae–based wastewater treatment process has been considered one of the most promising technologies for the advanced treatment of wastewater. Some of these examples are illustrated in Table 5. For optimal microalgae growth in wastewater, the effluent should have the following conditions: (i) low turbidity, ensuring the light penetration, if conducted under photoautotrophic and mixotrophic conditions, (ii) absence of pharmaceuticals and/or heavy metals pollutants, if the biomass is intended for food and feed applications, (iii) low/moderate microbial contamination, mainly by bacteria and/or predators, such as protozoa, for example (Nagarajan et al., 2017; Sánchez Zurano et al., 2020; Wollmann et al., 2019). A mutual and synergistic effect maybe found between microalgae and bacteria in a co–existing wastewater treatment system (Ma et al., 2014). Algal–bacterial consortia could enhance the assimilation of nutrients, notably nitrogen and phosphorus, resulting in a higher biomass productivity (Jiménez-Pérez et al., 2004; Unnithan et al., 2014). Generally, bacteria assimilate organic carbon for its growth and provide CO_2, which in turn is favorable for microalgae, especially if photoautotrophic metabolism is applied. On the other hand, microalgae produce oxygen and other nutrients that could be assimilated by bacteria (Nagarajan et al., 2019; Unnithan et al., 2014). This consortium could enhance the nitrogen and phosphorus removal, producing a biomass that can be used as biofertilizers for agriculture added with primary nutrients.

4. Microalgae biotechnology as a factor to assist United Nations Sustainable Development Goals

In 2015, the United Nations adopted 17 global priorities and 169 targets through its Sustainable Development Goals (SDG), which were set to be implemented over the next 15 years. The 17 SDGs aim to achieve a better and more sustainable future for both people and planet at regional, national and global scale via interlinked and integrated SDGs (United Nations, 2015).

With over 200,000 microalgae species discovered, and only a handful number of microalgal species used for a relatively small number of industrial applications, this highly diverse group of microorganisms can assist some of the SDG's achievements. Examples of the role and the impact of microalgae biotechnology in contributing with the achievement of the UN SDGs are shown in Table 6 and Fig. 3, respectively. More specifically, contributions of microalgae to support six SDGs are discussed below (Sutherland et al., 2021):

- SDG 1 – No poverty: seeks to eradicate poverty in all its forms and everywhere by 2030. Expanding microalgae cultivation in low-income countries may be a strategy that could help to eradicate poverty by creating new jobs and generating new sources of income for the society.
- SDG 2 – Zero hunger: seeks to address adequate human nutrition and food security, while promoting sustainable agricultural practices. Protein is an essential key macronutrient for humans, and microalgae, including both eukaryotic algae and cyanobacteria, have long been recognized as source of protein with all essential amino acids required in the human diet. They are also valuable source of lipids, fatty acids, vitamins and minerals, helping to boost not only the human nutrition but also aquaculture practices.
- SDG 3 – Good health and well–being: seeks to improve human health and quality of life. Secondary metabolites from microalgae, such as carotenoids, can be used in the enriching of foods and beverages promoting bioactive activities – function foods. Moreover, several other molecules found in microalgal biomass can be used for development of new drugs for the treatment of viruses, bacteria, fungi, and tumors.
- SDG 6 – Clean water and sanitation: focuses on addressing the global availability of water, including reducing pollution through increased wastewater treatment as well as water recycling. The use of microorganisms like microalgae to clean nutrient enriched wastewaters has been successfully demonstrated.

Table 5
Removal of pollutants by microalgae cultivated in different types of wastewaters.

Microalga	Wastewater	Main composition	Culture system	Ref.
Arthrospira platensis	Desalination concentrate	Ca^{2+}, $CaCO_3$, Cl^-, Fe^{3+}, K^+, Mg^{2+}, Na^+, SO_4^{2-}	100-L fiber photobioreactor, 4000-L raceway pond	Matos et al. (2021)
Chlorella sp. (MUR 270)	Anaerobically digested abattoir effluent	COD, NH_4–N, TN, TP	250 mL Erlenmeyer flasks	Vadiveloo et al. (2020)
Chlorella vulgaris (CA1)	Dairy anaerobic digestion	NH_4–N, TC, TN, TP	250 mL Erlenmeyer flasks	Pang et al. (2020)
Chlorella vulgaris (UTEX 2714)	Municipal centrate	COD, TN, TP, NH_4–N	250 mL Erlenmeyer flasks	Ma et al. (2014)
Galdieria sulphuraria (CCMEE 5587.1)	Urban wastewater	NH_4–N, TN, TP	Enclosed polyethylene bag (3 m^2)	Selvaratnam et al. (2014)
Nannochloropsis gaditana (Clone 130)	Desalination concentrate	Ca^{2+}, $CaCO_3$, Cl^-, Fe^{3+}, K^+, Mg^{2+}, Na^+, Si, SO_4^{2-}	500 mL Erlenmeyer flasks, inverted conical photobioreactor (3.5-L)	Matos et al. (2015)
Scenedesmus sp.	Primary domestic	Ca^{2+}, Cl^-, COD, Mg^{2+}, Na^+, NO_3^-, PO_4^{3-}, SO_4^{2-}	Thin-layer cascade reactor (32 m^2)	Sánchez Zurano et al. (2020)
Scenedesmus sp. (LEA 01)	Sanitary landfill leachate	Heavy metals (Cd, Ni, Cu, Zn), NH_3–N, NO_2^-, NO_3^-	1000 mL Erlenmeyer flasks	de Souza et al. (2021)
Scenedesmus intermedius	Pig manure	NH_4–N, TN, TP	500 mL Erlenmeyer flasks	Jiménez-Pérez et al. (2004)

Table 6
Examples on how microalgae biotechnology can assist with the achievement of the United Nations Sustainable Development Goals (SDG).

Sustainable Development Goal	SDG objective	Microalgal role	Microalgal purpose	Ref.
SDG – 1	No poverty	Cultivation of microalgae in system with low-energy and -investment requirements that do not require technical skills (such as open ponds, aerated tanks, and raceway ponds).	Microalgae cultivation can create new jobs and improve the local economy from low-income countries from Africa and South America.	Ahiahonu et al. (2021)
SDG – 2	Zero hunger	Source of protein, amino acids, polyunsaturated fatty acids, vitamins, minerals and pigments.	Support human nutrition, nutraceutical and functional food, dietary supplement, feed supplement for agriculture and aquaculture.	Matos (2020)
SDG – 3	Good health and well-being	Metabolites from microalgal biomass used to prevent diseases and/or development of new drugs	Support human health, nutraceutical and functional food, and potentially novel compounds for new drugs	Dantas et al. (2021)
SDG – 6	Clean water and sanitization	Wastewater treatment via nutrient uptake into microalgal biomass. Bioremediation of emerging contaminants such as heavy metals through bioadsorption. Biodegradation of pharmaceutical compounds. High rate algal ponds (HRAPs) driven by solar-UV mediated pathogen disinfection.	Microalgae to bioremediate wastewaters have been successfully demonstrated including full scale-systems. Clean water availability is vital for the environment, human health and economic development.	(Galès et al., 2019; Vassalle et al., 2020)
SDG – 7	Affordable and clean energy	Feedstock of third generation biofuels, including biodiesel, bioethanol, biogas (methane), biohydrogen, jet fuel	Biofuels from microalgae have been highlighted as an alternative renewable energy.	(Jacob et al., 2021; Oliveira et al., 2021a)
SDG – 12	Responsible consumption and production	Microalgal bioplastics produced by either from direct microalgal biomass or from cellular components.	Bioplastics from renewable natural source like microalgae are considered to be an environmentally friendly alternative.	Beckstrom et al. (2020)
SDG – 14	Life below water	Bioremediation of diffuse pollution intensified by run-off contaminants, such as nutrients (N/P), sediment and microorganisms.	Algal turf scrubbers, filamentous algae nutrient scrubbers and periphyton-based storm-water treatment areas have been tested for mitigation of nutrient pollution in waterways.	(Aston et al., 2018; Salvi et al., 2021)
SDG – 15	Life on land	Microalgae can be cultured in open ponds using non-potable water in places like arid and semi-arid regions, reversing possible land degradation.	Microalgal biomass can improve human livelihoods located in vulnerable regions like deserts.	(He et al., 2018; Oliveira et al., 2019)

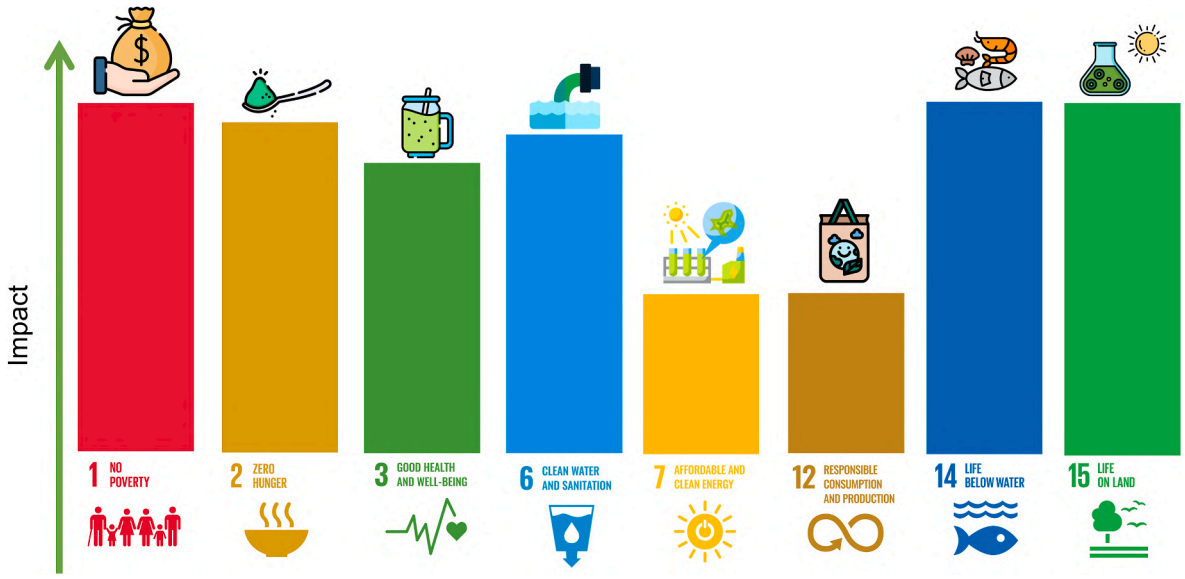

Fig. 3. Impact of the use of microalgae to the achievement of the United Nations Sustainable Development Goals.

- SDG 7 – Affordable and clean energy: seeks to ensure access to affordable, reliable sustainable and modern energy for all. Microalgal biomass is already considered as a suitable feedstock for 3rd generation biofuel production, with a plethora of studies found in the literature about biodiesel, bioethanol, butanol, biogas (methane), bio–oil, and biohydrogen production from microalgae.
- SDG 12 – Responsible consumption and production: seeks to ensure sustainable consumption and production patters. In particular, plastic is recognized as an important product in modern–day life, and algal–based bioplastics are considered to be an environmentally friendly alternative compared to conventional petro–plastics. Although algal bioplastics is currently in its infancy with the technology routes still under research, bioplastics such as polyhydroxyalkanoates (PHA) and polyhydroxybutyrate (PHB) manufactured from microalgal–derived intracellular components have received considerable interest by industry.
- SDG 14 – Life below water: conservation and sustainable use of oceans and marine resources are the key element of this goal. Agricultural run–off, contaminants, sediment, pathogens microorganisms, and nutrients, notably N and P, into waterways has resulted in eutrophication, affecting the aquatic life. Algal turf scrubbers (ATS) and periphyton ponds, for example, are some emerging technologies that can help to treat fresh and coastal marine water bodies

overloaded with diffused nutrients. Moreover, microalgae can help to increase sustainability in aquaculture.

- SDG 15 – Life on land: focuses on managing natural resources, including combat land affected by desertification, drought and floods. Large scale cultivation of microalgae on non–arable land using brackish water, seawater or wastewater is feasible, especially in arid and semi–arid regions where ample solar irradiance favors the photosynthetic capacity.

5. Future perspective and challenges

Although a significant amount of research on microalgae has been conducted in the last decades, it cannot be considered exhaustive. The various stages of development of microalgal biotechnology should serve as an incentive for old and new researchers in this multidisciplinary research field. For example, integrating microalgae cultivation and wastewater treatment, allowing dual function like removing contaminants from diverse effluents and generating biomass rich in biomolecules have been successfully demonstrated. This integrated process requires low technology, low capital and operational cost. The appropriate cultivation system will depend greatly on the desired final product. For example, nutraceutical compounds from microalgae cultured in photobioreactor require high technology and operational system, in addition to the further steps for extraction and by–product purification. On the other hand, simple biological processing of algal biomass via anaerobic digestion for biogas production is less expensive. Biomass harvesting, due to small cell size (3–30 μm), low cell concentrations (0.3–2.0 g L^{-1}) and neutral buoyancy of microalgae, is arguably the most energy–intensive step accounting for up to 30% of the total production cost. Further efforts towards optimizing nutrient loads, culture system, water recycling, harvesting techniques at industrial scale, byproduct extraction and purification can greatly advance the microalgal bioeconomy.

Even after several years of research, the downstream processing of microalgae biomass still requires significant advances to reach industrial maturity. The structural diversity and rigidity of microalgal cell walls complicate the standardization of an efficient downstream processing method for disruption of microalgal biomass and subsequent recovery of intracellular biocomponents. Even if downstream methods fail during application for a new microalga species and/or scale–up for industrial applications, reducing the efforts during operation can provide significant benefits in terms of investment and operating costs.

Furthermore, microalgae–based carbon sequestration is another important topic for sustainable development in many countries. The typical technologies for capturing CO_2 demand considerable financial costs, which can make their use unfeasible in low–income countries. With a view to developing affordable technologies, the US National Energy Technology Laboratory recommends a carbon capture cost of around US$ 40 per ton of CO_2 (Daneshvar et al., 2022). The use of microalgae for this application is part of this planning, as a potential, efficient, low–cost, and sustainable alternative in the world economic scenario. However, most approaches have focused on the selection of promising species for CO_2 sequestration at laboratory scale (Prasad et al., 2021).

Finally, although climatic conditions in several low–income countries in Africa, South America, and South Asia are favorable for microalgae cultivation (i.e., high levels of sunlight and high/moderate temperatures), there is very limited research on cultivation of microalgae in these regions (Ahiahonu et al., 2021). In order for microalgae to successfully impact the achievement of SDGs, policy actions and economic frameworks that are sensitive to these regions between now and 2030 will be required.

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Author contribution

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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4. Chapter 2 – Research articles

4.1. Article 4: Light induces peridinin and docosahexaenoic acid accumulation in the dinoflagellate *Durusdinium glynnii*

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Title	Light induces peridinin and docosahexaenoic acid accumulation in the dinoflagellate *Durusdinium glynnii*
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ENVIRONMENTAL BIOTECHNOLOGY

Light induces peridinin and docosahexaenoic acid accumulation in the dinoflagellate *Durusdinium glynnii*

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Abstract

Peridinin is a light-harvesting carotenoid present in phototrophic dinoflagellates and has great potential for new drug applications and cosmetics development. Herein, the effects of irradiance mediated by light-emitting diodes on growth performance, carotenoid and fatty acid profiles, and antioxidant activity of the endosymbiotic dinoflagellate *Durusdinium glynnii* were investigated. The results demonstrate that *D. glynnii* is particularly well adapted to low-light conditions; however, it can be high-light-tolerant. In contrast to other light-harvesting carotenoids, the peridinin accumulation in *D. glynnii* occurred during high-light exposure. The peridinin to chlorophyll-*a* ratio varied as a function of irradiance, while the peridinin to total carotenoids ratio remained stable. Under optimal irradiance for growth, there was a peak in docosahexaenoic acid (DHA) bioaccumulation. This study contributes to the understanding of the photoprotective role of peridinin in endosymbiont dinoflagellates and highlights the antioxidant activity of peridinin-rich extracts.

Key Points

- *Peridinin has a protective role against chlorophyll photo-oxidation*
- *High light conditions induce cellular peridinin accumulation*
- *D. glynnii accumulates high amounts of DHA under optimal light supply*

Keywords Antioxidant · Carotenoids · Light harvesting · Photosynthesis · *Symbiodiniaceae*

Introduction

Promoting sustainable development in the face of an increasing human population requires a continuous pursuit for new bioresources and bioprocesses. Recent studies have revealed that microalgae are one of the most promising bioresource towards a global sustainable development (Chen et al. 2020; Tang et al. 2020). New emerging microalgae-based products derived from its biomass include biofuels, food and feed, biofertilizers, and drugs. Marine dinoflagellates have attracted great attention for bioproduct development because of their capacity to synthesize a diverse range of high-value bioactive molecules (Assunção et al. 2017; López-Rodríguez et al. 2019). Although there is a great interest in bioactive molecules from dinoflagellates, the available information on the cultivation techniques of dinoflagellates, biomass processing, and its potential applications are scarce in the literature (Oliveira et al. 2020).

Dinoflagellates can produce different types of carotenoids, such as violaxanthin, fucoxanthin, zeaxanthin, and

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peridinin (Molina-Miras et al. 2018; López-Rodríguez et al. 2020; Zahedi Dizaji et al. 2021). The latter, in particular, has a dual role in the photosynthetic capability of phototrophic dinoflagellates: the first role in capturing blue-green light (470–550 nm range), outside the range accessible to chlorophylls, and the second one, in photoprotection in high light conditions (Hofmann et al. 1996; Di Valentin et al. 2016). Peridinin is present in the peridinin–chlorophyll–protein (PCP) soluble molecular complex (Dorrell et al. 2019), and this complex is a sensitive photosynthetic apparatus that can be modified depending upon culture conditions, which results in different peridinin to chlorophyll ratios (Langenbach and Melkonian 2019; Ara et al. 2020). Recently, Supasri et al. (2021) reported that purified peridinin extract has exhibited antioxidant, anti-inflammatory, and anti-cancer activities. Understanding these inherent effects is an initial and important step towards the establishment of dinoflagellate mass cultivation for pharmacological metabolite production. The effects of light on PCP complex synthesis and its cascading effects on growth performance and cellular biochemical composition have not yet been fully elucidated and require further investigations for the successful mass cultivation of dinoflagellates.

Endosymbiotic dinoflagellates of the family *Symbiodiniaceae* (formally known as the *Symbiodinium* clades) play a vital role in reef coral ecosystems (LaJeunesse et al. 2018; Eckert et al. 2020; Müller et al. 2021). Oxygenic photosynthesis performed by endosymbiotic dinoflagellates provides up to 95% of the host energy requirement and provides additional metabolites that can inhibit the growth of pathogenic bacteria (Baker 2003). This symbiotic relationship is complex, delicate, and susceptible, particularly in regard to projected changes in the marine environment, such as ocean acidification (Brading et al. 2011), temperature rise (Davies et al. 2018), availability of nutrients (Li et al. 2021), among other consequences (Ceh et al. 2013; Bernasconi et al. 2019). Due to the increased frequency of coral bleaching events, research efforts are being made to understand the underlying causes of that phenomenon, and to restore degraded coralline ecosystems (LaJeunesse et al. 2018; Oliveira et al. 2020). *Symbiodiniaceae* taxa are a source of several biomolecules, such as symbiospirols/super–carbon–chain compounds (SCCs), carotenoids, and eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively) with ample biotechnological applications due to their biological properties and potential use as a therapeutic agent against human diseases (Kita et al. 2005; Tsunematsu et al. 2009; Beedessee et al. 2019).

Changing environmental conditions can affect the biosynthesis of fatty acids, pigments, and other metabolites in microalgae (Oliveira et al. 2021b). Light is a mandatory requirement for photoautotrophic microalgae to perform oxygenic photosynthesis, but quality (color and source) and intensity (irradiance) can play secondary roles, affecting the biochemical composition of microalgae (Singh and Singh 2015; Lehmuskero et al. 2018). New technologies such as light-emitting diodes (LEDs) have been applied in the cultivation of microalgae with a general long-term reduction of associated economic costs (Teo et al. 2014; Ma et al. 2018; Molina-Miras et al. 2018; Jung et al. 2019). The use of LED lamps, despite having a more expensive installation cost, has a lower environmental impact than conventional lighting and emits less heat (Molina–Miras et al. 2018). Thus, the application of LED lamps for the culture of heat-sensitive marine dinoflagellates, like *Symbiodiniaceae* species, has the potential to improve the overall growth performance (Langenbach and Melkonian 2019).

In this study, the effects of irradiance on the culturing of an endosymbiotic strain of *Durusdinium glynnii* were investigated considering four main goals: (1) to evaluate the growth, biomass, and kinetics performance as well as nutrient uptake (nitrogen and phosphorus); (2) to determine the photosynthetic pigments in terms of chlorophyll-*a* (Chl-*a*) and *c* (Chl-*c*), total carotenoids, β-carotene, and peridinin; (3) to quantify the fatty acid content of *D. glynnii,* subjected to different irradiances; and (4) to examine the effects of irradiance on the production of antioxidant compounds.

Materials and methods

Microalgal strain

D. glynnii, clone BMK 211, was kindly provided by the Oceanographic Institute at the University of São Paulo (IO USP). The strain was maintained in seawater, previously filtered (0.45 µm) and sterilized (121 °C for 21 min), and enriched with f/2 medium at a salinity of 30 g L^{-1}. Cultures were kept in a room with a controlled temperature (22 ± 1 °C), under continuous illuminance (150 µmol photons m^{-2} s^{-1}) and transferred to a new fresh medium every week.

Culture conditions

A brief methodological flowchart of the culture conditions and analyses carried out in the present study is presented in Fig. 1.

To evaluate the effects of irradiance on growth, photosynthetic parameters, fatty acid profile, and antioxidant capacity of *D. glynnii* biomass, the dinoflagellate cultures were acclimated under light-emitting diode lamps. All treatments were performed in 250-mL Erlenmeyer flasks in triplicate, and incubated at room temperature 22 ± 0.5 °C, bubbled with atmospheric air (0.05 L_{air} L^{-1} min^{-1}). Initially, the cultures were acclimated for 7 days at their respective irradiance

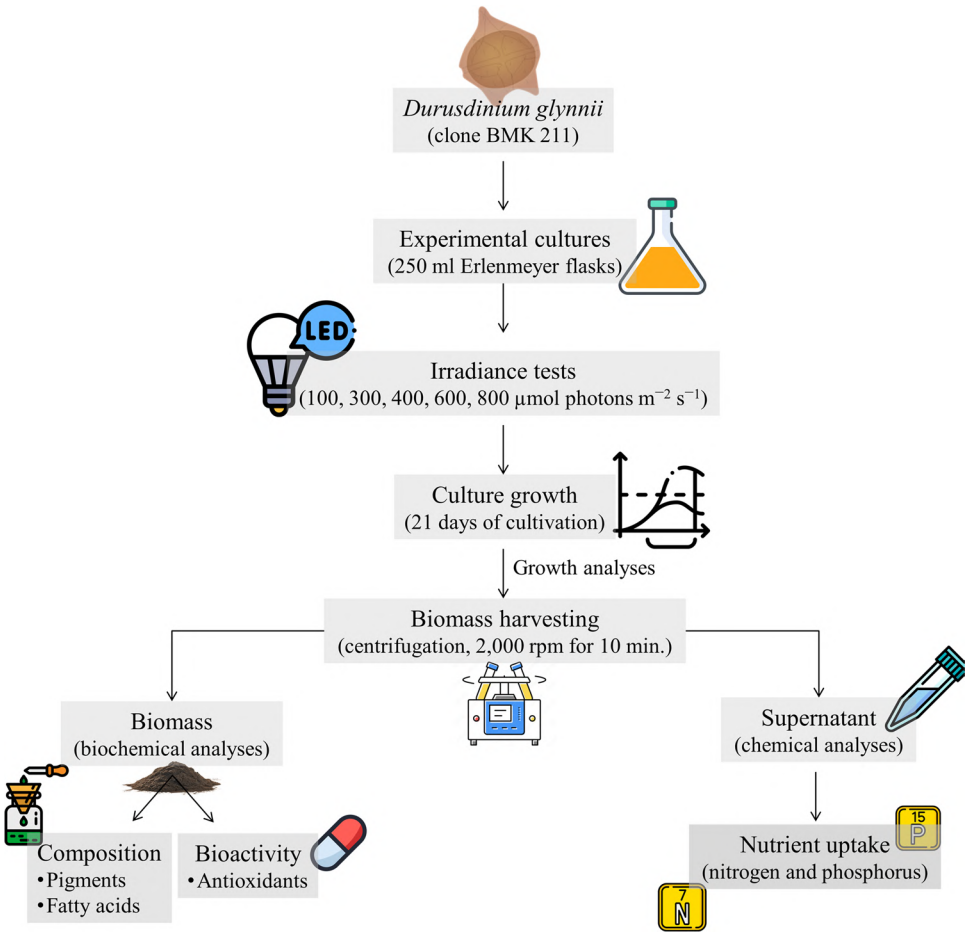

Fig. 1 Flow diagram for the cultivation of marine *Durusdinium glynnii* under different irradiance and subsequent analyses of the biomass and supernatant

treatments, due to the lower maximum quantum yield of photosynthesis reported for dinoflagellates when compared to other microalgae groups (MacIntyre et al. 2002). After the acclimation period, the cultures were diluted to standardize the cell concentration to a value of 50×10^3 cells mL^{-1}.

Experimental set-up

Firstly, a photosynthesis–irradiance (PE) curve was generated using irradiances varying from 0 to 1200 µmol photons m^{-2} s^{-1}, provided by 36 W LED panels, to determine the light-limited and light-saturated range. The photoinhibition model of Platt et al. (1980) was applied to fit the experimental data, based on Eqs. (1) and (2):

$$P^{B} = P_{s}^{B}\left(1 - \exp\left[-\frac{\alpha^{B}E}{P_{s}^{B}}\right]\right)\exp\left[-\frac{\beta^{B}E}{P_{s}^{B}}\right] \quad (1)$$

$$P_{m}^{B} = P_{s}^{B}\left(\frac{\alpha^{B}}{\left[\alpha^{B}+\beta^{B}\right]}\right)\left(\frac{\beta^{B}}{\left[\alpha^{B}+\beta^{B}\right]}\right)^{\beta/\alpha} \quad (2)$$

where P^B (mg C mg Chl-a^{-1} h^{-1}) is the photosynthetic rate at photosynthetically active radiation (PAR) I normalized to Chl-*a*, P_s^B (mg C mg Chl-a^{-1} h^{-1}) is the maximum photosynthetic rate in the absence of photoinhibition, and P_m^B is equal to P_s^B when β^B is zero. β^B [mg C mg Chl-a^{-1} h^{-1} (µmol photons m^{-2} s^{-1}) $^{-1}$] is a parameter that describes photoinhibition, α^B [mg C mg Chl-a^{-1} h^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$] is the initial slope of the PE curve at subsaturating irradiance, *E* (µmol photons m^{-2} s^{-1}) is the irradiance, and *a* and β are the constants. The cellular carbon content was indirectly calculated using the linear relationship between cell biovolume and carbon content, proposed by Menden-Deuer and Lessard (2000).

From the PE curve (Supplemental Fig. S1), the optimum irradiance for *D. glynnii* was assessed, and ranged from 117 µmol photons m^{-2} s^{-1} (E_k) to 800 µmol photons m^{-2} s^{-1}. Although it is not possible to state that above 800 µmol photons m^{-2} s^{-1} there was photoinhibition, the previous test on the PE curve indicates that in the irradiance close to 800 µmol photons m^{-2} s^{-1} there was excessive light, and therefore, it was estimated to be the optimum photosynthesis zone. Based on this previous test, we hypothesized that five different irradiances, i.e., 100, 300, 400, 600, and 800 µmol

photons m^{-2} s^{-1}, are the suitable irradiance conditions to conduct a robust photoacclimation study. These levels were adjusted using a quantameter and the distance of the cultures from the light source.

Biological, chemical, and biochemical analyses

Samples were taken for growth analyses on days 0, 1, 2, 3, 6, 9, 12, 15, 18, and 21 of each independent replicate from the different irradiance conditions. Samples taken from lag, exponential, and stationary phases were collected to determine nutrient concentrations (nitrogen and phosphorus) and cellular pigments (chlorophylls and carotenoids).

Growth and kinetics evaluation

To quantify *D. glynnii* growth, cell concentration and biomass, in dry weight, were analyzed. Biomass (mg L^{-1}) was estimated by the gravimetric method using 0.45-µm glass fiber microfilters (APHA 2005). Cell concentration (cells mL^{-1}) and cell biovolume (µm^3 normalized for ng $cell^{-1}$) of 1.5-mL samples were analyzed using a FlowCAM (Model C71 Syringe Pump, Fluid Imaging Technologies Inc., Scarborough, USA) equipped with a FC50 Flow Cell.

Cultures of *D. glynnii* exhibited asymmetric growth curves as reported for dinoflagellates. Thus, an asymmetric logistic equation was used for fitting the cell concentration (N(t)) *vs.* time (t) data in order to accurately determine the specific growth rate (µ, day^{-1}), according to Eqs. (3) and (4), respectively:

$$N(\mathrm{t}) = a + b\left[\exp(-0.5\frac{(t-c)^2}{d^2})\right] \tag{3}$$

$$\mu(\mathrm{t}) = \frac{1}{N(t)}\left(\frac{\mathrm{dN}(t)}{\mathrm{dt}}\right) \tag{4}$$

where *a*, *b*, *c*, and *d* are constants (Molina-Miras et al. 2018).

Nutrient uptake

Total nitrogen ($N-NO_3$) was determined through the persulfate digestion method on a Hach spectrophotometer using Reagent NitraVer (Hach ®, Loveland, USA) kits following standard protocols. Phosphorus ($P-PO_4^{3-}$) concentration was determined using the classical method of ascorbic acid (APHA 2005). The variations in nitrogen and phosphorus throughout the growth phases were used to calculate nitrogen uptake rate (NUR) and phosphorus uptake rate (PUR) during an interval time (Δt).

Pigment analysis

Samples from day 3 (lag phase), day 15 (exponential phase), and day 21 (stationary phase) of the experiment were taken to analyze intracellular pigments. After centrifuging 15 mL of algal culture for 10 min at 2000 rpm, the remained biomass was subjected to pigment extraction using acetone 90% (Strickland and Parsons 1972). Chl-*a* and Chl-*c* (*c*1 + *c*2) contents were calculated according to Jeffrey and Humphrey (1975), while carotenoid content (i.e., total carotenoids, β-carotene, and peridinin) were analyzed following the methods proposed by Carreto and Catoggio (1977) and Prézelin (1976). Values for all pigment concentrations were expressed as pg $cell^{-1}$.

Lipid extraction and fatty acid analysis

After acid digestion with 4 N HCl, intracellular lipids of *D. glynnii* were extracted by the Soxhlet method (963.15) with petroleum ether for 6 h, followed by concentration in a rotary evaporator. The samples were dried in an oven and subsequently weighed (AOAC 2005).

The fatty acid compositions of the microalgal lipid content were determined after the conversion of fatty acids to their corresponding methyl esters using the method of O'Fallon et al. (2007). The analysis of fatty acid methyl esters (FAME) was determined on a gas chromatograph (model GC-2014, Shimadzu, Kyoto, Japan), equipped with split-injection port, flame-ionization detector, and 105-m-long Restek capillary column (ID = 0.25 mm) coated with 0.25 µm of 10% cyanopropylphenyl and 90% biscyanopropylsiloxane. The temperatures of the injector and detector were both 260 °C. The oven temperature was initially set at 140 °C for 5 min, and then programmed to increase at 2.5 °C min^{-1}. The qualitative fatty acid composition was determined by comparing the peak retention times with those for respective fatty acid standards (Sigma, St. Louis, USA). The quantitative composition was determined by area normalization and expressed as a mass percent. The Class-GC10 software (https://www.shimadzu.com/an/products/gas-chromatography/gc-software/) was used to acquire and process the gas chromatograph data.

Antioxidant activity

Extracts of *D. glynnii* were obtained using 0.1 g of dried biomass resuspended and homogenized in 5 mL of 90% (v/v) dimethyl sulfoxide. Cells were incubated for 30 min in an ultrasonic bath (40 kHz) followed by homogenization for 2 h. Afterwards, the mixture was centrifugated at 1000 rpm for 10 min. The supernatant was collected subjected to serial dilution (20, 10, 5, 2.50, 1.25, 0.62, 0.31, and 0.16 mg mL^{-1}) for posterior antioxidant assays. The antioxidant activity (inhibition %) of the extracts was evaluated by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

($ABTS^{+\bullet}$) (Guedes et al. 2013), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) (Dantas et al. 2019), and phosphomolybdate (total antioxidant capacity, TAC) (Prazeres et al. 2019) methods. Trolox® and ascorbic acid were used as standards. The half-maximum inhibitory concentration (IC_{50}) of antioxidant activity of each method was calculated based on the linear regression of the % of inhibition vs. the sample concentration from serial dilution.

Data processing and statistical analysis

D. glynnii grown at each irradiance were transferred to three independent subcultures (i.e., a culture fraction was transferred to a fresh growth medium at the same condition). Then, the three subcultures were analyzed individually, generating three individual datasets. Multifactor ANOVA was performed to compare the three datasets in terms of growth performance (i.e., maximum cell concentration, μ_{max}, and biomass yield). Single comparisons were performed using one-way ANOVA (normality of the data and homogeneity of the variances were previously verified, by the Shapiro–Wilk and Levene tests, respectively), followed by Tukey's post hoc mean comparison test. In addition, non-linear regressions were calculated to plot different growth curves, as well as to study the photoacclimation of *D. glynnii* cultures subjected to different irradiances. For all analyses, a level of significance of 5% was adopted. All the analyses were performed using RStudio software (Version 3.1.1; https://www.rstudio.com/).

Results

The effect of irradiance on the growth kinetics

In this study, a strain of *D. glynnii* grew under saturating irradiances varying from 100 to 800 µmol photons m^{-2} s^{-1}. The data described herein suggest that this species had a better growth performance under moderate–light conditions (i.e., 300 and 400 µmol photons m^{-2} s^{-1}), but can be also high-light-tolerant (Fig. 2a; Table 1). The P^B_m ranged from 8.24 to 52.7 mg C mg Chl-a^{-1} h^{-1} at 100 and 800 µmol photons m^{-2} s^{-1}, respectively. High cell concentration ($702.5 \pm 20 \times 10^3$ cells mL^{-1}), μ_{max} ($0.49 \pm day^{-1}$), and NUR (40.4 ± 4.2 µM N day^{-1}) were found at 300 µmol photons m^{-2} s^{-1}. On the other hand, no significant difference ($p > 0.05$) was observed for PUR, with the value being around 1.2 µM P day^{-1}. The maximum biomass yield of 35.6 and 24.4 mg L^{-1} day^{-1} was obtained at a moderate irradiance, i.e., 300 and 400 µmol photons m^{-2} s^{-1}, respectively. When *D. glynnii* was cultured in low to moderate irradiance (i.e., 100 to 400 µmol photons m^{-2} s^{-1}), cell weight was close to 1 ng, while at high irradiance (i.e., 600 and 800 µmol photons m^{-2} s^{-1}), cell weight was halved (0.61–0.49 ng, respectively) than that observed under low irradiance condition. The photoacclimation model in terms of μ_{max} (Fig. 2b) was significant ($p < 0.05$; $r^2 = 0.79$) and confirmed the optimal range of photosynthesis optimum photosynthesis zone found in the PE curve (Supplemental Fig. S1).

The effect of irradiance on the photophysiological status

Chl-*a*, total carotenoids, β-carotene, and peridinin contents in *D. glynnii* cultures were significantly different ($p < 0.05$) between irradiance treatments (Table 2), with an extent and oscillatory pigment content observed. Total carotenoids had high oscillation (range 1.65 to 10.14 pg $cell^{-1}$), being composed mainly of peridinin (range 1.03 to 8.69 pg $cell^{-1}$) and β-carotene (range 0.55 to 3.25 pg $cell^{-1}$). Chl-*a* contents (range 0.76 to 4.28 pg $cell^{-1}$) were significantly ($p < 0.05$) higher than Chl-*c* levels (range 0.61 to 1.39 pg $cell^{-1}$). Briefly, higher contents of Chl-*a* were obtained in *D. glynnii*

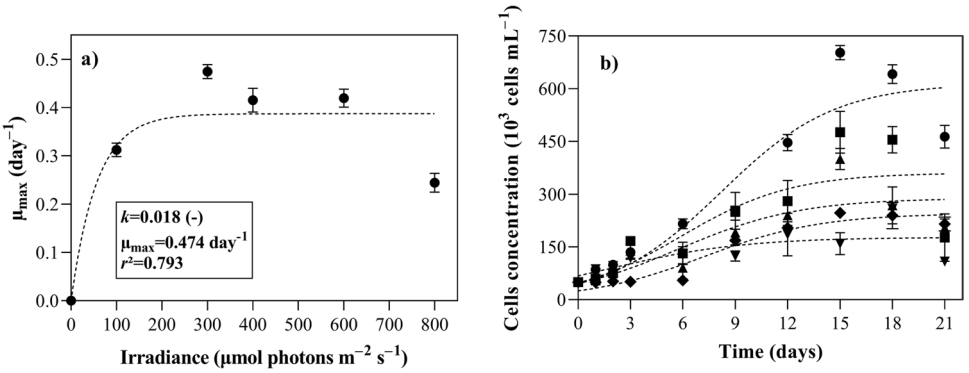

Fig. 2 Effect of irradiance on the maximum specific growth rates (**a**) and growth curves (**b**) of *Durusdinium glynnii* cultures subjected to different irradiances. Points are averages and vertical bars represent standard deviation of each subcultivation. Values denoted by a different lowercase letter at each point differ significantly ($p < 0.05$). The line represents the fit of the model to the experimental data. The best-fit values of k, μ_{max}, and determination coefficient are displayed. filled diamond 100, filled circle 300, filled square 400, filled triangle 600, and filled inverted triangle 800 µmol photons m^{-2} $s.^{-1}$

Table 1 Growth kinetics of *Durusdinium glynnii* cultures under different irradiances

Parameter	Irradiance (µmol photons m^{-2} s^{-1})					*p*–value
	100	300	400	600	800	
Maximum cells concentration ($\times 10^3$ cells mL^{-1})	246.7 ± 13.8^{c}	702.5 ± 20.0^{a}	476.3 ± 59.7^{b}	400.3 ± 29.5^{b}	258.8 ± 16.6^{c}	< 0.01
μ_{max} (day^{-1})	0.31 ± 0.01^{c}	0.49 ± 0.00^{a}	0.43 ± 0.05^{b}	0.42 ± 0.02^{b}	0.24 ± 0.02^{d}	< 0.01
Biomass yield (mg L^{-1} day^{-1})	12.1 ± 1.9^{c}	35.56 ± 16.29^{a}	24.44 ± 5.92^{ab}	11.11 ± 7.40^{c}	8.89 ± 2.96^{cd}	< 0.01
Cell weight (ng)	1.05 ± 0.12^{a}	1.04 ± 0.19^{a}	0.90 ± 0.32^{a}	0.49 ± 0.24^{b}	0.61 ± 0.09^{b}	< 0.01
P_m^B(mg C mg Chl-a^{-1} h^{-1})	8.24 ± 0.98^{a}	21.73 ± 6.02^{b}	24.87 ± 9.25^{b}	50.04 ± 5.51^{c}	52.74 ± 7.24^{c}	< 0.01
NUR (µM N day^{-1})	21.3 ± 3.3^{c}	40.4 ± 4.2^{a}	32.7 ± 3.1^{ab}	31.5 ± 4.2^{ab}	24.0 ± 2.4^{bc}	< 0.01
PUR (µM P·day^{-1})	0.9 ± 0.5	1.8 ± 0.7	1.4 ± 0.8	1.1 ± 0.7	1.0 ± 0.5	0.47

μ_{max} maximum specific growth rate; P_m^B light-saturated maximum rate; *NUR* nitrogen uptake rate; *PUR* phosphorus uptake rate

Data represents the mean ± standard deviation of three independent subcultivations for each condition. Different lowercase letters on the same line indicate a significant difference by Tukey's post hoc test ($p < 0.05$)

cultures subjected to lower irradiances (100 and 300 µmol photons m^{-2} s^{-1}), while no significant differences were found for Chl-*c* ($p > 0.05$). Maximum total carotenoids, peridinin, and β-carotene contents were majorly found when subjecting the *D. glynnii* culture to 600 µmol photons m^{-2} s^{-1}.

The ratios of Chl-*a*/Chl-*c* (Fig. 3a), total carotenoids/Chl-*a* (Fig. 3b), peridinin/Chl-*a* (Fig. 3c), and peridinin/total carotenoids (Fig. 3d) to assess the light-harvesting complex efficiency were calculated. The ratio of Chl-*a*/Chl-*c* decreased with increasing irradiance, while the ratios of total carotenoids/Chl-*a* and peridinin/Chl-*a* increased. In contrast, the ratio peridinin/total carotenoids remained stable regardless of irradiance, with an average peridinin content of 65.42 ± 0.20% of the total carotenoid content. For low irradiances, i.e., 100, 300, and 400 µmol photons m^{-2} s^{-1}, the mean ratio of peridinin/Chl-*a* was significantly lower (0.63 ± 0.23) than for high irradiance, i.e., 600 and 800 µmol photons m^{-2} s^{-1} (3.42 ± 2.66) ($p < 0.01$).

The effect of irradiance on lipid content and fatty acid composition

The lipid content was significantly higher ($p < 0.05$) in *D. glynnii* cells grown at irradiance ranging from 100 to 400 µmol photons m^{-2} s^{-1}, compared to the high irradiance (Table 3). The FAME composition of *D. glynnii* was also significantly different ($p < 0.05$) between the irradiances. Expressed as the percentage of the total FAMEs in *D. glynnii* biomass, the main fatty acid classes were saturated fatty acids (SFAs, 42.0–62.4%), followed by monounsaturated fatty acids (MUFAs, 20.6–30.2), and polyunsaturated ω-3 fatty acids (PUFA-ω3, 3.2–11.4%) and ω-6 fatty acids (1.57–3.38%). The overall predominant fatty acids were palmitic acid ($C_{16:0}$; 35.5–49.4%), oleic acid ($C_{18:1}$, 11.0–16.5%), and palmitoleic acid ($C_{16:1}$, 9.1–13.2%) and docosahexaenoic acid (C22:6ω3, DHA, 2.8–11.4%), in which the highest DHA concentration was detected for biomass grown at 300 µmol photons m^{-2} s^{-1} irradiance.

Table 2 Variations in photosynthetic pigments of *Durusdinium glynnii* cultures under different irradiances

	Irradiance (µmol photons m^{-2} s^{-1})				
	100	300	400	600	800
Chlorophyll-*a* (pg $cell^{-1}$)					
Mean	4.82^{a}	4.17^{a}	2.77^{b}	1.26^{c}	0.76^{d}
SD	1.13	0.82	0.54	0.85	0.33
Max	6.00	5.23	3.39	2.28	1.15
Min	2.60	3.53	2.34	0.45	0.38
Chlorophyll-*c* (pg $cell^{-1}$)					
Mean	0.96	1.10	1.39	0.97	0.61
SD	0.70	1.27	0.68	0.71	0.23
Max	1.98	3.11	2.46	1.77	0.87
Min	0.44	0.21	0.55	0.30	0.33
Total carotenoids (pg $cell^{-1}$)					
Mean	4.00^{a}	3.20^{a}	3.26^{a}	10.14^{a}	1.65^{ab}
SD	1.88	2.19	0.86	3.04	1.21
Max	6.08	6.11	4.08	13.91	2.88
Min	2.06	1.26	2.03	6.98	0.44
β-Carotene (pg $cell^{-1}$)					
Mean	1.11^{b}	1.02^{b}	1.06^{b}	3.25^{a}	0.55^{b}
SD	0.59	0.71	0.40	1.17	0.40
Max	2.02	2.04	1.47	4.66	0.96
Min	0.63	0.42	0.68	2.12	0.14
Peridinin (pg $cell^{-1}$)					
Mean	2.49^{b}	1.91^{b}	1.98^{b}	8.69^{a}	1.03^{b}
SD	1.17	1.29	0.75	2.17	0.76
Max	3.78	3.43	2.75	8.69	1.79
Min	1.29	0.78	1.27	2.12	0.27

Different letters on the same line indicate a significant difference by Tukey's post hoc test ($p < 0.05$)

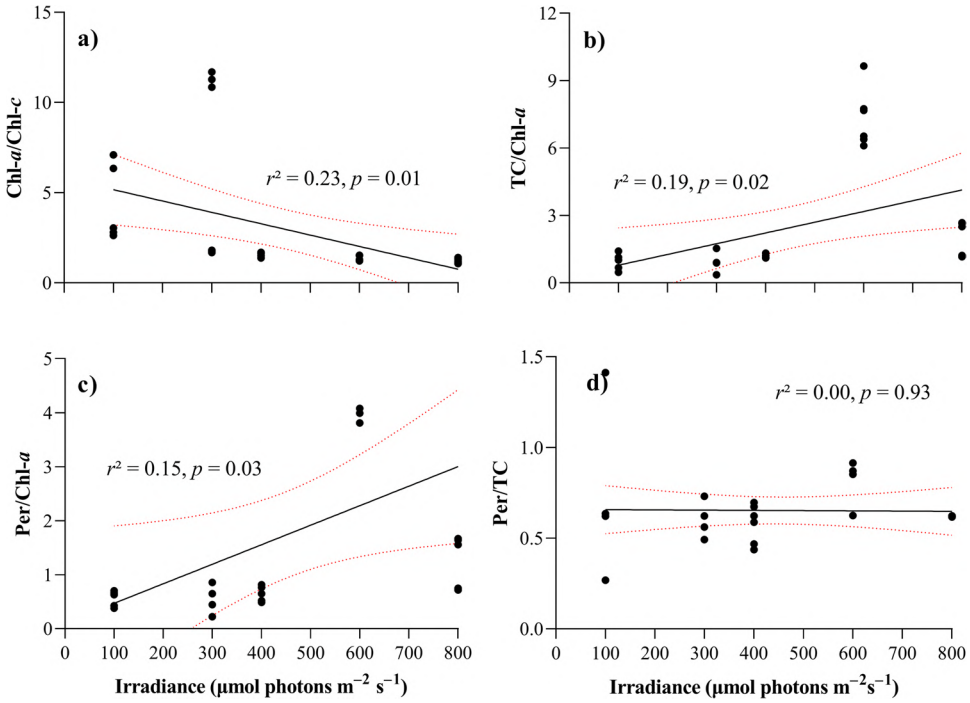

Fig. 3 The effects of irradiance on the ratios of chlorophyll-*a* to chlorophyll-*c* (**a**), chlorophyll-*a* to total carotenoids (**b**), chlorophyll-*a* to peridinin (**c**), and total carotenoids to peridinin (**d**) in cultures of *Durusdinium glynnii*. Points are means of each subcultivation. Red pointed lines represent 95% of confidence interval. Chl-*a* – chlorophyll-*a*; Chl-c – chlorophyll-*c*; TC – total carotenoids; and Per – peridinin

Table 3 Lipid content (%) and fatty acid composition (% of total fatty acids content) of *Durusdinium glynnii* cultured under different irradiances

FAMEs (%)	Irradiance (µmol photons m^{-2} s^{-1})				
	100	**300**	**400**	**600**	**800**
C12:0	1.76 ± 0.3^{a}	1.62 ± 0.2^{a}	1.18 ± 0.2^{b}	0.40 ± 0.1^{c}	0.95 ± 0.1^{b}
C14:0	7.49 ± 0.8^{a}	6.20 ± 0.6^{ab}	7.69 ± 0.9^{a}	1.44 ± 0.3^{c}	6.32 ± 0.5^{ab}
C16:0	42.78 ± 1.3^{b}	35.50 ± 1.1^{c}	49.38 ± 1.5^{a}	37.78 ± 1.2^{c}	49.55 ± 1.3^{a}
C18:0	1.20 ± 0.3	1.01 ± 0.1	1.77 ± 0.3	1.65 ± 0.3	1.96 ± 0.3
C21:0	3.80 ± 0.2^{b}	4.93 ± 0.3^{a}	0.77 ± 0.2^{c}	0.25 ± 0.1^{d}	0.98 ± 0.1^{c}
Other SFA	0.61 ± 0.2	0.57 ± 0.1	1.62 ± 0.1	0.53 ± 0.1	0.23 ± 0.1
Σ SFA	57.64 ± 1.0^{c}	49.83 ± 1.2^{c}	62.41 ± 1.1^{a}	42.05 ± 1.0^{d}	60.00 ± 1.0^{ab}
C15:1	0.35 ± 0.1	0.17 ± 0.1	0.31 ± 0.1	0.20 ± 0.1	0.23 ± 0.1
C16:1	12.18 ± 0.6^{a}	10.84 ± 0.5^{b}	11.67 ± 0.9^{ab}	13.16 ± 0.6^{a}	9.06 ± 0.8^{b}
C18:1	14.17 ± 0.7^{a}	13.30 ± 0.6^{a}	15.66 ± 1.0^{a}	16.53 ± 0.8^{a}	11.00 ± 0.9^{b}
Other MUFA	0.45 ± 0.1	0.60 ± 0.1	0.96 ± 0.1	0.30 ± 0.1	0.32 ± 0.1
Σ MUFA	27.15 ± 1.1^{b}	24.91 ± 1.3^{b}	28.60 ± 0.5^{ab}	30.19 ± 0.7^{a}	20.61 ± 0.6^{c}
C18:3 ω3 (ALA)	0.31 ± 0.1	0.27 ± 0.1	0.36 ± 0.1	0.24 ± 0.1	0.31 ± 0.1
C22:6 ω3 (DHA)	5.71 ± 0.6^{b}	11.38 ± 0.6^{a}	2.85 ± 0.3^{c}	7.18 ± 0.8^{b}	6.66 ± 0.6^{b}
Σ PUFA-ω3	6.02 ± 0.7^{bc}	11.65 ± 0.4^{a}	3.21 ± 0.2^{d}	7.42 ± 0.6^{b}	6.97 ± 0.4^{b}
C18:2 ω6 (LA)	0.85 ± 0.1^{a}	0.88 ± 0.1^{a}	0.55 ± 0.1^{b}	0.20 ± 0.1^{c}	0.50 ± 0.1^{b}
C18:3 ω6 (GLA)	1.07 ± 0.2^{a}	1.12 ± 0.2^{a}	0.74 ± 0.2^{ab}	0.51 ± 0.1^{b}	0.80 ± 0.1^{a}
C20:4 ω6 (AA)	0.61 ± 0.3^{b}	1.38 ± 0.3^{a}	0.28 ± 0.3^{bc}	1.02 ± 0.2^{a}	1.50 ± 0.2^{a}
Σ PUFA-ω6	2.53 ± 0.2^{b}	3.38 ± 0.2^{a}	1.57 ± 0.3^{c}	1.73 ± 0.2^{c}	2.80 ± 0.2^{b}
ω3/ω6	2.27	3.44	2.04	4.28	2.48
Total lipid	16.63 ± 1.0^{a}	18.35 ± 1.2^{a}	18.04 ± 1.3^{a}	10.70 ± 0.8^{b}	11.17 ± 0.9^{b}

SFA saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

SFAs not shown in the table: pentadecanoic acid C15:0, behenic acid C22:0

MUFAs not show in the table: margaroleic acid C17:1, erucic acid C22:1

Different letters on the same line indicate a significant difference by Tukey's post hoc test ($p < 0.05$)

The effect of irradiance on antioxidant production

The $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and TAC radical scavenging assays were used to assess the antioxidant activity of extracts from *D. glynnii* biomass cultured under different irradiances (Fig. 4) in terms of percentage of inhibition (Fig. 4a) and IC_{50} (mg mL^{-1}) (Fig. 4b). In the present study, the antioxidant activities were positively associated with SFA ($r^2 = 0.76$) and negatively associated with PUFA ($r^2 = -0.61$) contents (Fig. 4c).

Discussion

Growth performance

Photoautotrophic cultivation of marine dinoflagellates at pilot and large scale is restrained by a number of problems related to low growth, generally related to contamination and sensitivity to shear and heat stresses (Camacho et al. 2011; Langenbach and Melkonian 2019). Recently, a *Symbiodinium microadriaticum* strain was successfully cultured in twin-layer porous substrate photobioreactors (Tsirigoti et al. 2020). The authors remarkably reported 5.21 g m^{-2} day^{-1} biomass productivity at 200 µmol photons m^{-2} s^{-1} provided by fluorescent lamps. However, these photobioreactors used for the scale-up process are hampered by high-cost issues (Langenbach and Melkonian 2019). For this reason, raceway ponds and bubble column photobioreactors have been explored for the scale-up process of dinoflagellate cultivation (Molina-Miras et al. 2018, 2020; Lim et al. 2020).

Photoacclimation of *D. glynnii* cultures, in terms of μ_{max}, showed that at 100 µmol photons m^{-2} s^{-1} irradiance there was a reduction in the specific growth rate of the cells due to the low availability of photon molecules (photolimitation). On the other hand, at 800 µmol photons m^{-2} s^{-1} or higher irradiances, algal cells could not properly dissipate the excessive light energy, resulting from a high number of photon molecules which negatively affected culture growth (photoinhibition).

Higher growth performance (cell concentration, μ_{max}, and biomass yield) were reported at 300 and 400 µmol photons m^{-2} s^{-1} irradiances. For example, the higher values for growth parameters are consistent with previous studies of other dinoflagellate species, such as *Akashiwo sanguinea*, *Prorocentrum micans*, and *Scrippsiella trochoidea* (Islabão et al. 2016), and *Karenia brevis* (Tilney et al. 2019), but are lower than those reported for other microalgae group, such as marine diatoms

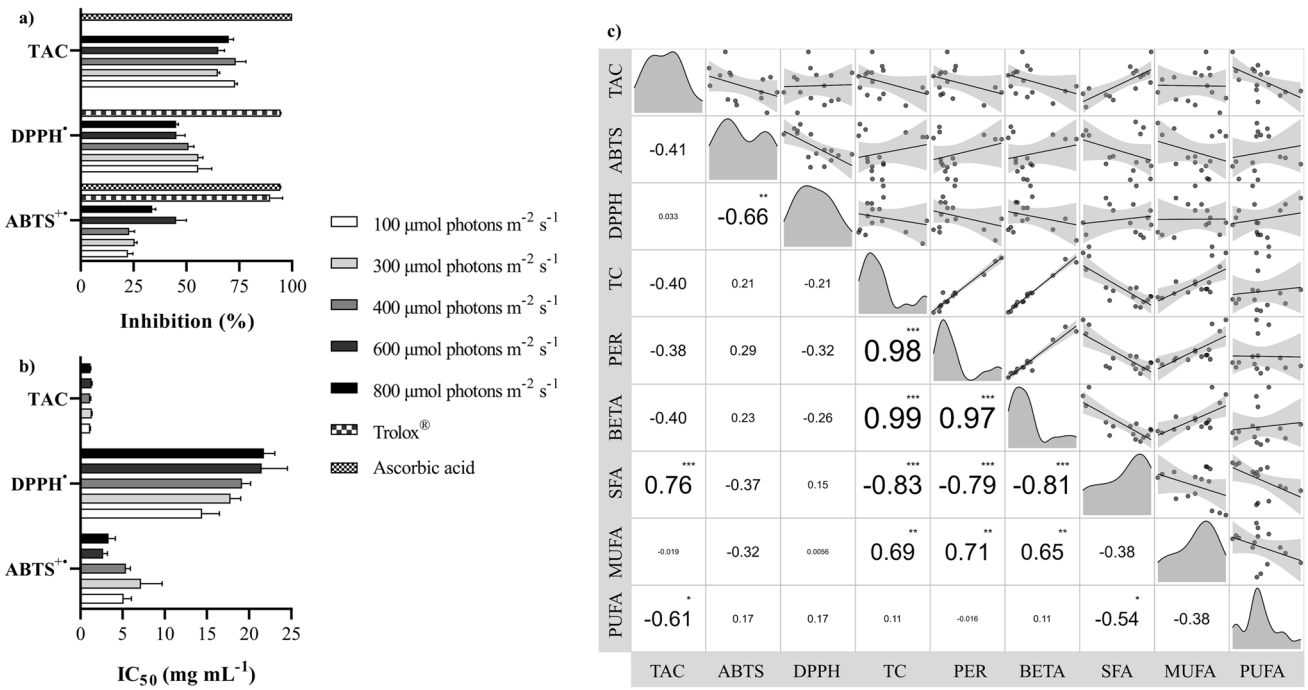

Fig. 4 Antioxidant activity (**a**), 50% inhibition of free-radical scavenging (**b**), and Pearson's correlation coefficients between antioxidant activity and contents of carotenoids and fatty acids (**c**) of *Durusdinium glynnii* extracts. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; TAC – total antioxidant capacity; ABTS – 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH – 2,2-diphenyl-1-picrylhydrazyl; TC – total carotenoids; PER – peridinin; BETA – β-carotene; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. Trolox® and ascorbic acid are positive controls

(Laws et al. 2020; Zhou et al. 2021). Molina–Miras et al. (2018) reported a maximum specific growth (μ_{max}) of around 0.60 day^{-1} for marine dinoflagellate *Amphidinium carterae* growing under irradiances between 100 and 400 µmol photons m^{-2} s^{-1}, while Iwasaki et al. (2021) reported $\mu_{max} = 1.60$ day^{-1} for marine diatom *Chaetoceros muelleri* growing under 500 µmol photons m^{-2} s^{-1} irradiance. Thus, it is possible to affirm that the PCP complex, unique in photoautotrophic dinoflagellates (Dorrell et al. 2019), is two to four times less efficient than the fucoxanthin–Chl *a*/*c* complex (FCP), unique in diatoms. Besides the light-harvesting role of PCP in photoautotrophic dinoflagellates, peridinin is suggested to confer a secondary role to quench harmful photo-oxidizing singlet oxygen ($^1O_2$) that arises as an unwanted byproduct during photosynthesis (Alexandre et al. 2007). This can support the fact that there was no photo-oxidation in *D. glynnii* cells even when it was subjected to relatively high irradiance for a long period of cultivation time.

Some studies have reported that short photoperiods were favorable for achieving high μ_{max} (Kitaya et al. 2008; Wang et al. 2019; Kilcoyne et al. 2019). For example, Wang et al. (2019) reported that a small strain of *Alexandrium minutum* grew faster under shorter photoperiods ($\mu = 1.01$ day^{-1} at 8:16 light:dark), while a larger strain of *Alexandrium catenella* grew faster under longer photoperiod at low light ($\mu = 0.76$ day^{-1} at 24:0 light:dark). This relationship between light intensity and photoperiod, may justify the lower μ_{max} reported for cultures of *D. glynnii* under 800 µmol photons m^{-2} s^{-1} irradiance. Under high irradiance (i.e., 800 µmol photons m^{-2} s^{-1}), short photoperiods may be more favorable for the growth of *D. glynnii* that increases the division rate of smaller cells and in turn accumulates high amounts of Chl-*a*.

Pigment accumulation

In contrast to the accumulation of secondary carotenoids in microalgae, light-harvesting carotenoids, such as peridinin, accumulate at low light intensity with increasing size or number of antenna complexes (Owens et al. 1980; Langenbach and Melkonian 2019). However, in the present study, higher levels of peridinin were detected at 600 µmol photons m^{-2} s^{-1}, suggesting that cells tend to accumulate more peridinin to prevent photo-oxidation than to capture light energy under photolimitation. The important photoprotective role under high light conditions can prevent the formation of $^1O_2$, a harmful oxidizing species, from chlorophyll triplet states (Di Valentin et al. 2016). On the other hand, the lower levels of peridinin at 800 µmol photons m^{-2} s^{-1} may be the result of a partial photo-oxidation of cells, due to inadequate processing of excessive light energy. Therefore, our findings prove that peridinin is a vital photoprotective carotenoid in the photosynthetic pathways of endosymbiont dinoflagellates.

At high irradiance conditions, the production of reactive oxygen species (ROS) can oxidize some carotenoids, inducing for instance the biosynthesis of some volatile organic compounds. For example, under high irradiance, the production of $^1O_2$ leads to the oxidation of β-carotene to the synthesis of terpenoids β-cyclocitral, β-ionone, and dihydroactinidiolide (Laloi and Havaux 2015; Nader et al. 2022). Thus, it is more likely that the lower levels of β-carotene found in cells grown at 800 µmol photons m^{-2} s^{-1} were a result of the oxidation of this carotenoid in detriment to the synthesis of ROS cascade compounds.

Photosynthetic pigments are usually investigated to express the changes in photosynthesis efficiency under light stress conditions (Song and Pei 2018). Changes in photosynthesis are usually reflected in photosynthetic pigment contents (George et al. 2014; Coulombier et al. 2020). These changes may occur in response to nutrient concentration, temperature, and light. Regarding the ratios of Chl-*a*/Chl-*c*, a high ratio occurs in response to a reduction in light-harvesting related to the rate of photosystem II (PS-II) photochemistry reaction (Larkum et al. 1994; Song and Pei 2018). This reduction in light harvesting may be associated with a possible light limitation at 100 µmol photons m^{-2} s^{-1} irradiances, where the higher ratios were found. Similarly, an increase in the ratio of total carotenoids/Chl-*a* resulted in a reduction in the light-harvesting complex and PS-II activity. Thus, a peak in total carotenoids/Chl-*a* ratio at 600 µmol photons m^{-2} s^{-1} may be linked to a lower photosynthetic rate, and consequently, a lower growth performance. On the other hand, the reduction of this ratio at 800 µmol photons m^{-2} s^{-1} may reinforce that probably there was a partial photo-oxidation of chlorophyll at this irradiance.

Shi et al. (2018) reported stable peridinin/Chl-*a* ratios (1.3–1.5) over 48-h in non-photo-acclimated cultures of the marine dinoflagellate *Prorocentrum donghaiense* with irradiance ranging from 100 to 600 µmol photons m^{-2} s^{-1}. Similarly, peridinin/Chl-*a* ratios of 0.4 and 0.8 for *Heterocapsa* sp. and *Prorocentrum micans* have been reported under different culture conditions (Latasa and Berdalet 1994; Schlüter et al. 2000). In the present study, the ratios of peridinin/Chl-*a* varied from 0.44 to 5.67 pg $cell^{-1}$ at 300 and 600 µmol photons m^{-2} s^{-1}, respectively. These differences can be attributed to the long-term exposure to these irradiances, both to the 7 days of acclimation and to the three consecutive subcultivations, resulting in up to 70 days of exposure at the last sampling.

Fatty acid composition

The fatty acid profiles obtained in the present study are in accordance with those previously reported for *Symbiodiniaceae* taxa grown photoautotrophically (Kneeland et al.

2013; Mies et al. 2017). The variations in the *D. glynnii* fatty acid profile in relation to the different tested irradiance followed a general pattern that is well established in the microalgae culture found in the literature. For example, Remmers et al. (2017) and Conceição et al. (2020) reported a reduction of DHA level in the fatty acid fraction in the diatom *Phaeodactylum tricornutum* cultured in high irradiance. In the same way, at low growth rates, SFA and MUFA accumulation occurs due to the cellular accumulation of excess carbon during photosynthesis (Li et al. 2014).

Although the fatty acid profile of *D. glynnii* indicates good characteristics for biodiesel production, such as synthesis of SFA content and less than 12% of C18:3 (Oliveira et al. 2021a), it is economically unfeasible due to the low growth rate inherent of this microalga. On contrary, a high content of long-chain fatty acids (LCFA, i.e., > 12C) was detected in the *D. glynnii* fatty acid profile, regardless of their unsaturation degree. The LCFA acts in a number of intracellular signaling and metabolic pathways related to the pathogenesis of vasoproliferative and neurodegenerative disorders in human cells. Dietary supplementation of PUFA-ω3, mainly DHA and EPA, is an important agent in retinal function, and neuroprotection in humans (Zárate et al. 2017; Djuricic and Calder 2021).

The well-known LCFA such as DHA and EPA have attracted much attention due to a possible association with reduced risks of cancer, obesity, diabetes, and certain cardiovascular disease in human metabolism (Merendino et al. 2013; Arnoldussen and Kiliaan 2014; Kris-Etherton et al. 2019). The main source of these LC-PUFAs is fish oil, even if traits like odor or off-flavors are found in this oil as well as some health risks associated with contaminated fish consumption, notably caused by environmental pollutants, like toxins, biphenyls, and mercury, are considered undesirable by many consumers. The production of LC-PUFAs from marine microorganisms is already an industrial reality (moving annually about $200 million), where these compounds come from heterotrophic microorganism production (Khozin-Goldberg et al. 2011). However, the economic viability of photoautotrophic microalgae cultures for LC-PUFA production still requires substantial advances in photobiology, and the development of a robust and feasible biorefinery model is required. Taken together, the fatty acid profile verified in the *D. glynnii* cells exposed to different irradiances has demonstrated its potential application for LC-PUFA production.

Antioxidants

The industrial demand for biobased antioxidants is constantly increasing over the years. For example, the food and pharmaceutical industries use antioxidants to prevent skin damage caused by oxidative stress and by UV-irradiance, respectively (Conde et al. 2021). Generally, PUFAs are directly associated with antioxidant activity for freshwater and marine microalgae (Conde et al. 2021). But not only PUFAs confer antioxidant activity in microalgal biomass, carotenoids such as astaxanthin and lutein and β-carotene are also recognized for strong antioxidant activity (Young and Lowe 2018; Pérez-gálvez et al. 2020).

In a study conducted by Supasri et al. (2021), a purified extract of peridinin exhibited scavenging of up to 75% of free radicals at 72 µM by $ABTS^{\bullet+}$ method. In the present study, a lower percentage of free-radical scavenging was observed using the same method, due to the possible presence of other compounds in the extract that may reduce the antioxidant capacity. Moreover, light-harvesting carotenoids, for instance fucoxanthin and peridinin, usually contribute to the high antioxidant potential under anoxic conditions, whereas photoprotective carotenoids (i.e., astaxanthin, β-carotene, lutein, etc.) have a high performance in scavenging reactive radicals (Nomura et al. 1997; Supasri et al. 2021). Phenolic compounds and flavonoids generally have a higher relationship with antioxidant activities in the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods. Furthermore, these compounds may have a higher and positive relationship with $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods in terms of antioxidant activities (Dantas et al. 2019; Haoujar et al. 2019).

Some bottlenecks in the successful cultivation of marine dinoflagellates have caused the number of published studies on the biological activities of peridinin, and other secondary metabolites, to be substantially lower than bioactive compounds from green algae and diatoms, for example. However, in recent years, there have seen an advanced knowledge of *Symbiodinium voratum* nutrient demand (Tsirigoti et al. 2020), long-term cultivation of *A. carterae* in an indoor-LED-lighted raceway photobioreactor (Molina-Miras et al. 2018), and the investigation and effects of light irradiance on carotenoid and fatty acid production linked with antioxidant compounds. The development of massive cultures of marine dinoflagellates for the synthesis of valuable compounds with biological activity suggests the importance of investigating this under-explored algae group for potential use in the pharmaceutical industry, opening up new frontiers in microalgal biotechnology.

In conclusion, irradiance is a key factor in the growth of *D. glynnii* as well as for the synthesis of carotenoids, LC-PUFA, and antioxidant molecules. Our results show that moderate irradiance (300 µmol photons m^{-2} s^{-1}) is the best option for maximum biomass productivity. Peridinin and β-carotene are best obtained in *D. glynnii* when cultured under 600 µmol photons m^{-2} s^{-1}, while fatty acid composition varied greatly between irradiances. In sum, the marine dinoflagellate *D. glynnii* must be grown at low light irradiance to produce high amount of cells, followed by high-light stress to induce the synthesis of peridinin.

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Author contribution CYBO: conceptualization; investigation; methodology; data curation; formal analysis; writing—original draft. JLA: investigation, formal analysis. EPS: investigation, formal analysis. APM: resources; formal analysis; writing—review and editing. GT: resources, formal analysis. CDLO: data curation; formal analysis; writing—review and editing. BOV: data curation, formal analysis. RSB: resources, formal analysis. MNM: resources; supervision; writing—review and editing. AOG: resources; supervision; project administration; writing—review and editing.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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4.2. Article 5: New insights on the role of nitrogen in the resistance to stressors in an endosymbiotic dinoflagellate

Research in this field will be submitted to the following journal	
Title	New insights on the role of nitrogen in the resistance to stressors in an endosymbiotic dinoflagellate
Authors	**CYB Oliveira**, BCS Brandão, LGS Jannuzzi, DWS Oliveira, GT Yogui, MN Müller, AO Gálvez
Journal	Coral Reefs
Year	-
Volume	-
Pages	-
DOI	-
IF (JCR 2021)	4.640
Category	Aquatic Science (22/234)
Percentile	90

New insights on the role of nitrogen in the resistance to stressors in an endosymbiotic dinoflagellate

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Abstract

Endosymbiotic dinoflagellates provide the nutritional basis for marine invertebrates, especially reef–building corals. These dinoflagellates are very sensitive to environmental changes, and understanding the factors that can increase the resistance of the symbionts is crucial for the elucidation of the mechanisms involved with the coral bleaching. Here, we demonstrate how the endosymbiotic dinoflagellate *Durusdinium glynnii* is affected by the concentration (1760 and 440 μM) and source (sodium nitrate and urea) of nitrogen after light and thermal stressors. The effectiveness in the use of the two nitrogen forms was proven by the nitrogen isotopic signature. Overall, high nitrogen concentrations, regardless of source, increased *D. glynnii* growth and chlorophyll–*a* and peridinin levels. During the pre–stress period, the use of urea accelerated the growth of *D. glynnii* compared to cells grown using sodium nitrate. During the luminous stress, the high nitrate condition has increased the cell growth, but changes in pigments composition was not observed. On the other hand, during the thermal stress was observed by a steep and steady decline in cell densities over time, except for high urea condition, where there is cellular division and peridinin accumulation 72 h after the thermal shock. Our findings suggest peridinin has a protective role during the thermal stress, and the uptake of urea by *D. glynnii* can alleviate a thermal stress, eventually preventing a coral bleaching event.

Keywords: coral reefs, peridinin, stable isotopes, symbiosis, zooxanthellae.

1. Introduction

Dinoflagellates of the family Symbiodiniaceae play a crucial role in coral reef ecological balance providing chemical energy (in carbohydrate form) produced by photosynthetic pathways, enabling calcium carbonate accretion and growth. Furthermore, these dinoflagellates can produce several metabolites that can improve the coral reef health, and this resulted in recent interest to understand these host–Symbiodiniaceae relationships (Jiang et al. 2014). Additionally, these dinoflagellates have attracted great pharmacological attention, because many of these molecules have been proving as potential anti–inflammatory, analgesic, vasoconstrictor, cytotoxic, and antitumor compounds that can inspire new drugs (Bigham Soostani et al. 2021; Assunção et al. 2017). This has led to recent efforts to improve the cultivation techniques of these dinoflagellates (Sánchez-Suárez et al. 2021; Oliveira et al. 2020).

Endosymbiotic dinoflagellates have a complex life cycle composed by two stages: the motile mastigote stage and the non–motile coccoid one. In natural environment, Symbiodiniaceae cells grow as mastigotes during the light phase, and divide in the dark as coccoid cells. The coccoid cells of Symbiodiniaceae are spherical with an average diameter of 10 μm and, in this stage, they become intracellular symbionts inside of the coral and other hosts (Shah et al. 2020; Figueroa et al. 2021). On the other hand, motile mastigote cells (sometimes referred as free–living cells) have different dimensions of the epicone and hypocone among species, and they can be found in different marine ecosystems (Wham et al. 2017). The free–living mastigote cells are essential to about 80% of coral species that establish endosymbiotic relationships anew each generation or during an environmental change (e.g., salinity reduction and temperature rise) (Claar et al. 2020). In view this, it is clear that exploring the diversity and coral–specificity, the

nutritional strategies, the responses to stress of free–living Symbiodiniaceae is crucial to understand the functioning of coral reefs.

Nitrogen is an essential nutrient for microalgae growth and plays a fundamental role in biosynthesis of protein, lipid, and carbohydrate (Su 2021). Microalgae can assimilate nitrogen in the form of nitrate, nitrite, urea, and ammonium, nonetheless, although the latter is often most efficiently assimilated, at high concentrations (approx. at 25 μM) it may exhibit toxicity to cells (Yaakob et al. 2021). Intracellularly, the excess nitrogen might be stored in chemical and biochemical forms, such as free amino acids, proteins (especially Rubisco), and chlorophylls (Guilherme et al. 2019; Walker et al. 2018). On the other hand, under nitrogen deplete conditions, microalgae cells chance their carbon storage patterns in favor of neutral lipids, generally by the degradation of polyunsaturated fatty acids for triacylglycerol (Rodolfi et al. 2009; Wang et al. 2019). Although there is a number of studies on the effects of nitrogen–replete and –deplete in microalgae cultivation, these studies mainly focus on lipids profile and yield of biomass produced, generally aiming a boosting in biofuels production (Tarazona Delgado et al. 2021; Wei et al. 2022), and almost never to assess the physiological state of cells and their susceptibility to stress factors.

Understanding nutritional strategies that can improve resistance of endosymbiont dinoflagellates can contribute for optimization of large–scale cultivation of the endosymbiotic dinoflagellates and elucidation of their susceptibility to environmental stress resulting in coral bleaching events. Here, we described the physiological mechanisms of *Durusdinium glynnii* cells cultured under high and low nitrogen supply, using sodium nitrate and urea as nitrogen source. Our approach is based on two main hypotheses: (1) nitrogen–replete condition increases the resistance of *D. glynnii* to

stresses and; (2) urea is more efficient than nitrate to support cell growth and to improve the resistance to stresses.

2. Materials and methods

2.1. Biological material

Durusdinium glynnii was maintained in filter–sterilized seawater (salinity of 30 psu) enriched with f/2 medium–Si at 22 ± 1 ºC, under continuous lighting at 150 µmol photons m^{-2} s^{-1}. Cultures were kept in exponential growth by regular transfer to fresh media to avoid nutrient limitation.

2.2. Experimental set–up

A methodological flowchart of the experimental set–up is presented in Fig. 1. Experimental acclimated cultures of *D. glynnii* were performed in 250 mL Erlenmeyer flasks, under irradiance of 300 µmol photons m^{-2} s^{-1} provided by 36 W light–emitting diodes panels, and bubbled with atmospheric air. In order to assess the effects of concentration and source of nitrogen on the light– and heat–tolerance of *D. glynnii*, two concentrations (1760 and 440 µM resulting in a N:P ratio of 28:1 and 7:1, respectively) of nitrogen in form of sodium nitrate ($NaNO_3$) and urea (CH_4N_2O), normalized by nitrogen percentage in each form, were evaluated resulting in a bi–factorial (2×2) design with six independent replicates (n = 6) for each condition.

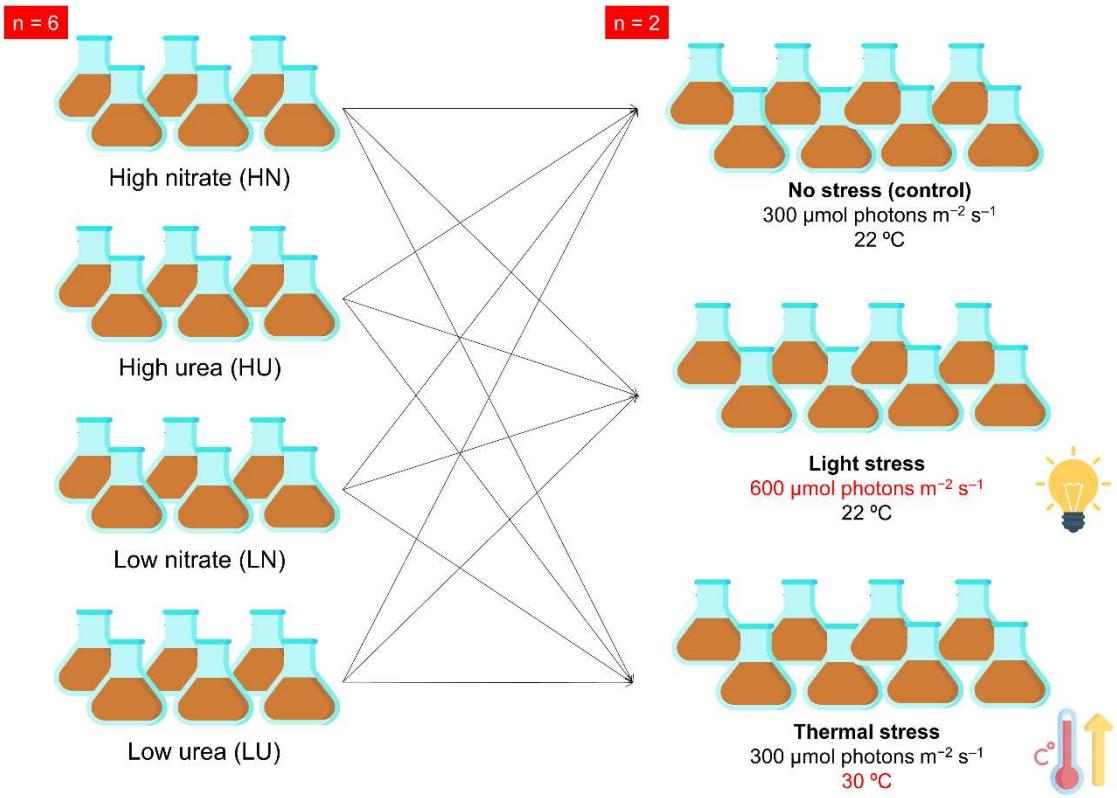

Fig. 1. Flowchart for the experimental set–up.

Initially, cultures grown for 96 h and then, they were diluted to a mean cell concentration of 21.4 ± 2.6 × 10^4 cells mL^{-1}. After dilution, the nitrogen concentration in each of the treatments was adjusted as close as possible to the time before dilution (after growth), when necessary. Then, two experimental units from each nitrogen condition were submitted for stress assays: (1) in the thermal stress, the cultures were transferred to a germination chamber with a thermostat adjusted to 30 ± 0.5 °C, under the same illumination regime (i.e., 300 µmol photons m^{-2} s^{-1}); (2) in the light stress, the irradiance subjected to the cultures was increased to 600 µmol photons m^{-2} s^{-1}, by adding a new LED panel, and adjusting using a quantameter and the distance of the cultures from the light source. The other cultures (control group) were maintained at the same conditions previously described (i.e., 22 ± 1 ºC and 300 µmol photons m^{-2} s^{-1}). The stress assays were maintained for 96 h. Stress levels were chosen based on previous photoacclimation

(Oliveira et al. 2022) and thermal stress (Lin et al. 2019) studies with Symbiodiniaceae species.

2.3. Growth analysis

Cell concentration (c, cells mL^{-1}) and cell type (mastigote and coccoid forms based on based on morphological characteristics reported in Kang et al. (2020)) were analyzed using a hemacytometer under an optical microscope (400 or 1,000× of magnification). An asymmetric logistic equation was used for fitting the cell concentration (C(t)) vs. time (t) data in order to accurately determine the specific growth rate (μ, day^{-1}), before (μ_c) and after the stress (μ_s) according to equation elsewhere described (Oliveira et al. 2022).

2.4. Photosynthetic pigments

At the end of the stress assays, samples from each experimental unit were taken to analyze intracellular pigments. After centrifuging 100 mL of algal culture for 10 min at 2000 rpm, the remained biomass was subjected to pigment extraction using acetone 90% (Strickland and Parsons 1972). Chlorophylls–*a* (Chl–*a*) and c (Chl–*c*; c1 + c2) contents were calculated according to Jeffrey and Humphrey (1975), while carotenoid content (i.e., total carotenoids, β–carotene, and peridinin) were analyzed following the methods proposed by Carreto and Catoggio (1977) and Prézelin (1976). Values for all pigment concentrations were expressed as pg $cell^{-1}$.

2.5. Stable isotopes analysis

2.5.1. Sample preparation

At the end of the stress assays, 50 mL from each duplicate treatment were pooled together and vacuum filtrated (~200 mbar) onto pre–combusted (450 °C for 4 h) GF/C glass

microfiber filters (pore size 1.2 μm), and stored in sterile glass Petri dishes. Filters were oven dried at 60 ºC for 24 h and decarbonated with concentrated HCl (12 M) for 4 h in a desiccator (Lorrain et al. 2003). Afterwards, the filters were cut and encapsulated in high purity aluminum disc.

2.5.2. Elemental and stable carbon isotope analysis

Cellular particulate organic carbon (POC) content, carbon stable isotope ratio ($\delta^{13}C$), nitrogen stable isotope ratio ($\delta^{14}N$) the stoichiometric ratio of particulate organic carbon to nitrogen (C:N) were determined with an elemental analyzer (EA, EuroVector, model EA3000 Single) coupled to an isotopic ratio mass spectrometer (IRMS, Thermo Scientific, model Delva V Advantage). The temperature in the EA furnace was maintained at 980 °C. Helium (purity: 99.99%), was used as a carrier gas at a flow rate of 93 mL min^{-1}. A pulse of 15 mL of oxygen (purity: 99.99%) was introduced into the reactor to facilitate combustion of the sample. The gases generated in the reactor were separated in a chromatographic column maintained in an isothermal oven (70 °C), and then transferred to the IRMS. The analysis time of a sample totaled five minutes. The IRMS was routinely calibrated with reference gases (CO_2 and N_2) traceable to an international isotopic standard (Vienna Pee Dee Belemnite – VPDB). A certified reference material (casein, Elemental Microanalysis P/N B2155) was employed for quality control. Analytical precision for $\delta^{13}C$ was 0.02 ‰. Results are presented according to the commonly used δ–notation Eqs. (1) and (2) expressed as per mil (‰) as follow:

$$\delta^{13}C = \left(\frac{\left({}^{13}C/{}_{12}C \right)_{sample}}{\left({}^{13}C/{}_{12}C \right)_{reference}} - 1 \right) \times 1000 \quad (1)$$

$$\delta^{13}\mathrm{N} = \left(\frac{\left({}^{13}N/_{12}N \right)_{sample}}{\left({}^{13}N/_{12}N \right)_{reference}} - 1 \right) \times 1000 \quad (2)$$

3. Results

In the first 96 h of *Durusdinium glynnii* growth in control conditions, the high urea concentration accelerated cell growth ($p < 0.01$, $n = 6$), reaching $91.83 \pm 16.83 \times 10^4$ cells mL^{-1} at 0.55 ± 0.05 day^{-1}, while the other nitrogen conditions grown at nearly 0.24 day^{-1}. In the 96 h after cultures dilution, without environmental changes, the high nitrogen concentration presented higher growth in comparison to the low one. During the light stress, in oppose to control conditions, the use of sodium nitrate showed a higher growth performance compared to urea, in both concentrations. In the temperature stress, a gradual reduction in cell concentration was observed for all nitrogen conditions, except the high urea concentration, resulting in negatives values of μ_s due to decline in cell densities (Fig. 2a–c, Table 1).

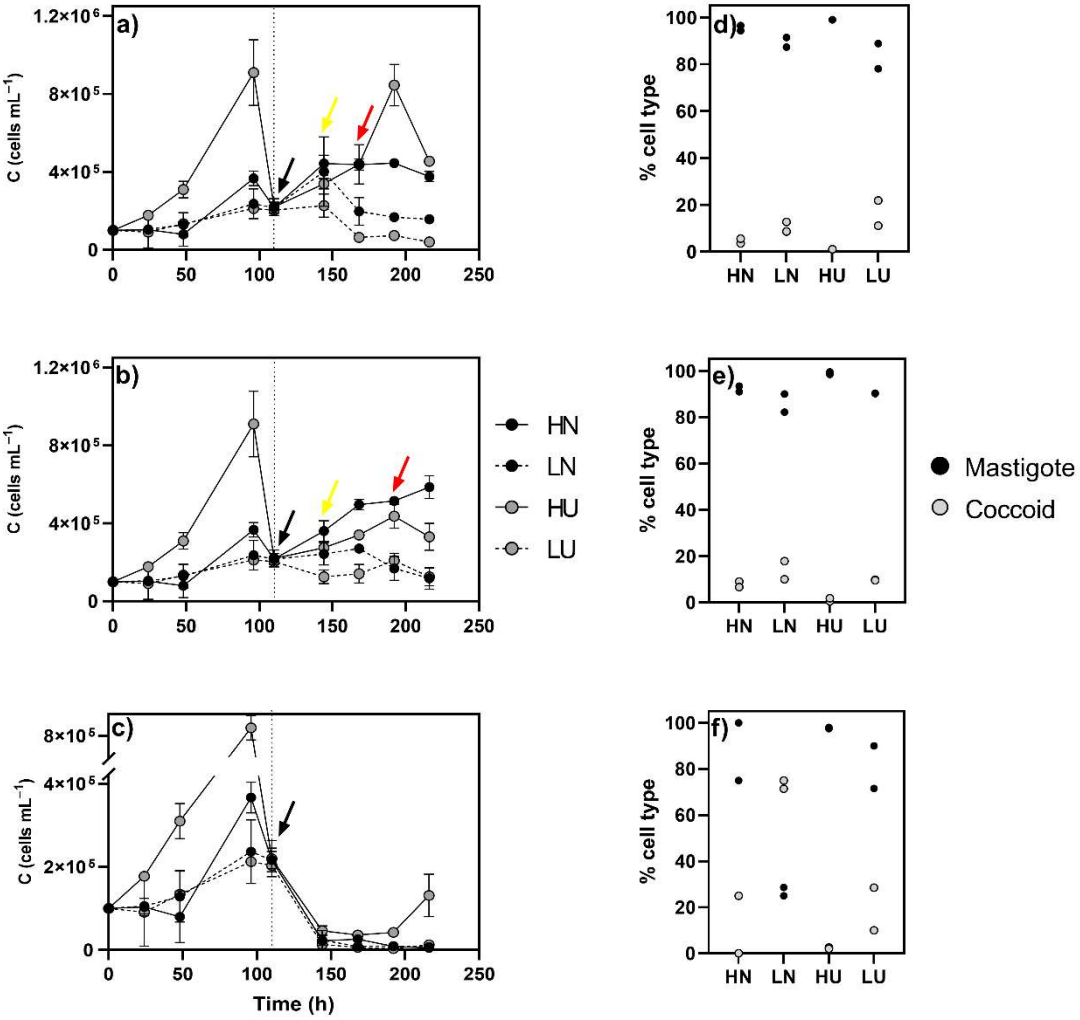

Fig. 2. Growth curves (a–c) and cell type proportion (d–f) of *Durusdinium glynnii* subjected to the light (b and e) and temperature (c and f) stress. Figures (a) and (d) represent the control. Black arrows indicate the dilution of the culture and the start of stress assay. Yellow and red arrows indicate the approximate timing of nitrogen depletion at low and high concentrations, respectively. Points plotted in cell type figures represents mean value from each experimental unit during the 96 hours of stress.

Table 1. Response of *Durusdinium glynnii* cultured using sodium nitrate and urea in terms of growth performance, stable carbon and nitrogen isotopes, and carbon–to–nitrogen ratio.

Parameter	Sodium nitrate		Urea	
	High	**Low**	**High**	**Low**
Control				
c (10^4 cells mL^{-1})	44.50±2.13	35.12±10.78	84.5±10.61	24.75±3.18
μ_c (day^{-1})	0.32±0.03	0.21±0.08	0.55±0.05	0.19±0.01
μ_s (day^{-1})	0.23±0.04	0.25±0.14	0.47±0.03	0.16±0.06
$\delta^{13}C$ (‰)	–14.10	–14.06	–15.90	–13.85
$\delta^{15}N$ (‰)	–29.44	–28.19	–3.03	–2.23
C:N	5.19	5.03	4.78	4.64
Light stress				
c (10^4 cells mL^{-1})	58.62±5.83	27.00±0.71	43.62±6.19	22.87±0.88
μ_c (day^{-1})	0.32±0.03	0.21±0.08	0.55±0.05	0.19±0.01
μ_s (day^{-1})	0.24±0.01	0.14±0.03	0.18±0.04	0.11±0.00
$\delta^{13}C$ (‰)	–14.71	–16.16	–14.07	–14.02
$\delta^{15}N$ (‰)	–28.96	–28.15	–1.90	–1.80
C:N	5.16	5.51	4.88	4.65
Temperature stress				
c (10^4 cells mL^{-1})	2.25±2.47	0.87±0.53	13.12±5.13	0.62±0.25
μ_c (day^{-1})	0.32±0.03	0.21±0.08	0.55±0.05	0.19±0.01
μ_s (day^{-1})	–0.69±0.41	–0.81±0.15	–0.12±0.10	–0.56±0.01
$\delta^{13}C$ (‰)	–16.45	–16.31	–14.52	–16.33
$\delta^{15}N$ (‰)	–28.50	–27.95	–1.76	–0.67
C:N	5.18	5.52	4.60	5.12

Samples for $\delta^{13}C$, $\delta^{15}N$, and C: N were pooled for analysis, resulting in single data for each treatment.

The cell morphotype of *Durusdinium glynnii* was mostly (over 80% of the total population) mastigote cells in the control conditions (Fig. 2d) and in the light stress (Fig. 2e) assay. On the other hand, the percentage of mastigote cells was lower (below 80%) during the temperature stress (Fig. 2f), except for the use of high urea concentration – above 95% of the population was mastigote cells. In addition, the population in the low urea concentration condition at the temperature stress was composed mainly by coccoid cells.

The cellular $\delta^{13}C$ values were relatively stable both with respect to the use of sodium nitrate and urea, their respective concentrations, and also when subjected to thermal and light stress, ranging from –16.45 to –13.85‰. For $\delta^{15}N$, a clear distinction between nitrogen sources was observed: the sodium nitrate source ranged from –29.44 to –27.95‰, while for urea one, ranged from –3.03 to –0.67. Furthermore, for urea source, lower $\delta^{15}N$ values were reported for both stress assays compared to control conditions. Finally, C:N ratio of cells grown using sodium nitrate (5.27 ± 0.20) as nitrogen source showed higher values ($p<0.05$; $n = 6$) compared to those grown using urea (4.76 ± 0.22) (Table 1).

Pigments contents in *D. glynnii* subjected to light and thermal stress showed differences compared to the control (Fig. 3). Overall, cells grown under high nitrogen condition had higher contents of chlorophyll–*a*. Cells subjected to the light stress had higher content of total carotenoids when grown using urea as nitrogen source at the two concentrations – it was also reflected in the contents of β–carotene and peridinin. In the thermal stress, the contents of all pigments in cells grown at high urea concentration were higher than other treatments.

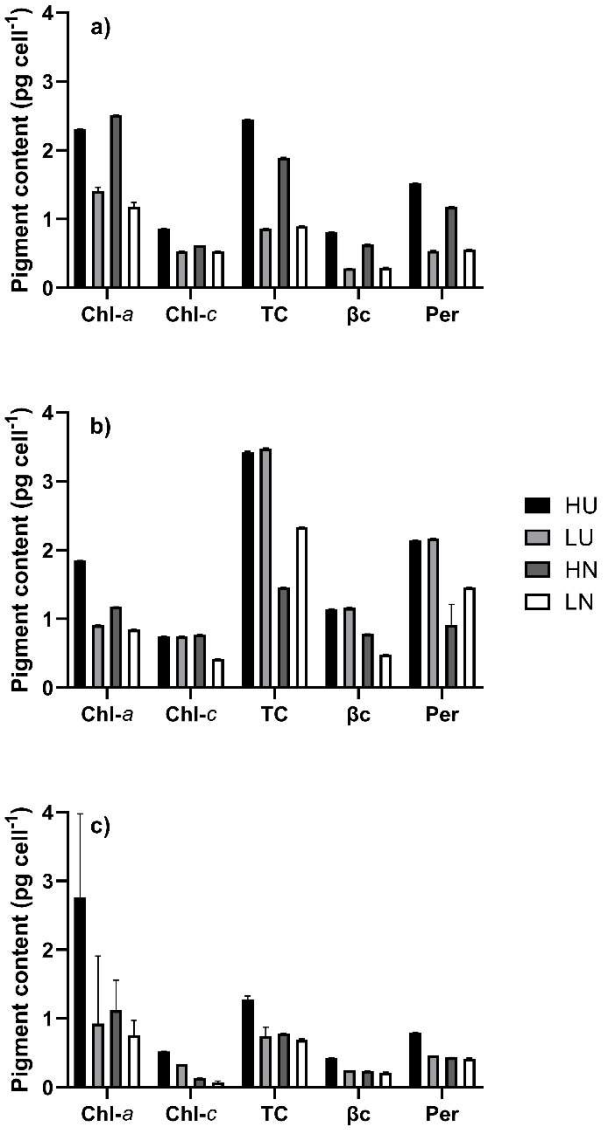

Fig. 3. Pigments content and composition of *Durusdinium glynnii* grown using sodium nitrate and urea as nitrogen source under control (a), light (b), and temperature (c) stress. HU – high urea; LU – low urea; HN – high sodium nitrate; LN – low sodium nitrate; Chl–*a* – Chlorophyll–*a*; Chl–*c* – Chlorophyll–*c*; TC – total carotenoids; βc – β–carotene; Per – Peridinin.

4. Discussion

The dataset here analyzed suggests the nitrogen overaccumulation can increase the tolerance of the endosymbiotic dinoflagellate *Durusdinium glynnii* subjected to light and

thermal stress. Moreover, the use of high urea concentration was the only condition that supported the cell growth and division of *D. glynnii* subjected to thermal stress. This may be associated with the fact that urea is more rapidly converted intracellularly into amino acids compared to nitrate–based compounds, as demonstrated in **Figure 4**. The use of urea as nitrogen source also has a less energetic cost during its assimilation (Su 2021).

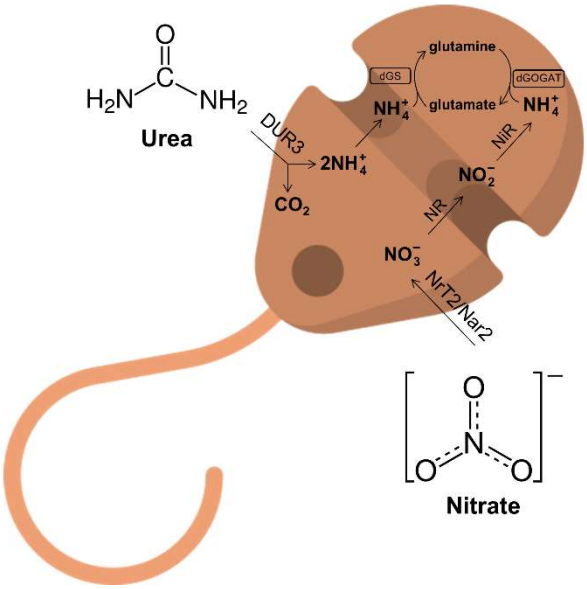

Fig. 4. Schematic representation of the inorganic nitrogen assimilation pathway and glutamate/glutamine cycle in *Durusdinium glynnii*.

Some authors suggest that the preferred order of nitrogen utilization by eukaryote microalgae is ammonium > nitrate > nitrite > urea (Perez-Garcia et al. 2011; Su 2021). However, some recent reports (e.g., Ou et al. 2019; Huang et al. 2020) suggest that urea is the most preferred nitrogen source by dinoflagellates, as reported by Matantseva et al. (2016) that the addition of urea to the nitrate–acclimated culture of the marine dinoflagellate *Prorocentrum minimum* led to noticeable suppression of the nitrate–nitrogen uptake. Thus, probably the preferred order of nitrogen assimilation for dinoflagellates is “ammonium > urea > nitrate > nitrite” or “urea > ammonium > nitrate

> nitrite", depending on species (Burford 2005). For decades, the use of urea as nitrogen–based fertilizer by farmers has pointed out as one of the main contributing to coastal eutrophication (Glibert et al. 2006). The escalating of using urea has associated with harmful dinoflagellate blooms species, due to the higher urease activity compared to other phytoplankton groups (Solomon and Glibert 2008; Jing et al. 2017). The findings of the present study corroborate this information, since *D. glynnii* presented high cell division when using urea as nitrogen source.

Regarding the isotopic signatures, no major changes were observed in the $\delta^{13}C$ values, and these values were within the range reported for *D. glynnii* under control conditions (Müller et al. 2021) and other C4–photosynthetic microalgae (Raven et al. 2020). On the other hand, a clear difference in the $\delta^{15}N$ signature was observed between the nitrogen sources, providing the effectiveness in the uptake of the different nitrogen source in the medium. The $\delta^{15}N$ signatures for *D. glynnii* cultured using urea as nitrogen source were similar to those reported by Bateman and Kelly (2007) for urea fertilizers from different manufacturers. Similarly, Freyer and Aly (1974) reported that the isotopic composition of sodium nitrate is much lower than that of other sources of inorganic nitrogen due to residual nitrogen oxides from the nitric acid process, resulting in $\delta^{15}N$ signatures close to –22 ‰. Although stable isotope analysis is routinely used in ecological studies of phytoplankton (e.g., Cai et al. 2019; Yang et al. 2020; Sabadel et al. 2022), it is rarely used to prove the effectiveness in absorption of dissolved compounds in microalgae cultures. Thus, this analysis can be successfully used to effectively track the uptake of inorganic and organic compounds by microalgae.

Contrary to our second hypothesis, the use of sodium nitrate as nitrogen source was more efficient than urea in terms of growth performance of *D. glynnii* subject to the light stress. This can be associated with the fact that light can stimulates the enzymatic activity

of nitrate reductase and glucose–6–phosphate dehydrogenase, which may have increased the rate of nitrate uptake and assimilation to protein (Tischner and Hüttermann 1978; Wang et al. 2022). Moreover, it may also be associated to increased nitrogen accumulation during the 96 h before the stress. At this moment, the cells cultured with high urea concentration presented a faster metabolism and this resulted in an intense cell division in the first days of cultivation. While the cultures with nitrate may be accumulating nitrate during this first moment.

The high content of peridinin in high nitrogen cultures can be linked to the fact that this carotenoid is associated with proteins, in form of peridinin–chlorophyll–protein complex, and thus, the nitrogen limitation may inhibit the peridinin biosynthesis (Di Valentin et al. 2016; Dorrell et al. 2019). During the thermal stress, the higher peridinin content in high urea condition may explain greater cellular health at this condition, while in the other conditions, the peridinin may have oxidized. The oxidation of intracellular metabolites (such as fatty acids and pigments) in Symbiodiniaceae subjected to thermal stress has also been previously reported. For example, Botana et al. (2022) reported an increase in oxy–polyunsaturated fatty acids in *Breviolum minutum* cells after heat sock (at 34 ºC). Here, we also provide evidence that peridinin may act as a protector against oxidative stress resulting from a temperature rise. In a previous study (Oliveira et al. 2022) we reported that under optimal irradiance for growth *D. glynnii* maintained a peridinin to chlorophyll–*a* ratio of approximately 1 (at 3.5 pg cell^{-1}), while under high–light exposure this ratio increased to approximately 4, before the photoinhibition zone. Here, this ratio was close to 1, in the high nitrate condition, but at a content of 1.5 pg cell^{-1}. These differences can be attributed to the cell concentration, since higher number of cells increases the light attenuation (Pruvost et al. 2015), reducing potential photo–oxidative stress.

4.1. Ecological implications

Our findings cannot be directly applied to natural environments for the obvious reason that artificial nitrogen enrichment would result in an invaluable environmental imbalance. Furthermore, the physiological responses of non–motile coccoid cells in endosymbiosis may be different from free–living mastigote cells. But it is worth noting that the presence of free–living cells of Symbiodiniaceae in the environment represent important pools for coral symbiont acquisition (Claar et al. 2020). Additionally, the results herein presented may help for understanding the Symbiodiniaceae susceptibility to changing environmental conditions, particularly those linked to global warming. Thus, a schematic drawing was constructed to summarize the response of *D. glynnii* related to a rise temperature or an exposure to high light (**Fig. 5**). Overall, our findings support the idea that corals from nutrient–poor waters (particularly in nitrogen) are more susceptible to bleaching events in a situation of temperature rise. Similarly, Symbiodiniaceae cells when using urea tend to accumulate the carotenoid peridinin to prevent photo–oxidation – these results may be related to the shallow water reefs.

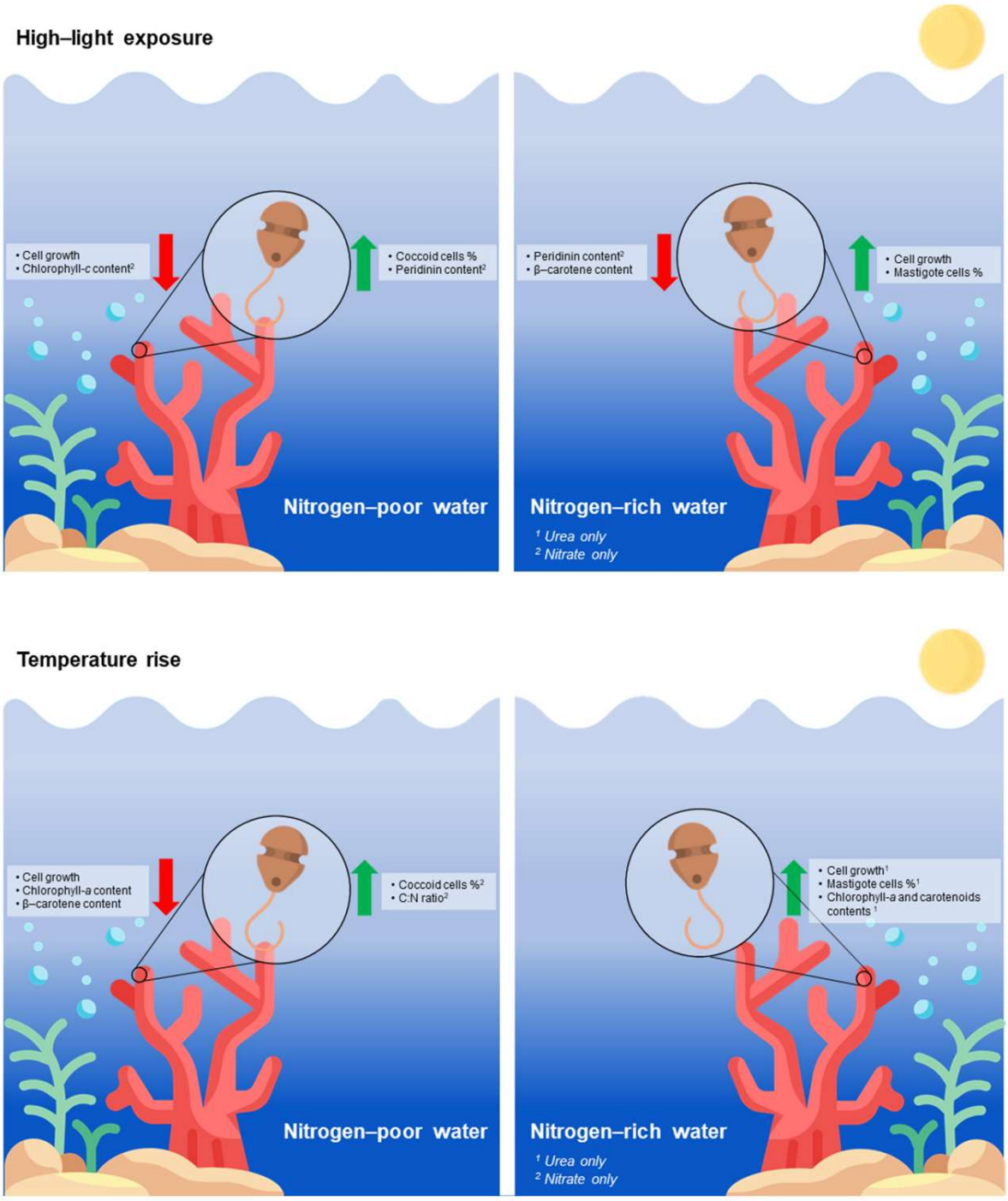

Fig. 5. Schematic illustration of the main ecological implications of high–light exposure and temperature rise for the endosymbiotic dinoflagellate *Durusdinium glynnii* in nitrogen–poor and –rich waters.

Undoubtedly, the temperature rise is one of the main stressors for endosymbiotic dinoflagellates. Here, we show that a well–established culture of *D. glynnii* gradually reduced its population over time after increasing temperature – except for the high urea condition. In the natural environment, a temperature rise may occur in combination with another environmental stressors, such as the presence of an emerging pollutant or a change in salinity, resulting in even more severe impacts (Coles and Jokiel 1978; Camp et al. 2016; Stien et al. 2020), and these multiple disturbances of the host–symbiont relationship can rapidly impact the homeostasis of reef ecosystems. Therefore, the synergism between various environmental stressors should be a priority issue, given the modern changing world.

Several factors, such as geographic (e.g., river inputs on coastal), anthropogenic (e.g., industrial wastewater disposal and eutrophication), temporal (e.g., seasonality of inorganic fertilization of agricultural land in coastal regions) and oceanographic (e.g., remineralization and lateral transport of nutrients), can influence the sources and dynamics of nitrogen in the oceans (Zehr and Ward 2002; Howarth 2008). This causes seas to be diversified in terms of nitrogen sources and concentrations, resulting in different coral susceptibilities depending among other factors, on main sources of nitrogen input (Roff and Mumby 2012; Cannon et al. 2021). However, it is worth noting that due to the diversity of Symbiodiniaceae taxa, the behavior reported here for *D. glynnii* may not be the same for other Symbiodiniaceae species.

4. Conclusions

Our approach showed nitrogen as a key nutrient involved with resistance to light- and thermal-stressors for *D. glynnii*. The availability of reduced nitrogen form, such as urea, can accelerate intracellular metabolism and alleviate environmental stressors (i.e.,

thermal stress). Additionally, our findings provide initial evidence that the carotenoid peridinin may have a thermal protective role for endosymbiont dinoflagellates. However, future interspecific and molecular investigations (assessing the regulation of correlated genes) still need to be conducted.

Compliance with Ethical Standards

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Conflict of Interest

The authors declare no competing interests.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution

CYBO: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. **BCSB:** Formal Analysis, Investigation. **LGSJ:** Data Curation,

Formal Analysis. **DWSO:** Formal Analysis. **GTY:** Data Curation Formal Analysis, Resources. **MNM:** Resources, Supervision, Writing – review & editing. **AOG**: Project administration, Resources, Supervision, Writing – review & editing.

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4.3. Article 6: A holistic approach to produce anti-*Vibrio* compounds using wastewater from shrimp culture

Research in this field will be submitted to the following journal	
Title	A holistic approach to produce anti-*Vibrio* compounds using wastewater from shrimp culture
Authors	**CYB Oliveira**, JL Abreu, BCS Brandão, DWS Oliveira, EP Santos, Costa GKA, SMBC Silva, Dantas DMM, MN Müller, AO Gálvez
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A holistic approach to produce anti-*Vibrio* compounds using wastewater from shrimp culture

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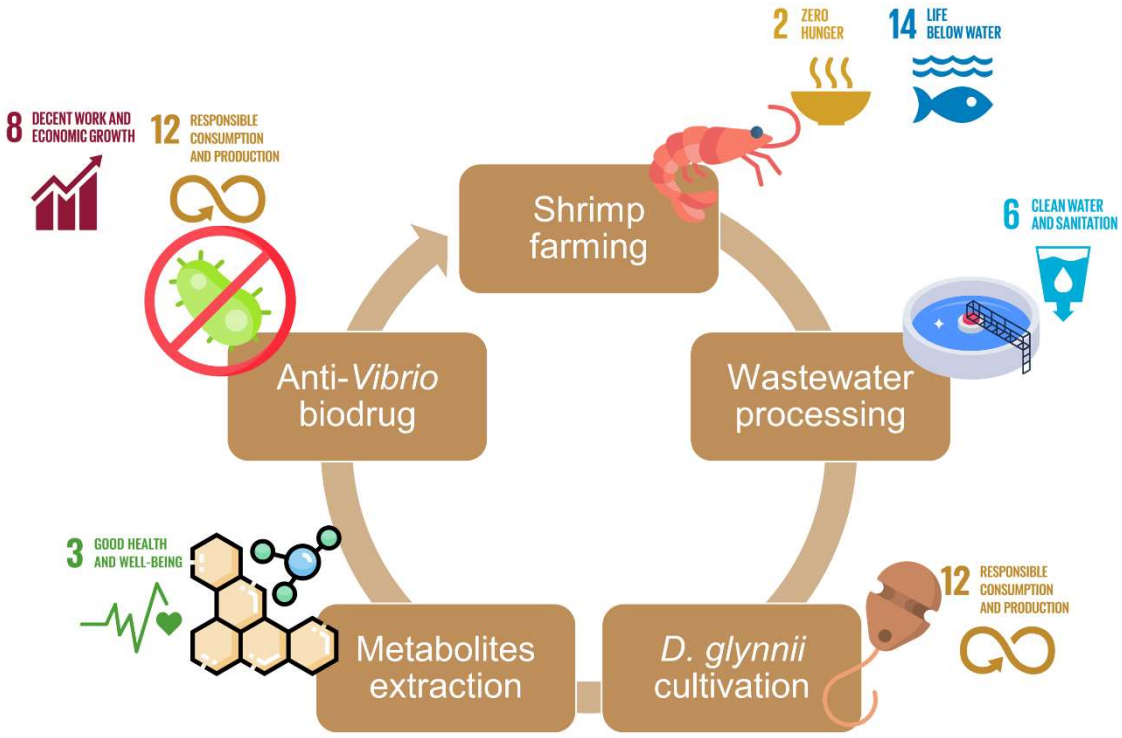
Abstract

Aquaculture industry requires green solutions to solve several environmental challenges, including adequate wastewater remediation and natural drugs to treat bacterial and virus diseases. In this study, feasibility of the endosymbiotic dinoflagellate *Durusdinium glynnii* cultivation in aquaculture wastewater from shrimp farming in synbiotic system (AWW–SS) diluted in different proportions of f/2 medium (FM) was investigated. Interestingly, *D. glynnii* grew better in any AWW–SS ratio than in the control (FM). The better proportions of AWW–SS and FM, in terms of growth performance were: 75% AWW–SS: 25% FM and 50% AWW–SS: 50% FM. The removal of total nitrogen and total phosphorus reached 50.1 and 71.7%, respectively, from the crude AWW–SS. Biomass grown on AWW-–S was able to inhibit the growth of *Vibrio parahaemolyticus* (inhibition zone of 10.0 ± 1.7 mm) and *V. vulnificus* (inhibition zone of 11.7 ± 1.5 mm). The results of this study revealed that *D. glynnii* is a potential dinoflagellate for development of circularity in aquaculture industry, particularly, by producing anti–*Vibrio* compounds with quasi–zero cost.

Keywords: aquaculture, dinoflagellate, peridinin, synbiotic.

Graphical abstract



Highlights

- Mixtures of aquaculture wastewater and f/2 were used as dinoflagellate growth media;
- Maximum *D. glynnii* growth was observed in a medium containing 50 or 75% of wastewater;
- *D. glynnii* removed 71.7% of total phosphorus;
- Acetonic extract from biomass grown in wastewater inhibited the growth of *Vibrio* strains;
- Inhibition zone of *V. parahaemolyticus* was positively correlated with carotenoid peridinin.

1. Introduction

Aquaculture plays a key role in achieving the sustainability development goals (SDGs) set out by United Nation in the 2030 Agenda, particularly in the SDGs 1 – No poverty, 2 – Zero hunger, 3 – Google health and well–being, 8 – Decent work and economic growth, 12 – Responsible consumption and production, 13 – Climate action, and 14 – Life below water (Hambrey, 2017; Stentiford et al., 2020). In recent years, fish production by aquaculture has surpassed fishing, and sustainable fish production can help restore overexploited fish stocks around the world without compromising food security (FAO, 2022). However, new practices and actions still need to be developed and/or improved to solve the old problems of aquaculture, i.e., new sources of proteins and oils suitable for fish nutrition (Alhazzaa et al., 2019; Jones et al., 2020), suitable treatment of wastewaters rich in nitrogen and phosphorus (Maigual-Enriquez et al., 2019; Farzana et al., 2019), and use of sustainable drugs to treat diseases (Heal et al., 2021). *Vibrio* are Gram-negative pathogenic bacteria widely distributed in marine environments. These bacteria cause vibriosis disease that resulted in high mortalities, and are a major threat to aquaculture farmers worldwide (Ghosh et al., 2021). Furthermore, some *Vibrio* spp. showed resistance to some traditional antibiotics used to control vibriosis, such as cotrimoxazole, chloramphenicol, and streptomycin, therefore, new anti–*Vibrio* agents with low resistance risk are of great interest to aquaculture sector (Kumara et al., 2018; Ghosh et al., 2021).

Microalgae are considered the best alternative to solve these aquaculture problems (Oliveira et al., 2022a). This is because they can efficiently treat wastewater, absorbing up to 99% of nitrogen and phosphorus sources (Guo et al., 2013; Tejido-Nuñez et al., 2019), and producing a valuable biomass that can be rich in lipids and proteins suitable for fish nutrition, and other bioactive metabolites (Shah et al., 2018). This circular process enables to recycle waste in a holistic approach with zero or quasi–zero residue production.

The circular bioeconomy is based on the application of green and sustainable chemistry principles in order to replace fossil–based materials by biologically–based resources. The circularity in aquaculture has the potential to improve profitability and sustainability through the valorization of by–products and wastes (solids and liquids) (Regeiro et al., 2021). Although several studies have already proved the ability of microalgae to grow in different types of aquaculture wastewater (e.g., Gao et al., 2016; Cardoso et al., 2020; Oliveira et al., 2020), few studies have been concerned with the application of this biomass as an aquaculture input, proving the effectiveness of a circular and holistic process.

Microalgae are capable of synthesizing primary and secondary metabolites and, according to Lopes da Silva et al. (2019), contribute to the global bioeconomy due to their ability to produce marketable value–added products from liquid, gaseous, and solid wastes. The primary metabolites are those necessary for cell growth, i.e., protein, carbohydrates, and lipids, while the secondary metabolites have vital functions in ecological interactions and adaptive strategies (Stirk and van Staden, 2020). Particularly, carotenoids from microalgae have a number of biological activities, including anticancer, antioxidants, antifungal, and antibacterial (Koyande et al., 2019; Stirk and van Staden, 2020). In recent years, in addition to the popular carotenoids synthesized by green microalgae, such as astaxanthin, lutein, and β–carotene, two other emerging carotenoids (i.e., fucoxanthin and peridinin) have gained prominence for their interesting biological activities (Lourenço-Lopes et al., 2021; Supasri et al., 2021). Peridinin is an apocarotenoid exclusively found in phototrophic dinoflagellates that play roles in light harvesting and can also protect cellular photosynthetic machinery from photo–oxidative damages by the scavenging free radical (Oliveira et al., 2022b). However, due to the

difficulties in dinoflagellates isolating and cultivation, this carotenoid has not yet been widely studied in terms of biological activities.

Herein, we examined the wastewater treatment capacity from shrimp culture in synbiotic system by the dinoflagellate *Durusdinium glynnii*. Firstly, the growth and nutrient uptake by *D. glynnii* was investigated, and the biomass produced was characterized in terms of (i) secondary metabolites and (ii) anti–*Vibrio* activity.

2. Materials and methods

2.1. Shrimp production

Pacific white shrimp *Penaeus vannamei* was cultured in a circular fiberglass tank (10 m^{-3}) under a super intensive system without water changes for 3 months. The culture was conducted with seawater (salinity of 35 PSU) that received inorganic fertilization with urea (4.5 g m^{-3} N), triple superphosphate (0.3 g m^{-3} P), and sodium silicate (0.23 g m^{-3} Si). After two days, organic fertilization was begun through 12 applications of product for 24 h in an anaerobic phase followed by an aerobic phase (24 h). The organic fertilizer was composed of rice bran (20 g m^{-3}), molasses (2 g m^{-3}), sodium bicarbonate (4 g m^{-3}) and a bacteria-based product (0.05 g m^{-3}), containing *Bacillus subtilis*, *B. licheniformis*, *Saccharomyces* sp. and *Pseudomonas* sp. at a total of 5.5 to 6.5 × 10^7 CFU g^{-1}. This synbiotic system has a final C:N ratio of 4.13. The shrimp production results were described elsewhere (de Andrade et al., 2021).

2.2. Dinoflagellate strain and culture conditions

Durusdinium glynnii (clone BMK 211) was obtained from Culture Collection of Laboratório de Produção de Alimento Vivo (Brasil). The strain was maintained in seawater (30 PSU), previous filtered (0.45 μm) and sterilized (121 ºC for 21 min),

enriched with f/2 medium without Si. Cultures were kept in a room with controlled temperature (22 ± 1 ºC), under continuous illuminance (150 μmol photons m^{-2} s^{-1}) and transferred to a newly fresh medium regularly.

The experimental cultures were grown under controlled conditions in 250–mL Erlenmeyer flasks, at the same temperature and under 300 μmol photons m^{-2} s^{-1} at an integral photoperiod. They were aerated at a rate of 0.05 vvm without CO_2 addition to not increase production costs and to allow the application on a large–scale.

Wastewater was submitted to solids sedimentation for 30 min and supernatant double filtered (1 μm). Moreover, the water chlorinated (at 50 ppm) and neutralized (25 ppm of thiosulphate solution). The seawater used to prepare f/2 medium was submitted to a single filtration, and the same purification, disinfection, and sterilization procedures.

2.3. Experimental design

Four proportions of aquaculture wastewater from synbiotic system (AWW–SS) were evaluated: 25 (25% AWW–SS), 50 (50% AWW–SS), 75 (75% AWW–SS), and 100% (100% AWW–SS), the remainder, i.e., 75, 50, 25, and 0%, respectively, was completed with f/2 culture medium (FM). In addition to these four treatments, a positive control (without wastewater addition) was also conducted. All treatments and the control were performed with three independent replicates, and the experiment was conducted in a completely randomized design.

2.4. Biological, chemical, and biochemical analyses

Samples were taken at day 0, 1, 2, 3, 6, 9, 12, and 15 of each independent replicate for growth analyses. Samples taken from 0, 7, and 15 were collected to determine nutrients

uptake (nitrogen and phosphorus) and pigments (chlorophylls and carotenoids) produced by *D. glynnii* during algal growth.

2.4.1. Growth evaluation

Biomass (mg L^{-1}) was estimated by the gravimetric method using 0.45 μm glass fiber microfilters (APHA, 2005). Moreover, cell concentration (c, cells mL^{-1}) was determined using a Neubauer hemocytometer under an optical microscopy. The cell concentration was used to determine the specific growth rate (μ, day^{-1}) in the exponential growth phase as described in Oliveira et al. (2022b).

2.4.2. Nitrogen and phosphorus analyses

Aliquots from biomass filtering were collected and subjected to the ammonia−N (NH_4^+-N; APHA, 2005), nitrite−N (NO_2^-−N; Fries, 1971), nitrate−N (NO_3^-−N; APHA, 2005), orthophosphate ($P–PO_4^{3-}$; APHA, 2005). The removal efficiency in % was calculated according to Ansari et al. (2017).

2.4.3. Pigments analysis

15–mL aliquots were centrifugated at 3,000 rpm for 10 min, and remained biomass was subjected to pigment extraction using acetone 90% (Strickland and Parsons, 1972). For the photosynthetic pigments, *i.e.*, Chlorophylls (Chlorophyll–*a* + *c*) contents were calculated according to Jeffrey and Humphrey (1975), while carotenoid contents (*i.e.*, total carotenoids, β–carotene, and peridinin) were analyzed by following the methods proposed by Carreto and Catoggio (1977) and Prézelin (1976). Values for all pigment concentrations were normalized as mg g^{-1} biomass.

2.5 Antibacterial activity

Vibrio strains (i.e., *V. parahaemolyticus* and *V. vulnificus*) were cultured onto thiosulfate citrate bile salt agar (TCBS) plates and incubated at 37 °C. The antimicrobial susceptibility of *V. parahaemolyticus* and *V. vulnificus* strain was determined using acetonic extract of *D. glynnii* biomass through Kirby–Bauer method according to the guideline of the Clinical and Laboratory Standards Institute (CLSI, 2016). A bacteria broth (10^8 CFU mL^{-1}) was prepared, and then was distributed in sterile Petri plates (140 × 15 mm) using a sterile swab. Sterile blank paper discs (6 mm diameter) impregnated with 20 µL of extracts carried out using dried algae (10%, w v^{-1}) were added onto agar plates. The disc with solvent was negative control. Plates were incubated at 37 ℃ for 24 h. A transparent ring around the paper disc revealed antibacterial activity, and transparent diameter was measured using a digital caliper to determine the inhibition zone.

2.6 Economic analysis

An economic analysis was conducted assuming only differences on the cost of culture medium. Culture medium costs were calculated according to the final concentration of each element used for production of one kilogram of dry biomass. The prices of f/2 medium used was based on Faé Neto et al. (2018). The cost for the treatment of synbiotic wastewater was considered zero as the wastewater processing was similar to the seawater, and no further addition of reagents in this medium.

2.7 Statistical analysis

Single comparisons were performed using one–way ANOVA, followed by Tukey's *post–hoc* mean comparison test (normality of the data and homogeneity of the variances were

previously verified, by the Shapiro–Wilk and Levene tests, respectively). In addition, linear and non–linear regressions were calculated to plot correlation between inhibition zone and peridinin content and growth curves of *D. glynnii* cultures subjected to different proportions of wastewater from shrimp culture, respectively. For all analyses, a level of significance of 5% was adopted.

3. Results

3.1. Growth performance

In the present study, the endosymbiotic dinoflagellate *Durusdinium glynnii* can grow in the pure AWW–SS, and partial replacement above 25% improved the growth performance of *D. glynnii* in comparison to the control one (Fig. 1a). The onset of the exponential growth phase (on the 5th day) was faster for the 50– and 75%– AWW–SS. Cultures conducted in 25%– and 100%–AWW–SS showed a shorter exponential growth phase. All treatments, and the control, reached the maximum cell density on the 12th day of cultivation. The partial replacement (i.e., 50 or 75%–AWW–SS) of FM by AWW–SS, resulted in higher values of μ (0.56 ± 0.01 and 0.58 ± 0.01 day^{-1}, respectively) of *D. glynnii* cultures. The replacement of 75% improved the B_{max} (0.51 ± 0.04 g L^{-1}) and P (57.0.04 ± 4.63 mg L^{-1} day^{-1}), in comparison to other treatments and the control (0.33 ± 0.03 g L^{-1} and 37.00 ± 3.02 mg L^{-1} day^{-1}) (Fig. 1b).

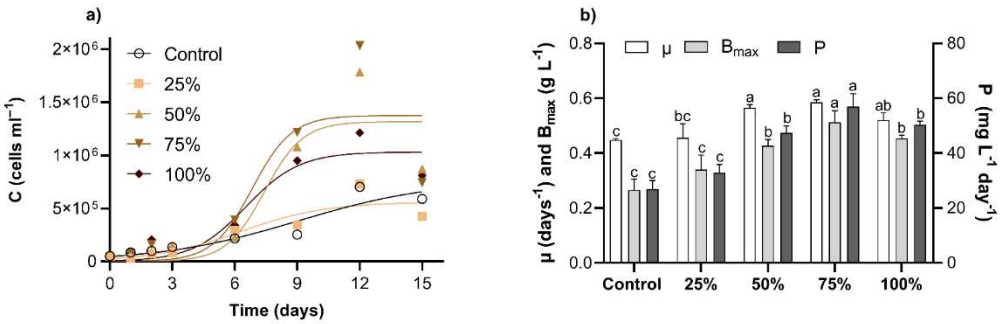


Fig. 1. Logistic growth curves (a) and growth parameters (b) of *Durusdinum glynnii* cultured in different proportions of wastewater from shrimp culture in a synbiotic system.

μ – specific growth rate (day^{-1}), B_{max} – maximum biomass reached ($g\ L^{-1}$), P – daily biomass productivity ($mg\ L^{-1}\ day^{-1}$). Different letters indicate significant differences ($p < 0.05$) between treatments by Tukey's *post-hoc* test.

3.2 Nutrient uptake

The different AWW–SS proportions evaluated in the present study resulted in different initial concentrations of NH_4^+-N, NO_2^-–N, NO_3^-–N, and P–PO_4^{3-} (Fig. 2). NO_3^-–N was completely absorbed in the control and the 25%–AWW–SS, while for NO_2^-–N, low or no absorption was observed. In the 50%–AWW–SS, the initial level of NH_4^+-N was considerably higher than at the final. Finally, a high phosphorus uptake was observed in all treatments.

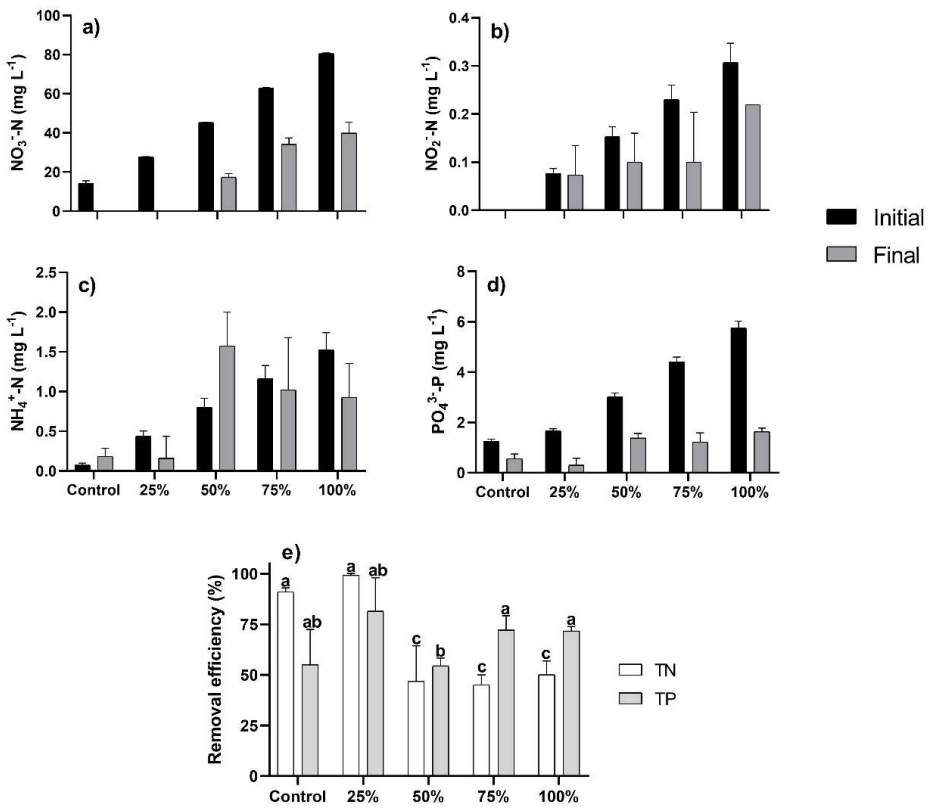


Fig. 2. Nutrients uptake (a-d) and efficiency removal (e) by *Durusdinium glynnii* cultured in different proportions of wastewater from shrimp culture in a synbiotic system. Different letters indicate significant differences ($p<0.05$) between treatments by Tukey's *post-hoc* test.

3.3 Pigments composition

Overall, lower oscillation in all pigments were found at day 7 compared to day 15. At day 15, higher levels of chlorophyll were found in cells grown on 50% AWW–SS compared to the control and 25% AWW–SS (Fig. 2a). Similarly, the high content of total carotenoids, β–carotene, and peridinin were found in cells grow at 50% AWW–SS at the end of the cultivation (Fig. 2b-d).

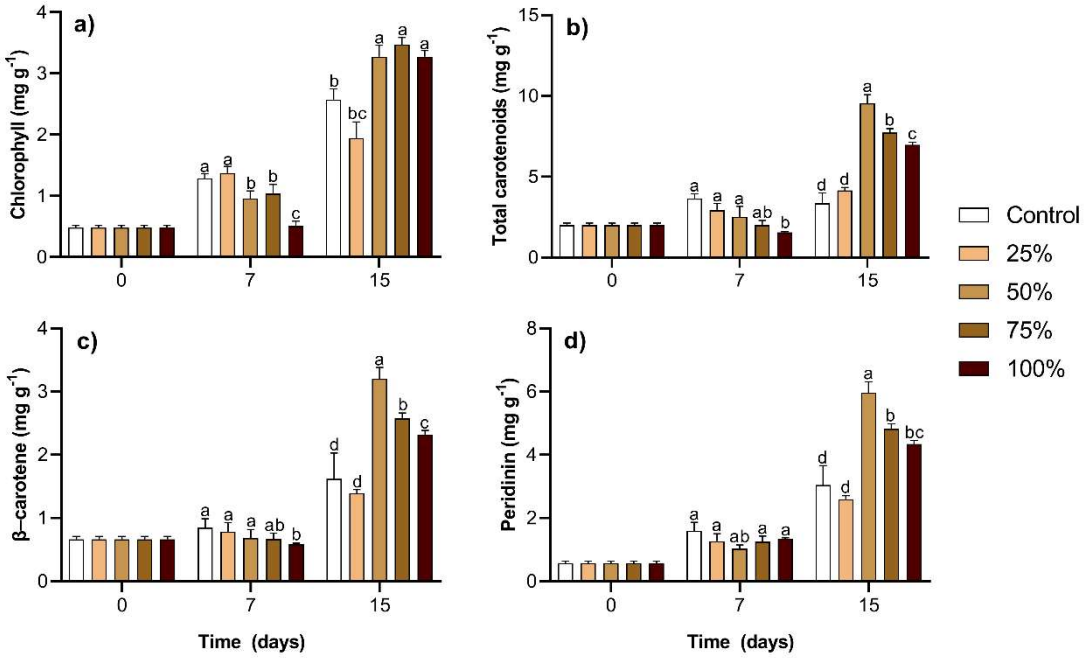


Fig. 3. Pigments content of *Durusdinum glynnii* cultured in different proportions of wastewater from shrimp culture in a synbiotic system. Different letters indicate significant differences ($p<0.05$) between treatments by Tukey's *post-hoc* test.

3.4 Antibacterial activity

The acetonic extracts obtained from *D. glynnii* biomass showed inhibitory effects on the *Vibrio parahaemolyticus* and *V. vulnificus* (Fig. 3). An inhibition zone of about 10 mm for *V. parahaemolyticus* was reported for all the extracts regardless of the growth medium. For *V. vulnificus*, an inhibition of about 6 mm was observed for the control, 25% and 50% AWW–SS, while a higher inhibition (close to 10 mm) was observed for the extracts from the biomass grown in 75% and 100% AWW–SS. Finally, the inhibition

zone of *V. parahaemolyticus* was positively correlated ($p < 0.05$) with the peridinin content; while the same correlation was no significant (p = 0.47) for *V. vulnificus*.

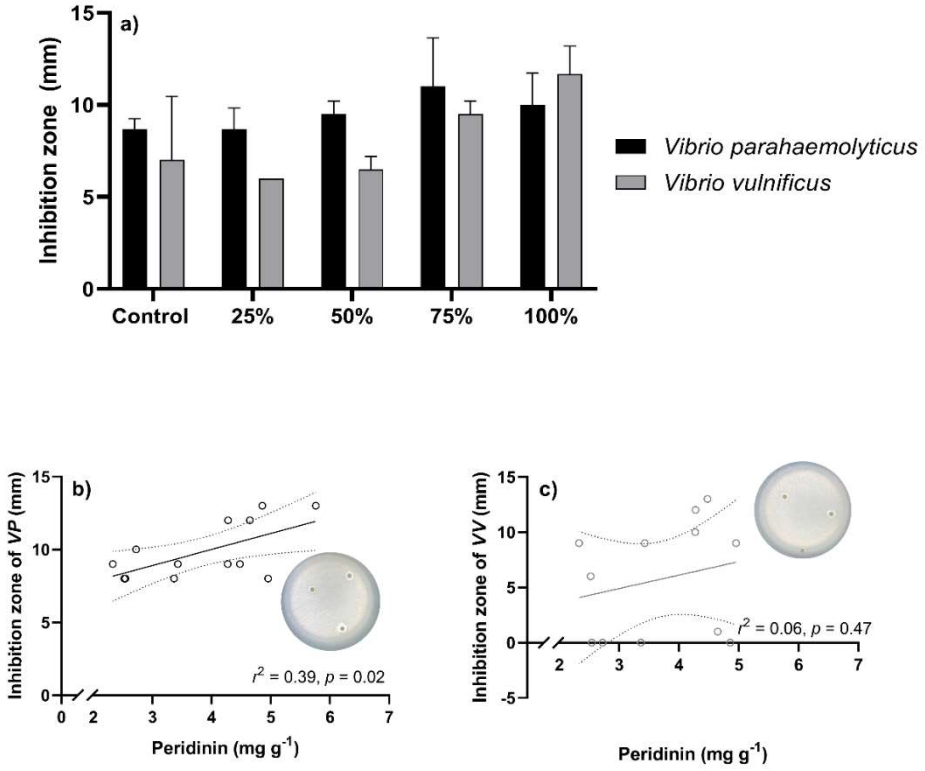


Fig. 4. Inhibition zone (a) of extracts from *Durusdinium glynnii* biomass and correlations (b and c) with peridinin against *Vibrio parahaemolyticus* and *V. vulnificus* bacteria strains. *VP – Vibrio parahaemolyticus*; *VV – Vibrio vulnificus*.

3.5 Economic analysis

Based on the growth data, peridinin content, and culture medium cost, the economic analysis was conducted and summarized in Table 1. As the percentage of wastewater use increases, the cost of producing biomass and peridinin decreases. The treatment using only wastewater from shrimp culture was the cheapest, but, due its low biomass yield in comparison to the 75% AWW–SS treatment, it has a longer production time.

Table 1. Economic analysis of *Durusdinium glynnii* production using different proportions of culture medium and wastewater from shrimp farming in a synbiotic system.

Treatment	Culture medium cost (US$ m^{-3})	Biomass production (g m^{-3})	Peridinin content (g kg^{-1})	US$ per Kg biomass	US$ per g peridinin	Production time (days)*
f/2 medium	15.6	266.0±39.7	3.0±0.6	59.5±8.7	20.2±5.3	37.4±4.1
25%	11.7	340.0±52.9	2.6±0.1	35.0±5.9	13.5±2.6	30.6±2.7
50%	7.8	426.7±23.1	6.0±0.3	18.3±1.0	3.1±0.0	21.1±1.2
75%	3.9	513.3±41.6	4.8±0.2	7.6±0.6	1.6±0.2	17.6±1.4
100%	0	453.3±11.5	4.3±0.1	0	0	19.9±0.5

* Time for production of 1 kg of biomass using a system with useful volume of 1 m^3.

4. Discussion

The ability of microalgae to grow in aquaculture wastewater represents an important mechanism towards development of aquaculture circular models. A number of microalgae species have already been effectively used to convert nitrogen and phosphorus from marine and freshwater aquaculture effluents into biomass, and some of these reports are listed in Table 2. However, most of these studies do not present a real usability of microalgal biomass, promoting circularity in aquaculture. Herein, the extracts from the microalgal biomass cultured using wastewater from a synbiotic system has presented *in vitro* antibacterial activity against two pathogenic *Vibrio* bacteria strains. In addition to the peridinin, other carotenoids, i.e., β-carotene, lycopene and fucoxanthin, have been documented earlier was antibacterial agents (Cucco et al., 2007; Karpiński and Adamczak, 2019), thus, it is likely that other carotenoids in *D. glynnii* extract exhibited higher anti–*V. vulnificus* than peridinin.

Table 2. Main characteristics of microalgae cultivation in various types of aquaculture wastewater.

Microalga species	**System**	**Target species**	**TN (%)**	**TP (%)**	**Ref.**
Durusdinium glynnii	SS	Pacific white shrimp	50.1	71.7	This study
Chaetoceros muelleri	BFT	Pacific white shrimp	-	100	Magnotti et al. 2016
Chlamydomonas sp.	-	Tilapia	79.6	96.0	Morando-Grijalva et al. 2020

Chlorella minutissima	RAS	Salmon	88.0	99.0	Hawrot-Paw et al. 2019
	BFT	Tilapia	84.3	48.3	Oliveira et al. 2020
Chlorella vulgaris	RAS	Tilapia	99.8	82.7	Gao et al. 2016
	-	Pacific white shrimp	86.1	82.7	Gao et al. 2016
	-	Flathead grey mullet	95.4	92.0	Andreotti et al. 2017
Isochrysis galbana	-	Flathead grey mullet	66.0	91.9	Andreotti et al. 2017
Nannochloropsis oculata	BFT	Pacific white shrimp	83.0	100	Magnotti et al. 2016
Picochlorum maculatum	-	Pacific white shrimp	66.7	92.8	Dinesh Kumar et al. 2018
Platymonas subcordiformi	-	Flounder	100	100	Guo et al. 2013
Spirulina sp.	-	Tilapia	81.1	100	Cardoso et al. 2020
Tetradesmus obliquus	RAS	Tilapia	99.7	99.6	Tejido-Nuñez et al. 2019
	RAS	Tilapia	80.1	~100	Ansari et al. 2017
Tetraselmis chuii	BFT	Pacific white shrimp	87.0	100	Magnotti et al. 2016

BFT – Biofloc system; RAS – Recirculating Aquaculture System; SS – Synbiotic System; TN – Total Nitrogen; TP – Total Phosphorus.

The levels of nitrogen and phosphate compounds in aquaculture wastewater vary greatly between production systems, feed offered, animal density, etc. (Magnotti et al., 2016; Ansari et al., 2017). The biofloc based system, i.e., heterotrophic, chemoautotrophic, and synbiotic system, accumulate large amounts of nitrate and orthophosphate over successive production cycles (El-Sayed, 2021). Although nitrate exhibits less toxicity to shrimp than other nitrogenous forms, concentrations above 220 mg L^{-1} can reduce shrimp growth and survival (Kuhn et al., 2010). Thus, nitrate decontamination is necessary to enable the reuse of water for several cycles. Various physical and chemical methods can be used efficiently to transform nitrate to harmless nitrogen gas, however, they are expensive and do not add value to aquaculture systems (Murphy, 1991; Liu et al., 2021). Herein, we demonstrated a reduction of nitrate, and other inorganic compounds, concomitant with the valorization of the produced biomass during the bioremediation.

The removal efficiency of TP found in the present study were similar to those reported for marine diatoms and chlorophytes (Ansari et al., 2017; Oliveira et al., 2020; Qian et al., 2022), but for TN the removal efficiency was relatively lower. For example, an efficiency removal of TN of 86.1% (6.81 to 1.17 mg L^{-1} of N) from the Pacific white shrimp farming by *Chlorella vulgaris* using a membrane photobioreactor (Gao et al., 2016). Although some comparisons on removal efficiency may be subjective, that is, effluents with low initial concentrations of N and P tend to have higher removal rates, the physicochemical characteristics of the effluent after microalgae growth are considerably better and more suitable for disposal to aquatic ecosystems. In the present study, the levels of TN were reduced from 82.3 to 41.1 mg L^{-1}, a nitrogen reduction 5 times higher than that reported by Gao et al. (2016).

In particular, dinoflagellates are recognized for their high capacity to absorb and accumulate phosphorus, including organic and inorganic forms (Kim et al., 2021). Thus, organic phosphorus available in synbiotic systems may have been remineralized by the bacterial community and made available in different forms to support the growth of *D. glynnii*. This fact may also support the higher growth of *D. glynnii* in wastewater-containing medium, compared to the control, since the enrichment of the phosphorus pool may contribute to the increase in the growth rate of dinoflagellates (Thomson et al., 2019; Mo et al., 2020).

Partial replacement of the culture medium by 50 or 75% of AWW–SS improved the growth performance of *D. glynnii* compared to control and other treatments. In general, marine dinoflagellates show low growth rates due to shear stresses and other nutritional issues not yet elucidated (Rifaie-Graham et al., 2021; Jeong et al., 2012). For example, the addition of soil extract to dinoflagellate culture medium is a traditional method to improve the growth of these microalgae (e.g., Berge et al., 2008; Müller et al.,

2019). Although the composition of the soil extract is almost never evaluated, it is likely that some of these oligoelements present in the soil extracts are also present in the biofloc wastewater. Furthermore, endosymbiotic dinoflagellates fed on organic matter, such as bacteria and small microalgae. Jeong et al. (2012) reported that free-living *Symbiodinium* spp. acquired more nitrogen from prey than the uptake of inorganic nitrogen from f/2 medium. Thus, organic residues smaller than 1 μm (filtration used in the present study) may also have served as feed for *D. glynnii*.

Vibriosis are threat bacterial diseases that affects aquaculture industry worldwide, and also the presence of *Vibrio* spp. can cause gastrointestinal problems in humans (Sheikh et al., 2022). Thus, new natural antibiotics are being investigated to contribute to the sustainable development of aquaculture, and some of these reports were summarized in Table 3 (Ghosh et al., 2021). Soto-Rodrigues et al. (2022) reported that aqueous extract from the diatom *Chaetoceros calcitrans* inhibited the growth of *V. parahaemolyticus*. In the present study, the inhibition of *V. parahaemolyticus* was positively correlated with the content of the carotenoid peridinin. The mechanisms of antibacterial activity of carotenoids include cytoplasm leakage, nucleic acid formation inhibition, and outer membrane permeability. In addition, other carotenoids, such as β-carotene and fucoxanthin, have been documented earlier as antibacterial agents (Cucco et al., 2007; Karpiński and Adamczak, 2019), thus, another carotenoid may have exhibited a greater influence on *V. vulnificus* inhibition than peridinin.

Table 3. In vitro activity of some biological anti-*Vibrio* spp. sources.

Source	**Type of inclusion**	**Dosage (μg mL^{-1})**	**Method**	***Vibrio* strain**	**Ref.**
Microalgae					
Durusdinium glynnii	AcE		KBM	*VP, VV*	This study
Chaetoceros calcitrans	AqE	70	LM	*VP*	Soto-Rodrigues et al., 2022
Seaweeds					
Caulerpa sertularioides	ME	1,000	MM	*VA, VP*	Esquer-Miranda et al., 2016

Gracilaria fisheri	CPE	50	BMD	*VP*	Boonsri et al., 2017
Gracilaria verrucosa	EE	2	AD	*VH*	Rudi et al., 2019
Ulva lactuca	ME	>1,500	MM	*VA, VP*	Esquer-Miranda et al., 2016
Plants					
Moringa oleifera	EE	64	BMPA	*VA*	Suhartono et al., 2019
Musa acuminata	AqE	1560	DD	*VP, VA*	Rattanavichai and Cheng, 2014
Ocimum basilicum	AqE	19	BMD	*VH, VP, VA*	Snoussi et al., 2016

AD – Agar disk; DD – Disk diffusion; KBM - Kirby-Bauer method; BMPA – Broth microtiter plate assay; LM – Liquid medium; MM – Microplate methods; AcE – Acetonic extract; AqE – Aqueous extract; CPE – Crude protein extract; EE – Ethanolic extract; ME – Methanolic extract; VP – *Vibrio parahaemolyticus; VH – Vibrio harveyi; VA – Vibio alginolyticus*; *VV – Vibrio vulnificus.*

5. Conclusions

Results of the present study clearly demonstrate that wastewater from a synbiotic system has adequate characteristics for the growth of the marine dinoflagellate *D. glynnii*, and it has shown higher biomass productivity when grown at a 75% wastewater. Nitrogen and phosphorus levels were reduced by 50.1 and 71.7%, respectively, from the wastewater, and this can make it possible to reuse the water for new shrimp production cycles, reducing negative impacts of accumulation of these harmful compounds to the animals. Moreover, metabolites from biomass produced using synbiotic wastewater can be used to control vibriosis during shrimp production. This circular approach represents a robust model towards development of circularity in aquaculture, contributing to the achievement of SDGs in 2030 Agenda.

Declarations

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Conflict of Interest

The authors declare no competing interests.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution

CYBO: Conceptualization, Investigation, Methodology, Data curation, Formal Analysis, Writing – original draft. **JLA**: Conceptualization, Investigation, Writing – review & editing. **BCSB**: Investigation, Formal Analysis, Writing – review & editing. **DWSO**: Investigation, Formal Analysis. **EPS**: Formal Analysis, Writing – review & editing. **GKAC**: Data curation, Formal analysis; **SMBCS**: Formal Analysis, Resources, Writing – review & editing. **DMMD**: Formal analysis, Writing – review & editing; **MNM**: Supervision, Writing – review & editing. **AOG**: Project Administration, Supervision, Resources, Writing – review & editing.

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4. General conclusions

With the works presented in this thesis, it can be concluded that:

- Dinoflagellate research is an active area of science with interests aimed at elucidating the harmful bloom phenomena and the endosymbiotic relationship with coral reefs;
- Studies with toxic dinoflagellates are of greater interest compared to non–toxic ones;
- Dinoflagellate biotechnology may become important organisms to achieve the SDGs set by the United Nations in 2030 Agenda;
- Light and nutrients (mainly carbon and nitrogen) are essentials requirements towards baseline research of underexplored microalgae;
- Light induces both primary and secondary metabolisms in the endosymbiotic dinoflagellate *D. glynnii*.
- *D. glynnii* can effectively uptake nitrogen and phosphorus compounds from synbiotic aquaculture wastewater, and to produce antibacterial compounds in a holistic with quasi-zero residue approach.
- High nitrogen supply can increase the light and thermal tolerance of *D. glynnii*.

5. Other R&D contributions

Aside from the published and in progress articles that compose the present thesis, several collaborations have been performed resulting in publications derived directly from the thesis. In addition, the PhD candidate has participated in several research projects and contributed as a reviewer on several occasions. Apart from the research activities, the PhD candidate has also been involved in foundation a multidisciplinary group of research, development, and innovation. All this information is summarized in this section.

5.1 Additional scientific production

During the development of the thesis, several collaborations with partners from the UFRPE and other national (such as Federal University of Pernambuco, Federal University of the São Francisco Valley, Federal University of Alagoas, Federal University of Maranhão, Federal University of Goiás, and Federal University of Santa Catarina) and international institutions (such as Indian Institute of Technology Delhi, Sathyabama Institute of Science and Technology, Vellore Institute of Technology – India; Palacký University – Czech Republic; University of Technology Sydney – Australia; Durban University of Technology – South Africa). This networking resulted in 24 publications in JCR journals. The topic of each of the publications was diverse, from microalgae biotechnology up to applied ecology.

1. Gonçalves Junior, G. F.; Santos, R. F. B.; **Oliveira, C. Y. B.**; Silva, Â. R. A.; Santos, E. P.; Bezerra, R. D. S.; Gálvez, A. O. The use of Artemia sp. conserved on larval performance of the Pacific white shrimp *Penaeus vannamei*. International Aquatic Research, v. 14, p. 1-11, 2022. https://doi.org/10.22034/IAR.2022.1965456.1320 (JCR: 1.055)
2. Oliveira, C. D. L.; Santos, L. V. R.; **Oliveira, C. Y. B**. Research on chimera fish with a thematical focus for life history: a scientometric analysis. Revista de Biología Marina y Oceanografía, v. 57, p. 1-9, 2022. https://doi.org/10.22370/rbmo.2022.57.Especial.3338 (JCR: 0.493)
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5.2 Conference papers

Due to the COVID-19 pandemic, the number of conferences in the period between 2020 and 2021 was lower than usual. Despite that, the PhD candidate participate in two internationals (XVII National Shrimp Fair and 1st Brazilian Symposium on Photosynthesis) and nationals (IV Biotechnology and Bioprocesses Meeting and XXI Fisheries Engineer Week) conferences, and six conference papers authored or co-authored were published:

1. Campos, C. V. F. S.; Nascimento, R. E. S.; **Oliveira, C. Y. B.**; Santos, E. P.; Brito, L. O.; Galvez, A. O. Biorremediação do efluente do cultivo de Tilápia em bioflocos a partir do consórcio *Chlorella-Daphnia* em diferentes salinidades. In: XVII FENACAM, 2021. Type of presentation: Poster. Place: Natal, RN, Brazil.
2. Nascimento, R. E. S.; Campos, C. V. F. S.; **Oliveira, C. Y. B.**; Santos, E. P.; Brito, L. O.; Galvez, A. O. Avaliação da qualidade de água no crescimento de *Daphnia similis* em diferentes salinidades utilizando efluente do cultivo de Tilápia em bioflocos. In: XVII FENACAM, 2021. Type of presentation: Poster, Place: Natal, RN, Brazil.
3. **Oliveira, C. Y. B.**; Abreu, J. L.; Santos, E. P.; Oliveira, D. W. S.; Brandao, B. C. S.; Dantas, D. M. M.; Muller, M. N.; Galvez, A. O. Effect of irradiance on the biomass production of the endosymbiont dinoflagellate *Symbiodinium* sp. cultured using light-emitting diode lamps. In: Aquaciência Digital 2021. Type of presentation: Oral. Place: Online.
4. Alves, G. V. P.; Carvalho, D. O.; Silva, M. S.; Silva, M. F. G.; Silva, W. W.; **Oliveira, C. Y. B.**; Silva, U. L. Contribuição do alimento natural no cultivo de pós-larvas de Tilápia-do-Nilo em diferentes tecnologias de cultivo. In: Aquaciência Digital 2021. Type of presentation: Oral. Place: Online.
5. Oliveira, D. W. S.; **Oliveira, C. Y. B.**; Abreu, J. L.; Silva, E. P.; Brandao, B. C. S.; Galvez, A. O. Efeitos de diferentes concentrações de nitrogênio nos parâmetros de crescimento do dinoflagelado *Symbiodinium* sp. In: XIX SEMAQUI - Congresso de Engenharia de Aquicultura, 2021. Type of presentation: Oral. Place: Online.
6. Gálvez, A. O.; Müller, M. N.; Oliveira, D. W.; Santos, E. P.; de Abreu, J. L.; Brandão, B. C.; **Oliveira, C. Y. B.** Marine dinoflagellate *Durusdinium glynnii* (Dinoflagellata, Symbiodiniaceae) as a promising protein source for aquafeeds. In: XX International Symposium on Fish Nutrition and Feeding, 2022. Type of presentation: Poster. Place: Sorrento, Italy.

5.3 Contribution as journal reviewer and undergraduate jury

Aside from published papers already mentioned, the PhD candidate served as a reviewer for several JCR journals, totaling a total of 33 reviews verified by Publons (Web of Science):

1. Biomass Conversion and Biorefinery (7 reviews)
2. Journal of Applied Phycology (5 reviews)
3. Environmental Science and Pollution Research (4 reviews)
4. Science of the Total Environment (4 reviews)
5. Renewable & Sustainable Energy Reviews (3 reviews)
6. Bioresource and Bioprocessing (2 reviews)
7. Frontiers in Food Science and Technology (2 reviews)
8. Reviews in Aquaculture (1 review)
9. Aquaculture (1 review)
10. Preparative Biochemistry & Biotechnology (1 review)
11. Journal of Limnology (1 review)
12. Brazilian Journal of Botany (1 review)
13. Acta Scientiarum. Technology (1 review)

Additionally, the PhD candidate has participated as a jury in four undergraduate examining committee:

1. Candidate: Cícero Carlos Vieira Lima. Title: Vivência em piscicultura de base familiar no sítio Jatobazinho, município de Betânia, PE. 2021. Course: Agricultural Technician with emphasis in Agroecology at Federal Rural University of Pernambuco.
2. Candidate: Abigail Jaynnara Gomes da Silva Almeida. Title: Produção de alevinos de Tilápia do Nilo (*Oreochromis niloticus*) na Cooperativa de Produtores do Vale de Itaparica (COOPVALE), Itacuruba, PE. Course: Animal husbandry at Federal Rural University of Pernambuco.
3. Candidate: Bruno Borba Santos Ferreira Costa. Title: Triploidia com ênfase em organismos marinhos: uma análise bibliométrica. Course: Fishing engineering at Federal Rural University of Pernambuco.
4. Candidate: Magna dos Santos Silva. Title: Alimento natural, qualidade de água e performance zootécnica de Tilápia alimentada com diferentes dietas proteicas em sistema de biofloco. Fishing engineering at Federal Rural University of Pernambuco.

Appendix A

Research in this field is supported by the following journal publication	
Title	A multidisciplinary review of *Tetradesmus obliquus*: a microalga suitable for large-scale biomass production and emerging environmental applications
Authors	**CYB Oliveira**, CDL Oliveira, R Prasad, HC Ong, ES Araujo, N Shabnam, AO Gálvez
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REVIEWS IN Aquaculture

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A multidisciplinary review of Tetradesmus obliquus: a microalga suitable for large-scale biomass production and emerging environmental applications

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Abstract

Microalgae biomass is among one of the most promising sustainable raw materials for many industrial sectors especially biodiesel production. Although a great diversity of microalgae species has been described and isolated, few have been used for large-scale cultivation. This review presents a multidisciplinary overview of studies on *Tetradesmus obliquus* – a freshwater microalga suitable for large-scale production and emerging environmental applications. It reviews the taxonomic history of *T. obliquus* and its potential commercial applications, including cultivations techniques and environmental parameters, production systems, harvesting and drying of biomass, and its biochemical composition. In addition, a model refinery for *T. obliquus* is proposed that combines the main productive bioprocesses. Finally, a bibliometric analysis is presented and opportunities for future research with *T. obliquus* are identified.

Key words: Acutodesmus obliquus, bibliometric analysis, biorefinery, renewable energy, Scenedesmus obliquus, wastewater treatment.

Introduction

Global microalgae biomass production, even after many years of research and a highly sustainable production chain, is low when compared to other aquaculture sectors (FAO 2018). Few species have been produced on a commercial-scale (*e.g. Chlorella* spp., *Arthrospira platensis* (Spirulina), *Haematococcus pluvialis* and *Dunaliella salina*), they are destined mainly for applications in the food industry (Higuera-Ciapara *et al.* 2006; Ben-Amotz *et al.* 2009; Fradique *et al.* 2010; Garrido-Cardenas *et al.* 2018). In aquaculture, live microalgae (*e.g. Chaetoceros calcitrans*, *Isochrysis galbana*, *Navicula* spp., and *Pavlova lutheri*) are used as hatchery and nursery feeds for shrimp, bivalve molluscs, larval finfish and also used to feed zooplanktons (Muller-Feuga 2000; Yarnold *et al.* 2019). In both cases, lipids (carotenoids and neutral lipids) and proteins are among the compounds of greatest interest.

Microalgae offer a wide range of applications, processes and products, including the following: renewable energies (third-generation biofuels), water/air decontamination and potential products for pharmaceutical, cosmetic and nutraceutical industries, making their utilization important for new business developing (Chisti 2007; Kumar *et al.* 2010; Safi *et al.* 2014; 't Lam *et al.* 2018; Collotta *et al.* 2018; Durán *et al.* 2018; Oliveira *et al.* 2020c). Furthermore, microalgae cultivation can also make use of water and land that is unsuitable for agriculture and, thus, not compete with (or affect) traditional agriculture (Lozano-Garcia *et al.* 2019; Serrà *et al.* 2020).

Among an estimated total of over 300 000 microalgae, species of the genus *Scenedesmus* (Sphaeropleales, Scenedesmaceae) are prominent. It is the world's third

most studied genus in terms of number of documents published – interestingly more than the genera *Spirulina* and *Nannochloropsis* (Garrido-Cardenas *et al.* 2018). *Scenedesmus* is cosmopolitan and one of the most common genera of green microalgae in freshwater environments. This genus has single-celled individuals capable of forming 2–32 cell coenobia, but usually forms a four-celled coenobium that are surrounded by a mucilaginous matrix. *Tetradesmus* (*Scenedesmus*) *obliquus* (Turpin) M.J.Wynne (Fig. 1), in particular, is recognized in Scenedesmaceae due to knowledge of the genetic coding of their mitochondria – which facilitates its identification by mtDNA analysis (Nedelcu *et al.* 2000).

This review systematically reveals key information about *T. obliquus*, which includes the following: cultivation and harvesting techniques, scientific information on taxonomy and morphology, biochemical composition and new insights and potential environmental applications in various industries. Finally, a bibliometric analysis is also presented to highlight the interest in the *T. obliquus* cultivations over the years in research and technological development areas.

History, taxonomy and morphology

Tetradesmus, *Acutodesmus*, *Desmodesmus* and *Scenedesmus* are green algae genera that have a relative morphological similarity. Modern genetic tools (*e.g.* ITS-1 e -2 rDNA, mtDNA) contributed to clarifying some taxonomic gaps in the family Scenedesmaceae, although in the 20th century beginning taxonomic impasses still prevailed for these four genera (Chodat 1913; Smith 1913; West 1915).

Tetradesmus obliquus (worldwide known as *Scenedesmus obliquus* and reclassified as *Acutodesmus obliquus*) was initially described taxonomically as *Achnanthes obliqua* (Turpin 1828) and since then some changes have been suggested. After the landmark creation of the genus *Desmodesmus*, when all species of *Scenedesmus* which thorns were classified to this genus, a recent change in the family Scenedesmaceae reclassified *T. obliquus*. After the reclassification of *T. wisconsinensis* as *Acutodesmus wisconsinensis* (see Tsarenko and Petlovanny (2001) supported by Hegewald *et al.* (2013)), Wynne and Hallan (2015) recalled that the generic name *Tetradesmus* had priority over *Acutodesmus* and for this reason, eleven taxa (including at that time *Acutodesmus obliquus*) were reclassified as *Tetradesmus*. Even though it is still referred to as *S. obliquus* in a number of current studies, *T. obliquus* was chosen in this review (including studies using *S. obliquus* and *A. obliquus*), because it is the current taxonomic classification for this taxon. The main and historical taxonomic classifications attributed to *T. obliquus* are shown in Box 1.

The cell wall of *T. obliquus* is mostly composed of neutral sugars (glucose, mannose, fructose and rhamnose) and amino acids (Blumreisinger *et al.* 1983). Each cell has a single chloroplast that fills the entire inner surface of the cell, a pyrenoid is also present near the centre of cells (Cepák *et al.* 2007; Wei *et al.* 2010). The species reproduces asexually by releasing autospores through rupture of the cell wall. Nonetheless, rare cases of sexual reproduction with biflagellate gametes have been reported (Trainor & Burg 1965). Under stress conditions like nitrogen depletion, chromium and thermal stresses, lumps of *T. obliquus* wrapped by a mucilage sheath are formed (Cain & Trainor 1976; Corradi *et al.* 1995). Another more easily discernible defence strategy in *T. obliquus* cells is the formation of colonies. The infochemicals released by different zooplankton taxa have been associated with this strategy where the colony size was proportional to the concentration of chemicals released by herbivores (Verschoor *et al.* 2004). In addition, morphological defence in *T. obliquus* is also affected by other microalgae species such as *Microcystis aeruginosa* (Zhu *et al.* 2015). Although these defence strategies may not be very commonly reported, they may seem to contribute to

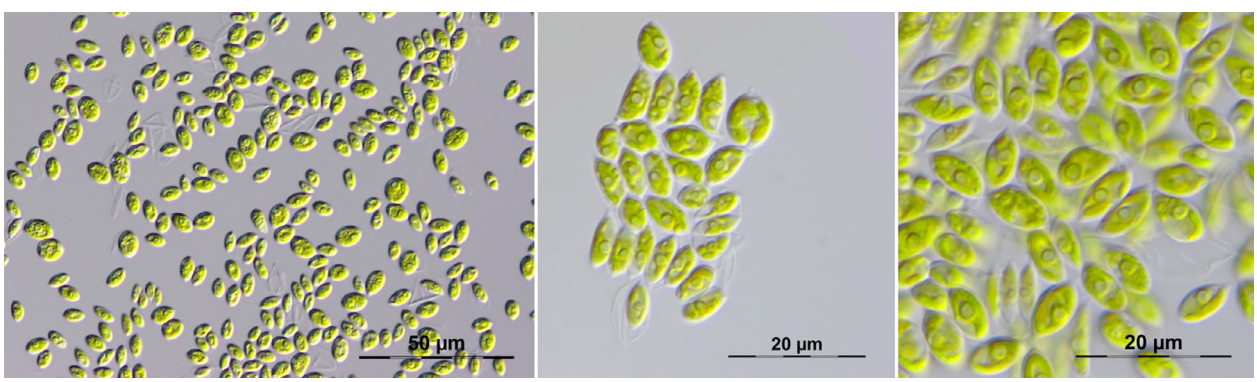

Figure 1 Micrographs of *Tetradesmus obliquus* cells on optical microscopy (images adapted from SAG (2020)).

Box 1. Taxonomic history of *Tetradesmus obliquus*

Empire: Eukaryota
Kingdom: Plantae
Subkingdom: Viridiplantae
Infrakingdom: Chlorophyta infrakingdom
Phylum: Chlorophyta
Subphylum: Chlorophytina
Class: Chlorophyceae
Order: Sphaeropleales
Family: Scenedesmaceae
Genus: *Tetradesmus*

Nomenclature	Publication details	Refs.
Achnanthes obliqua	Turpin 1828: 312	Turpin (1828)
Scenedesmus acutus	Meyen 1829: 775	Meyen (1829)
Scenedesmus bijugatus	Kützing 1834: 607	Kützing (1833)
Scenedesmus obliquus	Kützing 1834: 609	Kützing (1833)
Scenedesmus basiliensis	Chodat 1926: 136	Skrebovskaya *et al.* (2015)
Scenedesmus acutus f. *alternans*	Hortobágyi 1941: 164	Hortobágyi (1941)
Acutodesmus obliquus	Hegewald and Hanagata 2000: 156	Hegewald and Hangata (2000)
Tetradesmus obliquus	Wynne 2015: 84	Wynne and Hallan (2015)

the robustness of this species – making it promising for large-scale cultivations.

Microalgae production

Recent studies showed that the potential of *T. obliquus* for large-scale biomass production has not been exploited yet. This microalga has a fast growth rate and it is extremely resistant to adverse conditions, which is the key characteristic for large-scale cultivation. Potential of *T. obliquus* to grow in nitrogen and phosphorus rich wastewater has also been proven in several studies (Martínez *et al.* 2000; Hodaifa *et al.* 2008; Mata *et al.* 2012; Gupta *et al.* 2016; Ferreira *et al.* 2019). The ability of microalgae to grow in wastewater is attractive because they provide a pathway for converting chemical contaminants into biomass (Brennan & Owende 2010). *T. obliquus* is highly tolerant to high temperature (Yang *et al.* 2018) and irradiance (Hurtado *et al.* 2019). Moreover, it also can grow under heterotrophic and mixotrophic conditions (Shen *et al.* 2018; Di Caprio *et al.* 2019), and different nutrient strategies have been evaluated (Papazi *et al.* 2018; Qu *et al.* 2019). Therefore, the continuous study of *T. obliquus* cultivation techniques improvement is an important step for the validation of a productive technological package for this species. In this section, a brief description of the major advances in nutritional metabolisms and cultivation systems employed in cultivating *T. obliquus* is provided.

Photoautotrophic growth

Photoautotrophic growth is the most common means of cultivation for all microalgae species and is the cheapest method for large-scale production. Microalgae in photoautotrophic cultures carry out photosynthesis to convert light energy into chemical energy, which is conserved as adenosine triphosphate (ATP) and nicotinamide and adenine dinucleotide phosphate (NADPH) – subsequently these compounds are used in the CO_2 reduction for synthesis of carbohydrates and other organic compounds (Xia & Murphy 2016). In order to reach high cell densities cultures in photoautotrophic growth cultivation systems the main precautions must be directed to the irradiance and inorganic carbon (mainly CO_2) availability.

Raceway ponds

Raceway ponds are the most widespread and feasible cultivation systems for large-scale microalgal production despite having several limitations. The main limitations include higher susceptibility to contamination by protozoa, bacteria and other microalgae species, low productivity in systems with high depth, high costs with biomass harvesting (due to the low concentration cell and large volume) etc. (Safi *et al.* 2014). The water channel depth may vary between 10 and 50 cm, depending on the natural irradiance of the environment which results in a low illuminated surface-to-volume (S/V) ratio (García-González *et al.* 2003). In addition, high temperature and irradiance oscillations, and low CO_2 availability may also make the high growth rates of some species unfeasible in raceway ponds (Borowitzka 1999).

Photobioreactors

In general, laboratory cultures are maintained and grown in photobioreactors of different sizes and formats. Photobioreactors are closed systems that provide higher biomass production (1.5–4.0 g L^{-1}). There are less chances of contamination in photobioreactors compared to raceway ponds (Lee & Shen 2003). However, the cost of maintenance and production of photobioreactors makes it unfeasible if the desired end products do not have a high commercial value. Photobioreactor technology allows strict control of cultivation conditions and thus isolates a

variable (*e.g.* pH, irradiance, temperature, CO_2 concentration, culture medium etc.) to be studied. In intensive systems, Grobbelaar (2003) stated that atmospheric CO_2 is insufficient to meet the carbon demands of cells.

Thin-layer system

Thin-layer systems are perhaps an evolved version of raceway ponds (Tramontin *et al.* 2018). In these systems, cultures are maintained in a turbulent flow in a water column (of few millimetres) which allows higher volumetric and areal productivities (Masojídek & Prášil 2010; Morales-Amaral *et al.* 2015). Thin-layer systems, unlike other open systems, tend to be less susceptible to contamination by microorganisms, as high cell density prevails over contaminations (Masojídek *et al.* 2011). An important characteristic of this system is its high illuminated S/V ratio. This increases the photosynthetically active culture volume which is one of the main problems faced during operation of raceway ponds (Venancio *et al.* 2020). However, heat retention due to the thin layer of cultivation exposed to solar irradiance in the daytime allows survival of only thermotolerant strains in this system. In fact, thin-layer systems can be 12–100 times more productive when compared to photobioreactors and raceway ponds, respectively (Masojídek *et al.* 2011).

Heterotrophic growth

In heterotrophic cultivation, an organic carbon source is used to fulfil nutritional and energy requirements (Venkata Mohan *et al.* 2015). The non-dependence on light and disregard for the high illuminated S/V ratio, results in significantly higher biomass compared to photoautotrophic cultivation. Nevertheless, in contrast to the photoautotrophic cultivation where there is continuous oxygen production, in the heterotrophic cultivation oxygen is consumed which is related to the cellular respiration process that is regulated by energy demand in the ATP and NADPH form (Griffiths *et al.* 1960; Geider & Osborne 1989). The possibility of using industrial fermenters and reducing water use (due to high cell density) makes heterotrophic cultivations a viable alternative for the heterotrophic microalgae development. In addition, the heterotrophic cultivation would help to reduce harvest costs (Venkata Mohan *et al.* 2015).

Mixotrophic growth

The possibility of using organic carbon and light as an energy sources makes mixotrophic cultivation a promising strategy for ultrahigh cell density cultivations (Geider & Osborne 1989). Some studies have suggested that the microalgae growth rate under mixotrophic metabolism is approximately the sum of heterotrophic and photoautotrophic metabolisms, provided they be under the same conditions (*e.g.* temperature, culture medium, carbon source etc.); however, mixotrophic metabolic flow is more complex than a possible sum of metabolisms (Girard *et al.* 2014; Shen *et al.* 2018; Oliveira *et al.* 2021). A compilation of productive results of *T. obliquus* on different metabolisms and culture systems is listed in Table 1.

In a comparative study of the three nutritional metabolisms (photoautotrophic, heterotrophic and mixotrophic) conducted by Shen *et al.* (2018), the nitrogen assimilation rate in the photoautotrophic mode (5.7 ± 0.5 mg L^{-1} day^{-1}) by *T. obliquus* was about 2 and 4 times lower when compared to that under heterotrophic (10.7 ± 0.7 mg L^{-1} d^{-1}) and mixotrophic (22.7 ± 0.3 mg L^{-1} day^{-1}) metabolism, respectively, using acetate as carbon source. These authors also reported that the acetate assimilation rate was about three times higher in mixotrophic cultures (1.43 ± 0.04 mg L^{-1} day^{-1}). On the other hand, Vieira (2018) reported 14.77 g L^{-1} of biomass grew under mixotrophic mode using glucose as carbon source in a fed-batch culture. In addition, the heterotrophic cultivation, also using glucose, reached 7.9 g L^{-1} of biomass and this culture was limited by the oxygen availability into photobioreactor. Neither of these two studies evaluated the presence of bacteria in the cultures that manage to compete (and often to prevail) with microalgae for the organic substrate provided in the culture medium.

Tang *et al.* (2011) reported that *T. obliquus* could grow at 50% CO_2 (~0.7 g L^{-1}) but in fact grew well (~1.25 g L^{-1}) under CO_2 ranging from 5 to 20% at 25 ± 1°C and 180 μmol photons m^{-2} s^{-1}. These authors also reported a maximum carbon biofixation rate of 0.288 g L^{-1} day^{-1} at 10% CO_2. The results suggest *T. obliquus* have great potential to treat CO_2-rich gaseous effluents and potentially to convert the biomass into bioproducts.

The reduction in trace element concentration up to 1000-fold did not affect the growth performance of *T. obliquus* using the LCA-AD medium (adapted from Bold's Basal) (Oliveira *et al.* 2020a). The authors reported 4.2 g L^{-1} of biomass grown up at 0.3 g L^{-1} day^{-1} with irradiance increasing 360 to 1200 μmol photons m^{-2} s^{-1}. Growth of *T. obliquus* at high irradiance is also an important finding as this is a mandatory requirement for large-scale cultivations. The biomass productivity of *T. obliquus* reached up to 30 g m^{-2} day^{-1} in a solar tracked flat panel photobioreactor (Hindersin *et al.* 2014). The authors pointed out some advantages in solar tracked photobioreactors compared to static photobioreactors, such as increased light supply that enabled a year-round production of *T. obliquus* biomass, at unfavourable and fickle

Table 1 Growth performance of *Tetradesmus obliquus* in different cultivation systems and nutritional metabolisms

Strain	Nutritional mode	Cultivation system	Strategy	Biomass maximum ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ day^{-1}$)	References
T. obliquus SJTU-3	Photoautotrophic	Photobioreactor	Simple batch	1.84	0.155	Tang *et al*. (2011)
T. obliquus CNW-N	Photoautotrophic	Photobioreactor	Simple batch	3.51	0.292	Ho *et al*. (2010)
T. obliquus	Photoautotrophic	Photobioreactor	Simple batch	4.35	0.317	Oliveira *et al*. (2020a)
T. obliquus	Photoautotrophic	Thin-layer	Fed-batch	20.14	1.19	Venancio *et al*. (2020)
T. obliquus CPCC-5	Heterotrophic	Photobioreactor	Simple batch	2.7	0.208	Girard *et al*. (2014)
	Mixotrophic	Photobioreactor	Simple batch	3.5	0.269	
T. obliquus	Heterotrophic	Photobioreactor	Fed-batch	9.53	0.98	Vieira (2018)
T. obliquus	Photoautotrophic	Photobioreactor	Fed-batch	7.9	0.52	
T. obliquus	Mixotrophic	Photobioreactor	Fed-batch	14.77	1.24	
T. obliquus SAG 276.7	Photoautotrophic	Photobioreactor	Simple batch	4.92	0.57	Gris *et al*. (2014)
T. obliquus SAG 276-3a	Photoautotrophic	Photobioreactor	Simple batch	1.25	0.06	Mandal and Mallick (2009)
	Mixotrophic	Photobioreactor	Simple batch	5.11	0.51	
T. obliquus	Photoautotrophic	Hybrid photobioreactor	Simple batch	1.16	0.13	Tramontin *et al*. (2018)
T. obliquus NIES-2280	Photoautotrophic	Photobioreactor	Simple batch	0.56	0.09	Shen *et al*. (2018)
	Heterotrophic	Photobioreactor	Simple batch	0.68	0.11	
	Mixotrophic	Photobioreactor	Simple batch	2.20	0.37	

climatic conditions. Recently, Venancio *et al*. (2020) analysed the influence of S/V ratio in biomass production of *T. obliquus* in a thin-layer cascade system. They reported the 80 m^{-1} S/V (1.19 g L^{-1} day^{-1}) is more productive than 60 m^{-1} S/V (0.95 g L^{-1} day^{-1}) and it can affect the biomass reached, 20.14 g L^{-1} and 14.60 g L^{-1} for 80 and 60 m^{-1} SV, respectively. The authors attributed these differences to the photosynthetic and the carbon conversion efficiencies. Therefore, it is crucial to select production system and growth metabolism, given the wide variety of techniques successfully employed for the cultivation of *T. obliquus*. This can determine the cost-effective cultivation, no matter the size of the facility, or its geographical location.

Culture conditions

The culture conditions (availability, quality and source) can affect the growth and biomass accumulation, as well as biochemical composition of *T. obliquus*. The changes in the culture conditions can favour the production of a specific biocompound like lutein (Ho *et al*. 2015).

Carbon source

The carbon source varies depending on the nutritional metabolisms. Nevertheless, irrespective of the nutritional metabolism, carbon is the main nutrient required for the growth of microalgae since it represents about 50% of the dry cell weight. For most microalgae, CO_2 is the main carbon source in the photoautotrophic cultures, since this gas diffuses rapidly from the water into the cells (passive diffusion) and is used directly in the Calvin-Benson cycle (Chisti 2007). Moreover, use of sodium bicarbonate as a carbon source increased biomass production by 9% in *T. obliquus* (Mansouri & Hajizadeh 2018). Although it is stoichiometrically impossible to achieve high biomass using only atmospheric CO_2, in super-intensive cultures role of CO_2 is almost exclusively as a pH regulator than as a nutrient – which results in excessive expenditure of CO_2 and low conversion into biomass (Lee & Shen 2003).

Regarding organic carbon sources, glucose is the main substrate used for heterotrophic and mixotrophic cultures

and it is worth noting that many microalgae species cannot assimilate any other organic carbon source other than glucose, under heterotrophic or mixotrophic metabolisms. On the other hand, the study of alternative organic carbon sources (*e.g.* acetate, fructose, glycerol etc.) employed in the development of mixotrophic and heterotrophic cultivations can contribute to the economic viability of theses cultivation modes (Combres *et al.* 1994; Yang *et al.* 2014; Bagchi & Mallick 2016; Katiyar *et al.* 2017; Song & Pei 2018). Comparing the results of maximum biomass reached in the heterotrophic and mixotrophic cultivations reported by Shen *et al.* (2018), using acetate, (0.68 and 2.5 g L^{-1}, respectively) and Vieira (2018), using glucose, (7.9 and 14.77 g L^{-1}, respectively), it is possible to conclude that glucose is more suitable for biomass production of *T. obliquus*.

In addition, glycerol may be an organic carbon source suitable for biomass production of *T. obliquus*. It is a by-product of biodiesel production (Anitha *et al.* 2016) which offers a range of commercial applications. However, its excessive production due to the rapid expansion of biodiesel plants throughout world poses a major problem to these plants and the industry (Mario Pagliaro 2008). The cultivation of microalgae using glycerol has recently been used for different species through an integrated chain (Paranjape *et al.* 2016; Salati *et al.* 2017) but despite this, we not found reports on *T. obliquus* biomass production using glycerol as organic carbon source.

Nitrogen source

Nitrogen plays a vital role in microalgae cell growth and synthesis of amino acids and lipids which make up about 10% cell dry weight (Wijffels *et al.* 2010). Ammonia, nitrate and nitrite are the most commonly used nitrogen sources for microalgae growth and each will have a different effect on cell growth and biochemical composition (Lourenço *et al.* 2004). Compared to nitrate, ammonia supplementation accelerates nitrogen metabolism (since nitrate needs to be reduced to ammonia intracellularly) and is also economically efficient (since ammonia-based compounds are cheaper than nitrate-based compounds). However, high concentration of ammonia exhibits toxicity to cells (Gutierrez *et al.* 2016).

The nitrogen source can influence the biomass and lutein productivities in *T. obliquus* (Tang *et al.* 2011). The authors reported biomass production using nitrate as nitrogen source was around 2–3-fold higher than that obtained from using ammonia and urea, indicating that nitrate is the favourable nitrogen source for the biomass production of *T. obliquus*. On the other hand, the lutein content increased gradually along with the consumption of ammonia and urea, while the maximum lutein content was obtained at the beginning of nitrate depletion, while the lutein content started to decrease under the nitrate depletion condition.

A consolidated strategy for increasing the lipid yield in *T. obliquus* (and also other microalgae) is nitrogen depletion; however, this strategy reduces the biomass production, and consequently the overall lipid yield (Breuer *et al.* 2013; Chu *et al.* 2014; Shen *et al.* 2018). Thus, a balanced culture medium must be provided to improve biomass production followed by low nitrogen content conditions to increase the lipid synthesis.

Light

Light source is an important requirement in microalgae photoautotrophic (and also mixotrophic) cultures. But, with increase in cell density, the availability of light is a limiting factor for intensive cultures due to cell self-shading. On the other hand, if cells cannot efficiently distribute excess amounts of light, it accumulates in the photosystem causing photo-oxidation (Carvalho *et al.* 2011; Deng *et al.* 2019). To avoid chlorophyll photo-oxidation, it is essential that the irradiance is gradually increased as the number of cells increases. In this sense, a suitable model of increased irradiance related to the total chlorophyll content of *T. obliquus* cells was proposed by Oliveira *et al.* (2020a). Nevertheless, quality (colour) and duration (photoperiod) of light can affect the growth and photosynthetic pigments of *T. obliquus*. The growth of *T. obliquus* decreased when different colours of lights were used in the order red > white > blue > green (Cepák *et al.* 2006). In short, green is the only light in the visible spectrum that algae chloroplasts do not use, simply because green light is completely reflected. Biomass yield of *T. obliquus* as a function of light ($g_{biomass}$ mol_{photon}^{-1}) was similar in the 14:10 and 12:12 photoperiods (light: dark) (León-Saiki *et al.* 2018). Therefore, establishing an ideal light condition is a necessary measure to reduce costs (in indoor systems) and prevent any damage to cells.

Gris *et al.* (2014) investigated the effect of various irradiance conditions on growth, productivity and biochemical composition of *T. obliquus*; their investigation showed that maximum growth rate at 150 μmol photons m^{-2} s^{-1}. They suggested that light intensity above 150 μmol photons m^{-2} s^{-1} inhibit the growth of microalgae but the biochemical composition did not show significant variation under different illumination conditions. Interestingly, Vendruscolo *et al.* (2019) reported that photoperiods/light dark cycle directly affected *T. obliquus* growth, as well as protein, lipid and chlorophyll content. Cultivation under constant illumination was favourable to cell development and protein production, while cultivation with dark periods (12:12) induced higher lipid and chlorophyll production. These authors also reported that content of metabolites, organic acids, amino acids and fatty acids (FAs) was

influenced by different photoperiods and cell growth phases. The levels of proteins, lipids chlorophyll and biomass were significantly altered under different cultivation conditions.

Salinity

Water is fundamental to agricultural activity. Nonetheless, water scarcity in arid and semi-arid regions, as well as competition of the use of water from other fields (such as agriculture and livestock), lower the acceptability of microalgae cultivation by the agricultural farmers. Microalgae do have some advantages for development in regions unsuitable for agriculture (*e.g.* infertile or sandy soil, low availability of freshwater) (Chisti 2007). An attractive and viable alternative is the use of brackish waters (which are non-potable and often unsuitable for agriculture and livestock) for microalgae cultivation. In this regard, freshwater microalgae species may be more likely to be grown in brackish waters than marine microalgae species (Shetty *et al.* 2019). According to Pandit *et al.* (2017), the growth of *T. obliquus* was reported even at salinity of 23.4 g L^{-1} although the best biomass production was reported at a 3.5 g L^{-1}. In addition, these authors observed that sodium chloride (NaCl) favoured the lipid yield and also increased the palmitic acid content. Gan *et al.* (2016) reported a possible biological desalination, using *T. obliquus*, and lipid production using brackish water, at salinity ranging from 1.2 to 8.8 g L^{-1} of NaCl. The use of microalgae biological desalination is more advantageous than conventional desalinizers that use electricity and inevitably generate a salt-rich waste.

Temperature

The temperature of the culture affects the growth rate of microalgae. The ideal temperature to provide high biomass production varies between 15 and 30°C. Low temperatures (15–20°C) can reduce or limit growth and are commonly used in the strains maintenance – where a high growth rate is not desired. On the other hand, relatively high temperatures (20–30°C) accelerate the metabolic rate, resulting in an increase in chemical energy for cellular increase and multiplication (Ras *et al.* 2013). According to Guedes *et al.* (2011a), biomass productivity of *T. obliquus* was almost three times higher at 30°C (0.834 ± 0.054 g L^{-1} day^{-1}) when compared to 20°C (0.37 ± 0.017 g L^{-1} day^{-1}). Martínez *et al.* (1999) did not observe differences in the growth of *T. obliquus* at 30 or 35°C. In addition, a culture of *T. obliquus* in a thin-layer system, where temperature reached 40°C (ranged from 24 to 40°C), did not show any effect on growth performance (Venancio *et al.* 2020). These findings have proved that *T. obliquus* as a thermotolerant microalga and promising candidate for the development of intensive cultivations in tropical, semi-arid and desert areas – which are, in most cases, unsuitable for agriculture.

Microalgae harvesting and biomass processing

Harvesting, drying and storage are the major challenges in downstream microalgal processes. It is estimated that the cost involved in these processes can overcome 30% of the total production cost (Li *et al.* 2020). Basically, microalgae harvesting is the separation process involving a heterogeneous mixture composed by a discontinuous solid (microalgae cells) and a continuous liquid (culture medium). It is already known that the harvesting process must be fast, low energy demanding and should not contaminate the biomass (or leave waste). On the other hand, biomass drying represents a step prior to storage/purpose. Although studies have already been done on microalgae harvesting (*e.g.* flocculation, flotation, electrophoresis, filtration, centrifugation etc.) and drying (*e.g.* freeze-drying, spray-drying, oven-drying, sun-drying etc.) processes, these processes still represent a limiting step to the microalgae biomass production chain (Oliveira *et al.* 2018; De Melo Aguiar *et al.* 2019; Xue *et al.* 2019; Zhou *et al.* 2019; Liu *et al.* 2019a). This is mainly because large-scale cultivations often occur in large volumes and with low productivity – which makes it difficult for efficient harvesting (Roselet *et al.* 2019).

The flocculation efficiency using Fe_3O_4@PEI (nano-Fe3O4 coated with polyethyleneimine) was higher for *T. obliquus* when compared to *Chlorella pyrenoidosa* (Liu *et al.* 2019b). The authors reported a lower flocculation time (15 and 20 min) and dose (16- and 20-mL L^{-1}) for reaching an 98% efficiency in recovery of *T. obliquus* cells grown in urban sewage. In another study carried out by Dias *et al.* (2021), green flocculants have showed efficiencies greater than 60% and 80% for tropical trees *Guazuma ulmifolia* and *Moringa oleifera*, respectively, on the recovery of *T. obliquus* BR003 biomass. The use of efficient natural flocculants is necessary, mainly for pharmacological and food industries, because they do not present risks to human health or the environment (Singh & Patidar 2018). The choice of an appropriate flocculant is, therefore, essential not only for the efficiency of the coagulation-flocculation process but also for the sustainable development of the microalgae production chain (Houser *et al.* 2014).

Recently, two studies reported the effect of harvesting and drying methodologies on the energy recovery of *T. obliquus* biomass (Wang *et al.* 2019; Oliveira *et al.* 2020b). Wang *et al.* (2019) reported that flocculation of *T. obliquus* biomass using ferric sulphate was equally efficient in centrifugation and increased the biodiesel production (91.7 to 112.6 mg g^{-1} dw). In the same way, Oliveira *et al.* (2020b) found similar biodiesel productivities (somewhere around 140 mg g^{-1} dw) between flocculation, using a cationic polyacrylamide, and centrifugation; however, using the oven-drying process caused the biodiesel yield to

drop considerably (50.67 mg g^{-1} dw). It was more likely that the high oven temperatures may have favoured the bonding of the polymers to the cell walls, acting as a physical barrier against the efficient extraction of the apolar fraction from the *T. obliquus* biomass. In addition, the findings by Oliveira *et al.* (2020b) showed the effects of drying methodologies on the fatty acid methyl esters (FAMEs) composition extracted from *T. obliquus*. Briefly, a non-dried biomass (wet biomass) presented a suitable FAMEs profile for biodiesel production (lower unsaturated FAs content); on the other hand, dried biomasses (in a freeze- or oven-drying) showed a reduction in saturated (SFA) and monosaturated fatty acids (MUFA) contents and an increase in polyunsaturated fatty acids (PUFA) content.

These studies represent a major breakthrough, especially for the microalgae-derived biodiesel industries, since flocculation is shown to be an efficient and non-predictive method for biodiesel quality (considering only the FAs profile). In addition, the fact that non-dried biomass has higher SFA and MUFA levels (which give greater energy release) also represents a time and cost reduction, perhaps making microalgae-derived biodiesel competitive in the world scenario.

Biochemical composition

Microalgae are natural producer of biochemical compounds such as carbohydrate, protein, FAs and photosynthetic pigments (Dantas *et al.* 2019). It is well documented that biochemical composition of microalgae varies from species to species; simultaneously, it also depends on various factors, that is growth condition and composition of culture media. Carbohydrates are the common energy and carbon storage products in algae which play important role in their metabolism. The carbohydrate content in algae depends on various factors. A carbohydrate content ~27.7% has been reported in *T. obliquus* cultivated in Bristol media under controlled condition (Khatoon *et al.* 2019). The ranges of gross biochemical composition of *T. obliquus* are shown in Table 2.

Table 2 Gross biochemical composition (min-max) of *Tetradesmus obliquus*

	% (*w*/*w*)	Ref.
Carbohydrates	10–69	Harun *et al.* (2009) and Oliveira *et al.* (2020a)
Total lipids	10–56	Qu *et al.* (2020) and Oliveira *et al.* (2020a)
Crude protein	19–56	Becker (2007) and Oliveira *et al.* (2020a)
Ashes	1–3	Oliveira *et al.* (2020a)

Fatty acids

The free fatty acids (including steroids and pigments, which cannot be converted into biodiesel) are the group of the highest interest within microalgae lipids. SFA and MUFA are preferable for biodiesel production as they release higher calorific value, some long-chain PUFA known as essential fatty acids (EFAs), exhibit biological activities and are considered relevant for the treatment of diseases (Mendes *et al.* 2009; D'Alessandro & Antoniosi Filho 2016). They are essential because your body cannot produce them on its own so they must come from the diet. FAs composition and yield of *T. obliquus* varies depending on salinity (Salama *et al.* 2013), nitrogen source (An *et al.* 2020), nutrient stress (Chu *et al.* 2014), cultivation mode (Shen *et al.* 2018) or the way in which biomass is processed (explained in Microalgae harvesting and biomass processing section). The main FAs reported for *T. obliquus* biomass are listed in Table 3.

Shen *et al.* (2018) reported the FAME contents of *T. obliquus* for the photoautotrophic, heterotrophic and mixotrophic cultures with sufficient nitrogen supply were 11.0, 12.0 and 14.6%, respectively, while under nitrogen deplete, they were 15.3, 47.1 and 44.1%, respectively. The oil contents of algae from three cultures were all improved by nitrogen starvation. Moreover, the FAME content in heterotrophic nitrogen-deficient mode was higher than the mixotrophic nitrogen-deficient mode. Finally, the authors also reported variation on the FAMEs composition of *T. obliquus*. Under nitrogen-repletion conditions, the dominant FAMEs of heterotrophic and mixotrophic cultivations were similar (C18:1, C16:0, C18:3), and they were different with photoautotrophic cultivation (C18:3, C16:0, C18:2). In addition, under nitrogen-depletion conditions, C18:1 accounted for 50.6% and 53.4% of the total FAMEs in the heterotrophic and mixotrophic cultures, respectively, the data were also much higher than that from photoautotrophic culture (32.7%).

Ji *et al.* (2015) reported the increasing in SFA (mainly C16:0) and MUFA (mainly C18:1) contents using 14.1% CO_2 compared with 5% CO_2 in a culture medium containing wastewater. In addition, the FAs content of *T. obliquus* cultured in 1 or 2% wastewater supplemented with 10 or 14.1% CO_2 showed a highest concentration of C16:0 (36–38%). The use of wastewater for production of biomass convertible into biodiesel, it is an alternative to be studied economically since biomass produced in conventional culture media is extremely expensive to become competitive with fossil fuels.

Amino acids

Amino acids (or α-amino acids) are organic structural components that have two different functional groups (a

Table 3 Fatty acids profile of *Tetradesmus obliquus*

Fatty acid	Common name	Representations	% (w/w)
Saturated			
Tetradecanoic	Mirystic	C14:0	0–1.7
Hexadecanoic	Palmitic	C16:0	9.2–29.5
Octadecanoic	Estearic	C18:0	0.2–1.2
Docosanoic	Behenic	C22:0	0.2–1.7
Tetracosanoic	Lignoceric	C24:0	0–0.9
Monounsaturated			
cis-9-tetradecenoic	Myristoleic	C14:1c9 or C14:1ω5	0–0.5
cis-9-hexadecenoic	Palmitoleic	C16:1c9 or C16:1ω7	0.1–13–6
cis-9-octadecenoic	Oleic	C18:1c9 or C18:1ω9	3.0–41.1
cis-9-eicosenoic	Gadoleic	C20:1c9 or C 20:1ω11	0–0.9
Di-unsaturated			
cis-7, cis-10-hexadecadienoic	–	C16:2 c7, c10 or C16:2ω6	0.2–0.7
cis-9, cis-12-octadecadienoic	Linoleic (L)	C18:2 c9, c12 or C18:2ω6	0.1–15.7
Tri-unsaturated			
cis-6, cis-9, cis-12 octadecatrienoic	γ-linolenic	C18:3 c6 c9 c12 or C 18:3ω6	1.2–3.8
cis-9, cis-12, cis-15 octadecatrienoic	α-linolenic (Ln)	C18:3 c9 c12 c15 or C18:3ω3	4.5–41.2
Polyunsaturated			
cis-6, cis-9, cis-12, cis-15 octadecatetraenoic	Stearidonic	C18:4 c6 c9 c12 c15 or C18:4ω3	0.7–4.4
cis-5, cis-8, cis-11, cis-14, cis-17 eicosapentaenoic	Eicosapentaenoic (EPA)	C20:5 c5 c8 c11 c14 c17 or C20:5ω3	0.1–53.6
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19 docosahexaenoic	Docosahexaenoic (DHA)	C22:6 c4 c7 c10 c13 c16 c19 or C22:6ω3	0–2.1

According to Makulla (2000), Salama *et al*. (2013), Girard *et al*. (2014), Shen *et al*. (2018), Oliveira *et al*. (2020b) and An *et al*. (2020).

carboxyl and an amino) and are building blocks of proteins. Algae (microalgae and seaweeds) are responsible for the synthesis of essential amino acids in the aquatic environment that are transferred to higher links in the food chain (Romero García *et al*. 2012). Commonly, 17 α-amino acids are reported in *T. obliquus* cells (Table 4), of which Tryptophan is the only essential unreported amino acid (Omar 2002; Osman *et al*. 2004; Liu *et al*. 2019c).

The immobilized *T. obliquus* cells exhibited higher performances than that of free form in the ammonia bioconversion, from wastewater, into protein (Liu *et al*. 2019c). In addition, some amino acids contents varied according to nutritional metabolism, that is Asp decreased only under mixotrophic cultivation and Lys increased only under photoautotrophic cultivation. These results indicated that these two amino acids were greatly sensitive to the type of carbon source and these changes might be explained by its specific metabolic pathways. The contents of the others amino acids had no regular changes compared with the control (BG-11 medium, sodium nitrate as nitrogen source), which confirmed that these kinds of amino acid in *T. obliquus* were not sensitive to nitrogen source.

Similarly, Piasecka *et al*. (2020) confirmed the effect of nutritional metabolism on protein content of *T. obliquus*. The authors showed that *T. obliquus* cells had high-protein content in the mixotrophic cultivation compared to photoautotrophic and photoheterotrophic cultivations using molasse as carbon source. The control of metabolism and targeting the metabolic pathways towards synthesis and accumulation of specific amino acids can be an opportunity for development of food and feed industries using the *T. obliquus* biomass.

Afify *et al*. (2018) have highlighted the importance of *T. obliquus* protein hydrolysates as antioxidant and antiviral agents. This research group has shown that the quality of amino acids extracted from *T. obliquus* cells can be improved from treatments with specific enzymes. Amino acids such as methionine and arginine showed excellent

Table 4 Amino acids profile of *Tetradesmus obliquus*

Amino acid	Abbreviation (3-; 1-letter)	Polarity	Classification	Content (g/100 g biomass)
Alanine	Ala; A	Nonpolar	Nonessential	4.8–5.14
Arginine	Arg; R	Basic polar	Nonessential	3.85–6.4
Aspartate/ Aspartic acid	Asp; D	Acid polar	Nonessential	6.99–9.9
Cystine	Cys; C	Nonpolar	Nonessential	0.08–0.6
Glutamate	Gln; Q	Polar	Nonessential	6.01–10.8
Glycine	Gly; G	Nonpolar	Nonessential	2.92–5.8
Histidine	His; H	Basic polar	Essential	1.86–2.9
Isoleucine	Ile; I	Nonpolar	Essential	4.1–4.97
Leucine	Leu; L	Nonpolar	Essential	5.44–8.5
Lysine	Lys; K	Basic polar	Essential	4.24–7.4
Methionine	Met; M	Nonpolar	Essential	1.2–2.2
Phenylalanine	Phe; F	Nonpolar	Essential	3.12–6.5
Proline	Pro; P	Nonpolar	Nonessential	3.28–4.1
Serine	Ser; S	Polar	Nonessential	2.72–3.2
Threonine	Thr; T	Polar	Essential	2.95–3.2
Tyrosine	Tyr; Y	Polar	Nonessential	2.07–3.1
Valine	Val; V	Nonpolar	Essential	3.2–3.91

According to Omar (2002), Osman *et al*. (2004) and Liu *et al*. (2019a).

antioxidant activity and high antiviral activity against Coxsackie B3 virus. The authors also point out that further studies are needed to demystify the antiviral mechanisms of these hydrolysates.

Pigments

Pigments are photosynthetic essential compounds that participate in the light-harvesting process and protect the photosynthetic apparatus from photo-oxidative damage (Bartley & Scolnik' 1995). The most abundant pigments in *T. obliquus* are the chlorophylls (*a* and *b*) and lutein (Wiltshire *et al.* 2000; Table 5). *T. obliquus* also contains significant amounts of other relevant carotenoids (β-carotene and astaxanthin) widely used in various industries, such as aquaculture and pharmacology (Guedes *et al.* 2011b).

The lutein productivity of *T. obliquus* FSP-3 was increased three times (from 1.39 to 4.15 mg L^{-1} day^{-1}) at 300 μmol photons m^{-2} s^{-1} irradiance provided by a fluorescent lamp (Ho *et al.* 2014). The lutein can prevent or ameliorate cardiovascular diseases (Dwyer *et al.* 2001), contribute partly to the induction of some tumour cells growth inhibition (Gong *et al.* 2018) and lead to improvement of visual episodic memory in young and middle-aged adults (Nouchi *et al.* 2020). Although the astaxanthin market (from *Haematococcus pluvialis*) is larger (over $240 million per year) than that of lutein, its consumption of related products reached $150 illion in the USA (Cerón-García *et al.* 2008). Moreover, lutein production is expected to grow rapidly at 3.6% annually (Sun *et al.* 2016a).

Environmental applications

Apart from the application of *T. obliquus* for biofuel production, this species has gained increasing attention from researchers and industrial developers in recent years as biomaterial and as biocomponent for the formulation of functional composites for emerging environmental applications such as in wastewater treatment (Mata *et al.* 2012), biosensors development (Wei *et al.* 2010), plant nutrition (Renuka *et al.* 2016) and aquaculture (Tejido-Nuñez *et al.* 2019).

Table 5 Pigments profile of *Tetradesmus obliquus*

Pigment	μg g^{-1} (w/w)	References
Astaxanthin	22–75[†]	Mansouri and Hajizadeh (2018)
Chlorophyll *a*	800–18 000	Maroneze *et al.* (2019) and Oliveira *et al.* (2020a)
Chlorophyll *b*	895–4348	Maroneze *et al.* (2019) and Singh *et al.* (2020)
Lutein	63–3630	Ho *et al.* (2014) and Maroneze *et al.* (2019)
Neoxanthin	36–322	Maroneze *et al.* (2019)
Violaxanthin	2–109	Maroneze *et al.* (2019)
β-Carotene	7–560	Maroneze *et al.* (2019) and Singh *et al.* (2020)

[†]In fresh weight.

Wastewater treatment

The pollution of available potable water in the world is one of the main environmental problems today. A large portion of water contamination is a result of the disposal of excrement conveyed in sewers in aqueous effluents and inappropriate disposal from industrial waste which include dyes, heavy metals, pharmaceutical products etc. These substances interfere with the ecological cycle of species and alter the availability and quality of vital elements for living beings. It has been postulated that the world will face a 40% water shortage by 2030 (Sun *et al.* 2016b) thus posing a serious challenge for sustainable development.

Although the conventional wastewater treatment technologies (such as sedimentation, coagulation, aerobic activated sludge-based treatment, nitrification-denitrification and phosphorus removal) are widely used, they have several limitations and drawbacks like high energy consumption, carbon emission, excess sludge discharge and considerable cost. Microalgae-based wastewater treatment system attracted the attention in the recent years due to their carbon sequestration potential, utilization of algal biomass for production of biofuel, feed, pigments and other value-added, combined with its high availability and low cost. In particular, *T. obliquus* has been used for various types/levels of wastewater treatments and its resulting biomass has been largely used for biofuel production. It is evident from Table 6 that this species shows efficacy in removing nitrogen and phosphorus from the aqueous medium under different pollution conditions.

Various strategies have been developed for wastewater treatment using *T. obliquus* as biomaterial and biocomponent of functional composites. Urrutia *et al.* (1995) investigated potential of *T. obliquus* immobilised on polyurethane and polyvinyl foams for nitrogen removal of nitrogenous fertilizer wastewater. The investigation showed that the nitrogen-starved cells of *T. obliquus* were highly efficient for nitrogen removal. *T. obliquus* had the best performance in removing sulphate (36%) from wastewater, compared to microalgae such as *Chlorella vulgaris* (34%) and *Oocystis minuta* (27%) (Ajala & Alexander 2020). Furthermore, Ahmad *et al.* (2019) presented that *T. obliquus* can remove about 94% phosphate from municipal wastewater as a result of the combined action between absorption capacity of its cells, and volatization and precipitation in the medium. Scarponi *et al.* (2021) analysed the ability of *C. vulgaris* and *T. obliquus* to remove ammonia from solid waste. The

Table 6 Main characteristics of *Tetradesmus obliquus* cultivations in various types of wastewater

Type of WW	COD		TN		TP		Cultivation period (d)	Biomass yield (g L^{-1})	Lipid (%)	Ref.
	Initial (mg L^{-1})	% removal	Initial (mg L^{-1})	% removal	Initial (mg L^{-1})	% removal				
Brewery	3635	57.5	54	20.8	–	–	14	0.9	–	Mata *et al*. (2012)
Piggery	–	–	1280	58	4.3	69	40	0.02	27	Ji *et al*. (2013)
Municipal	–	–	21.8	97	2.15	82	6	0.005	19.7	Ji *et al*. (2015)
Municipal	–	–	40	99	6.41	99	14	1.46	36.26	Zhang *et al*. (2014)
Municipal	–	–	20.09	–	10.67	–	25	1.20	49	Álvarez-Díaz *et al*. (2015)
Raw sewage	320.07	76.3	52.23	98.54	8.47	97.99	15	–	28.36	Gupta *et al*. (2016)
Poultry	3694.7	96	122.7	97.1	27.9	99.3	–	3.8	11.4	Oliveira *et al*. (2019)
Municipal	141.25	–	63.35	95	5.41	81	–	0.92	16	Han *et al*. (2019)
Aquaculture	33	88.9	32	94.4	1.85	90.2	5	0.25	–	Liu *et al*. (2019b)
Aquaculture	96	42	51.51	78.4	8.82	100	14	1.25	30.85	Ansari *et al*. (2019)

COD, chemical oxygen demand; TN, total nitrogen; TP, total phosphorus.

authors showed that *T. obliquus* and *C. vulgaris* can remove 98 and 99% of ammonia available in sludge, respectively.

Renuka *et al*. (2016) reported that wastewater grown *T. obliquus* microalgal biomass can be utilized as a biofertilizer. In this study, the researchers showed that the wastewater grown microalgae-based biofertilizer has higher yield as compared to artificial medium. *T. obliquus* biomass, produced in brewery wastewater, produced 67.1 mL of bioH_2 (in terms of volatile solids) after dark fermentation. In addition, other energy products were also obtained from this biomass: bio-oil (64%), biochar (30%) and biogas (6%) (Ferreira *et al*. 2019). Similarly, Mata *et al*. (2012) reported the biomass yield 0.9 g L^{-1} day^{-1} when *T. obliquus* was cultivated in brewery wastewater for 9 days. Chemical oxygen demand (COD) and total nitrogen (TN) removal efficiency of 57.5% and 20.8% were reported, respectively. It is evident from the various studies that *T. obliquus* has potential to grow in different types of wastewater (low- or no-cost culture media) and produce bioproducts, but these studies are limited to batch- or pilot-scale operations.

Recently, Cengiz Sahin and Aksu (2017) analysed the use of *T. obliquus* biomass (in the form of activated carbon) as an adsorbent material of dyes in the textile industry. They reported that chemically activated carbon removes three times the amount of dye removed with its use in physical activation form. The authors attributed that the better results obtained with chemical activation of biomaterial are related to its superior surface area and total pore volume. The ability of *T. obliquus* to adsorb dyes has also been evaluated for the removal of methylene blue. *T. obliquus* biomass (1.2 g L^{-1}) was treated with $CaCl_2$ to obtain a fast adsorption of 70% of the dye, after 10 min of adsorbent action (Ghafar *et al*. 2017).

The Kotzabasis' group (Papazi *et al*. 2012) showed that *T. obliquus* can be used to degrade phenolic compounds present in wastewater generated from the manufacture of various products such as disinfectants, antiseptics, fumigants, medicines, synthetic resins, photographic developers, paint and varnish removers, explosives etc. These researchers reported that degradation of toxic phenolic compounds in wastewater was accompanied with an increase in the biomass of *T. obliquus*. These findings represent a significant aspect for biotechnological applications of this species, as discussed above.

Other works, such as that of Monteiro *et al*. (2009), reported the application of *T. obliquus* in removal of heavy metals. Generally, adsorption of heavy metal molecules occurs from the electrostatic attraction between the opposite charges of the functional groups present in the cell wall of *T. obliquus* and the metallic/heavy metal pollutant. Escapa *et al*. (2017) compared the efficiency of *T. obliquus* with *C. vulgaris* for the removal of by-products of pharmaceuticals – paracetamol and salicylic acid. The authors concluded that *T. obliquus* is more efficient than *C. vulgaris* for removing paracetamol (>40% versus >21%) and salicylic acid (>93% versus >25%) in batch culture, under the same environmental conditions. Likewise, Santos *et al*. (2017) found that *T. obliquus* removes about 98% (in batch culture) of the by-products of the diclofenac production, while *C. vulgaris* did not show considerable ability to remove this substance.

Aquaculture

Among the various applications, a large sector of microalgae biomass is intended for the feeding of aquatic organisms (Neori 2011). Aquaculture is considered the fastest

growing and most efficient food production sector; in fact, the production of aquatic organisms has already surpassed fishing (FAO 2018). Hence, *T. obliquus* can play impressive roles in aquaculture industry. Tejido-Nuñez *et al.* (2019) reported that the growth of *T. obliquus* in non-sterile wastewater from aquaculture recirculation systems was 22% more higher than *C. vulgaris*. Also, presence of protozoans negatively affected the growth of *C. vulgaris*, but not that of *T. obliquus*, which was attributed to the potential of *T. obliquus* to form cenobia in response to the infochemicals released by herbivorous pressure (a subject already commented in section History, taxonomy and morphology).

Biofloc technology is an undisputed sustainable alternative, especially for fish larviculture and shrimp farming (Crab *et al.* 2012), however, as it is a relatively newer technology, some improvements are still needed. Addition of *T. obliquus* and *C. vulgaris* has been shown to have positive effects on the immune response of Nile tilapia grown in biofloc systems, the technology was named Autotrophic biofloc technology (ABFT) (Jung *et al.* 2017). Nile tilapia's biochemical composition raised in ABFT also showed higher lipids and proteins content than those reared in the conventional system, and this fact may be a response by the microalgae provide complementary nutrients more efficient in the enhancement of the biochemical composition of fish than those provided by bacteria (Sandhya *et al.* 2020). Apart from exploiting various measures to improve the techniques used in aquaculture, exploring the processes that use by-products is also important in order to reduce production costs. This is more important since the aquaculture feed corresponds to about 50% of the total cost (Goddard 1996). A recent study evaluated the use of residual biomass (defatted biomass) of *T. obliquus* as a source of protein and other nutrients for *Rhamdia quelen* (Teleostei, Pemelodidae). The findings of this study revealed that defatted biomass prevented oxidative damage in brain via an enhanced antioxidant response (Marques *et al.* 2019). The recovery of other metabolites would improve the sustainability and economy of bioprocesses in the microalgae chain.

Recently, Piasecka *et al.* (2020) exploited the application of agro-industrial by-products in culture of *T. obliquus* for the production of high levels of EFAs, that is EPA and DHA. The cultivation technique of the species was related to the adopted nutritional strategy which proved to be low-cost and environmentally friendly, compared to other ways of obtaining them. These lipids are considered of great importance in biotechnology and aquaculture industries due to their role in the treatment of heart disease, cancer, type 1 diabetes and other diseases and also produce feed (Mendes *et al.* 2009). It is important to note that fish oil is the main source of EFAs. However, the use of fish oil to produce feed is unsustainable and, for this reason, microalgae are the most suitable source of EFAs for the sustainable aquaculture development.

Biosensors

Microalgae cells, owing to their sensitivity to environmental variables, are also used for developing biosensors for detecting pollutants in aqueous media (Becker 2007). *T. obliquus* has gained attention of researchers for development of biosensors for detection and monitoring of organic molecules in water. These sensors have been presented as an alternative to conventional analytical methods which are generally expensive, time-consuming, and difficult to adapt for the detection of emerging pollutants such as pesticides and by-products of drug production (Chouler *et al.* 2019; Gonzalez & Lorenzo 2019).

Gonzalez and Lorenzo (2019) presented a cost-effective photosynthetic biological fuel cell (pBFC) as an electrochemical biosensor for monitoring water quality in real time. The authors developed a cathode using *T. obliquus* cells and evaluated its potential for the detection of pesticides in water; the resulting anaerobic sludge from the process was disposed at the anode. The results obtained with *T. obliquus* cell-based cathode were compared with cathode made of graphite and indium tin oxide (ITO) surface to evaluate the relative efficiency. The *T. obliquus* pBFC device showed excellent sensitivity and fast response to environmental changes. The electrical response of this devices exhibited a photosynthetic cyclic pattern, characterized by an increase in electrical current during the day and a decrease in the night period. Moreover, the output electrical current showed a linear dependence on the level of oxygen dissolved in the cathode during the electrochemical process. This result is an indication that the *T. obliquus* pBFC device also has great potential to be used as a dissolved oxygen sensor in applications that require such monitoring.

Chouler *et al.* (2019) studied photosynthetic sensors based on microalgae grown in wastewater. The authors explored the use of *T. obliquus* in microbial fuel cells and for the first time developed a portable bioelectrochemical device for the in situ detection of formaldehyde (a toxic substance resulting from the oxidation of organic matter) in water. The devices work only in the presence of light, have a fast electrical response depending on the formaldehyde concentration, with almost 70% sensitivity. *T. obliquus* has been tested for the monitoring of heavy metals such as mercury, zinc and cadmium and their performance were far superior to other potential species such as *Chlorella pirenoydosa* and *Chlamydomonas reinhardtii* (Li *et al.* 2012). These results make *T. obliquus* a potential candidate for the development of new devices for water

biomonitoring with fast response, low cost and environmentally friendly applications.

Biofuels

Third-generation biofuels derived from microalgae are considered as an alternative to fossil fuels and biofuel crops such as soybeans, corn and other lignocellulosic raw materials (Safi *et al.* 2014; Goh *et al.* 2019). Microalgae can produce about 150 000 L of oil per hectare, which is three times higher than other oil feedstock (Li *et al.* 2020). Although much progress has been made, the production cost of biofuel from microalgae is still high (up to 20 times more expensive than biodiesel derived from soy, for example) and thus are less popular than other biofuels (Milano *et al.* 2016; Severo *et al.* 2019). However, soybean production increases at a slower rate than expected, and, thus, competition of microalgae with other oil crops may be still viable due the other high-value by-products present in microalgal biomass (such as pigments) (Chia *et al.* 2018; Severo *et al.* 2019). As previously mentioned, *T. obliquus* has the potential to produce high amounts of lipids (which can be transesterified for the biodiesel production) and carbohydrates (which can be fermented for the bioethanol production). Although some microalgae produce high amounts of lipid and carbohydrates, it does not necessarily mean high production of biodiesel and bioethanol, respectively. It was reported that *T. obliquus* could achieve 37.92% ethanol conversion (El-Sheekh *et al.* 2014) and 90.81% biodiesel-conversion (Guldhe *et al.* 2015) rates. The residual biomass after lipid or carbohydrate extraction is rich in proteins.

One of the most promising alternatives that have been discussed in algal biotechnology field to improve the yield of these valuable biochemical compounds is to control the cell growth of the species from its interaction with nanomaterials (materials whose dimensions are on the order of 10^{-9} m). As an example, He *et al.* (2017) showed that exposure of *T. obliquus* cells to low concentrations of iron (III) oxide nanoparticles (nano Fe_2O_3) improved the cell growth and enhanced synthesis of chlorophyll, protein and lipids. Interestingly, the lipid content of the cells exposed to nanoparticles was ~45% higher than control samples. This option of use of nanoparticles can serve as a potential alternative to the nitrogen-depletion technique since the interaction with the nanoparticles does not negatively affect the biomass growth (and consequently the overall lipid yield). On the other hand, a majority of nanoparticle-based products are toxic to aquatic organisms (Alves da Silva *et al.* 2018). Considering this fact, the use of nanoparticles can compromise the sustainability of biofuels from microalgae. Consequently, there is a need for continuing research, via eco-friendly routes (such as wastewater treatment to produce biomass and biorefinery models) to reduce mainly the harvesting cost.

Biological activities

The growing attention to functional food has driven huge research into microalgae biotechnology (Dantas *et al.* 2019). Microalgae are a promising source of bioactive compounds for new food products, which can be used to enhance the value-added of foods due to their potential anticancer, anti-diabetes, anti-inflammatory and antioxidant activities (Lauritano *et al.* 2016; Novoveská *et al.* 2019).

The supercritical fluids extraction (SFE) offered advantages in the extraction of vitamins from *T. obliquus* biomass. The phylloquinone (vitamin K1) content was higher in supercritical conditions and the menaquinone-7 (a homologous of vitamin K2) was isolated, which, otherwise, cannot be recovered by using traditional extraction procedures (Chronopoulou *et al.* 2019). SFE has been shown to be an efficient and clean technology to recover valuable components from microalgal biomass. However, high cost and scale up challenges impede the use of SFE in large-scale operations (Yen *et al.* 2015).

Montone *et al.* (2018) reported 25 sequenced peptides with potential antioxidant and ACE (angiotensin-converting enzyme) inhibitory activities were found in *T. obliquus* biomass. In particular, four of these peptides exhibited high DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (ranging from 56 to 70%). In addition, ACE-inhibitory activities reached up to 80% activity in one of these peptides.

Protein hydrolysates from *T. obliquus* biomass with papain (41.41%) and trypsin (40.62%) showed the highest antioxidant capacities among the hydrolysates evaluated. Moreover, all protein from *T. obliquus* and its hydrolysates exhibited high antioxidant capacity (up to 68.23%) using the 2,2′-azinobis 3-ethyl-benzothiazoline-6-sulphonate (ABTS) radical scavenging method (Afify *et al.* 2018). According to Mareček *et al.* (2017) both DPPH and ABTS methods are efficient tool for determination of antioxidant activity. In addition, Afify *et al.* (2018) also reported antiviral activity of proteins hydrolysed against the Coxsackie B3 virus, a pathogenic enterovirus that triggers gastrointestinal diseases to full-fledged pericarditis and myocarditis. *T. obliquus* protein hydrolysates exhibited inhibitory effect on Coxsackie B3 Virus (66%) at 100 μg mL^{-1}. The mechanisms behind action of the proteins hydrolysed were mainly attachment inhibition (69.6%), penetration inhibition (66.5%) and adsorption inhibition (53.5%).

Tetradesmus obliquus polysaccharides extracted under hot and cold conditions also presented antiviral activities

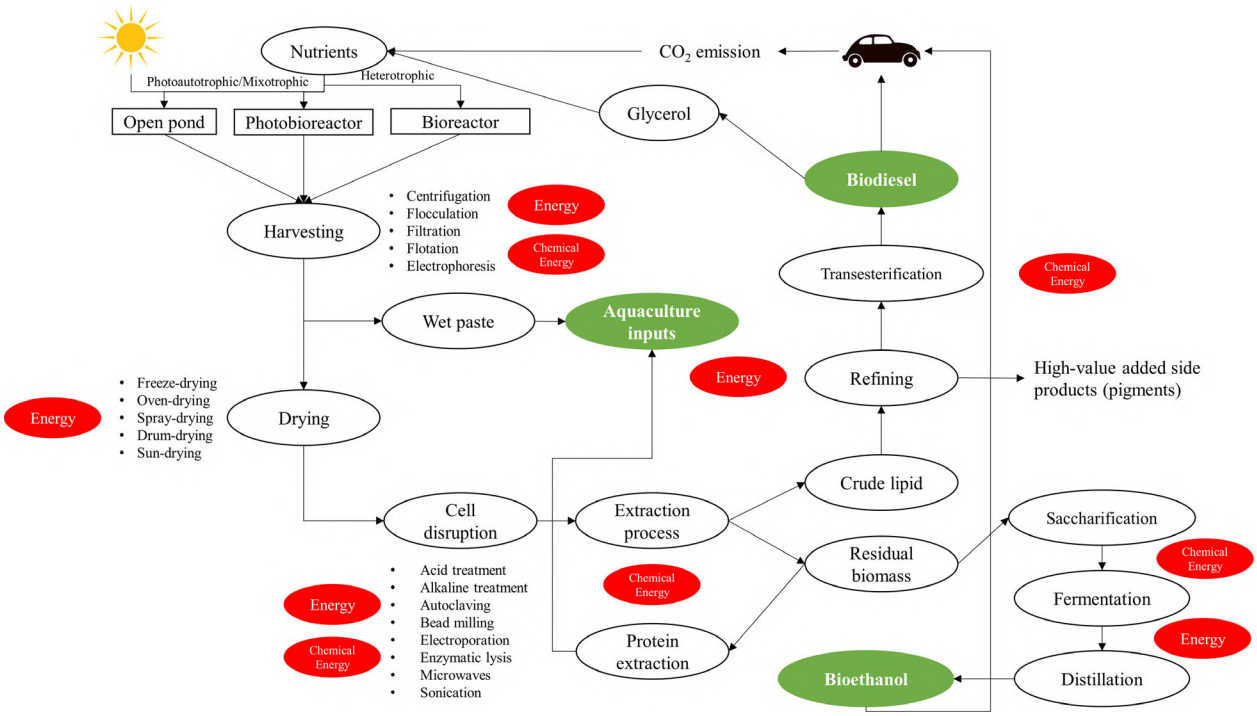

Figure 2 *Tetradesmus obliquus*-refinery concept.

against four virus strains (HCV (hepatitis C virus), HSV (herpes simplex virus), Coxsackievirus and Rotavirus) (Nasser *et al.* 2018). *T. obliquus* extract at 1.5 mg mL^{-1} (a nontoxic dose for some human cells) showed 40, 30, 10 and 40% reduction of the HCV, Rotavirus, HSP and Coxsackievirus, respectively. In addition, these authors reported that *T. obliquus* extract could also inhibit growth in 50.4% of human liver cancer cells (Hep G2) under *in vitro* assays.

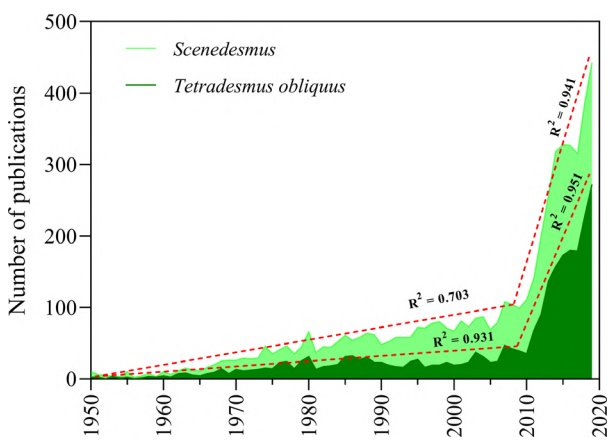

Figure 3 Trend in publications from 1950 to 2019.

Providing a diet enriched with 50% biomass of *T. obliquus* reduced the triglycerides content (70%), atherogenic index (80%) and serum glucose concentration (42%) compared to a diet without this microalga (based on casein). These results show that *T. obliquus* can represent a suitable source of functional and nutraceutical foods for potential treatment and prevention of dyslipidemia and diabetes (Silva *et al.* 2020). However, these authors did not correlate the improvement on blood parameters with specific biocompounds from the *T. obliquus* biomass.

Although some specific microalgae molecules (*e.g.* fucoxanthin, amphidinols, astaxanthin, etc.) have attracted more attention, and consequently, more studies have been conducted on these molecules, few biological activity assays conducted with *T. obliquus* showed an under-explored potential for the pharmaceutical industry of this microalga.

Tetradesmus obliquus-refinery concept

The concept *Tetradesmus obliquus*-based biorefinery (Fig. 2) is similar to a petroleum refinery which comprises of various process and unit operations (Safi *et al.* 2014). Algae-based biorefinery are integrated systems aimed at the reduction of cost and environmental impacts, for use of CO_2 and wastewater, and production of biomass and other value-added products. Recent advances in cultivation techniques, culture medium and development of low-cost

Table 7 Top 10 journals that published documents on *Tetradesmus obliquus*

Journal	*n*	Impact factor (2019)	CiteScore (2019)
Bioresource Technology	80	7.539	12.8
Algal Research	50	4.008	6.7
Journal of Applied Phycology	24	3.016	5.1
Chemosphere	21	5.778	8.8
Environmental Science and Pollution Research	19	3.056	4.9
Bioprocess and Biosystems Engineering	17	2.419	4.4
Ecotoxicology and Environmental Safety	16	4.872	6.2
Aquatic Toxicology	15	4.344	7.2
Journal of Hazardous Materials	13	9.038	13.1
Science of the Total Environment	13	6.551	8.6

photobioreactors based on linear-route production processes are insufficient to improve the economy of microalgae products. The principles of the circular chemistry can reinvent business and production models by integrating various processes into a circular zero-residue chain (Serrà *et al.* 2020; Oliveira *et al.* 2020d).

The integrated use of the various metabolites from microalgal biomass is a fundamental step for the microalgae chains to become economically competitive with the biofuels, pharmaceuticals and aquaculture industries, since in many cases, only interested in a single compound or group of compounds produced (*e.g.* astaxanthin in *H. pluvialis*, phycocyanin and protein content in *A. platensis*, β-carotene in *D. salina* etc.).

Furthermore, the emerging use of 'omics' approaches (such as genomics, transcriptomics, proteomics, lipidomics and metabolomics) can produce valuable massive data that could be deciphered using computational tools and software. *T. obliquus* has the complete mitochondrial DNA sequence published in 2000 (Nedelcu *et al.* 2000) and a draft whole-genome shotgun sequencing in 2017 (Carreres *et al.* 2017). This information can be used in predicting a cell model to optimize the synthesis of specific compounds. The concept of microalgal biorefinery is relatively new and, therefore, abundant literature on the subject is not available. Algal refinery approaches and cost-benefit analysis must be considered in future works.

Bibliometric analysis

In order to highlight the interest in *T. obliquus* into microalgae cultivations, a bibliometric analysis has been proposed. The information of scientific publications was based on Elsevier Scopus database (obtained on January 31, 2020). A detailed search was carried out using [TITLE-ABS-KEY (Scenedesmus); (Scenedesmus) AND (obliquus);

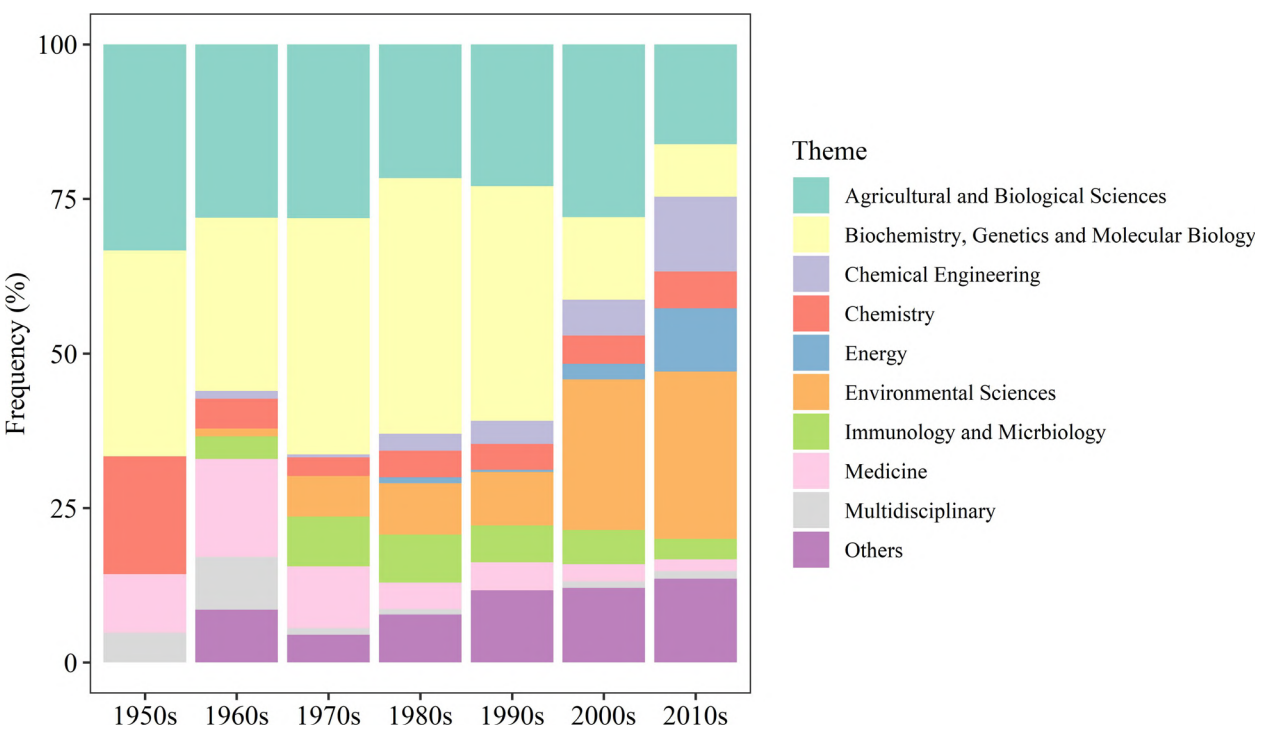

Figure 4 Documents by subject area per decade.

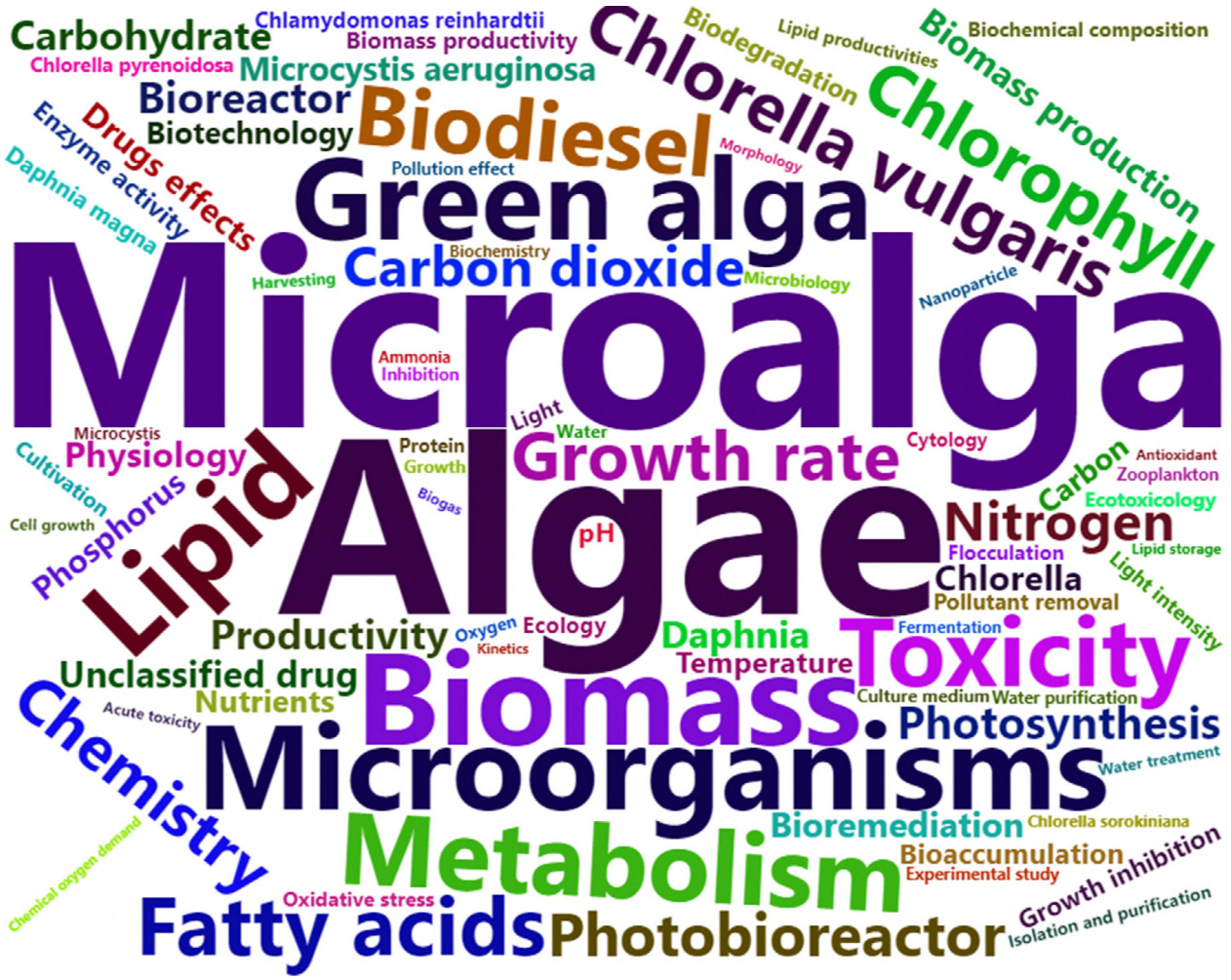

Figure 5 Word cloud representation of the main characteristics of documents on *Tetradesmus obliquus*.

(Acutodesmus) AND (obliquus); (Tetradesmus) AND (obliquus)]. The three specific searches were categorized as *T. obliquus*. The search results in 5434 and 2517 documents to *Scenedesmus* and *T. obliquus* (grouping the three taxonomic results) after limiting the search timescale from 1950 to 2019. In addition, three other parameters are analysed: (i) top ten journals in number of documents, (ii) number of documents by subject area, and (iii) main keywords found in the documents. The records obtained were conveniently processed using spreadsheets that were previously organized using the OpenRefine software. A possible transition in the trends of the keywords reported in *T. obliquus* studies was carried out using VOSviewer software (version 1.6.14).

There are a total of 2517 documents resulting from search carried out on the Scopus platform which were composed by 2376 articles (94.69%), 77 conference papers (3.1%), 30 review (1.19%) and 34 others (1.35%). Most documents were published in English (2351 ≅ 93.27%), Chinese (97 ≅ 3.85%) and German (31 ≅ 1.23%). In general, about 50% of the publications on the Scopus platform that have '*Scenedesmus*' on the title, abstract or keywords, are referred to the *T. obliquus*. Briefly, two trends in number of publications from 1950 and 2019 were observed (Fig. 3).

The total number of publications, the impact factor from the JCR (Journal Citation Reports) and the CiteScore from the SJR (SCImago Journal Rank) of the top 10 journals are listed in Table 7. *Bioresource technology* (80 documents) and *Algal research* (50 documents) were the journals that most published documents on *T. obliquus*. In contrast, *Journal of Hazardous Materials* is the journal with the highest Impact Factor and CiteScore of this ranking.

When organizing the documents by subject area, categorized by decade, it was possible to observe a change in the research profile using *T. obliquus* (Fig. 4). In the 1950s, the

documents were predominantly composed by *Agricultural and Biological Sciences* and *Biochemistry, Genetics and Molecular Biology*; these research fields were still representative until the last decade analysed. *Environmental Sciences* subject area showed a gradual growth over the analysed decades, and in the 2010s it was leading (27.04%) among the other subject areas. The growth of the *Environmental Sciences* subject area is mainly due to studies related to CO_2 mitigation, wastewater treatment and ecotoxicological approaches that have become intense in the last two decades. The 4-fold increase in the contribution of the *Energy* subject area (2.51% in 2000s and 10.25% in 2010s) is also notable, and it is due to the growing concern about depletion of fossil fuels and the need for new renewable energy sources (and microalgae are advantageous in this field). Therefore, it is likely that *Environmental Sciences* and *Energy* subject areas will continue to increase the contribution of research in *T. obliquus*. In addition, research of *T. obliquus* was also frequent in *Medicine, Immunology and Microbiology*, and *Chemistry* subject areas in the decades evaluated.

In addition, the word cloud concept was utilized to expose the main characteristics related to *T. obliquus* microalgae in the spotlight (Fig. 5). In this analysis, keywords '*Scenedesmus*' (1077), '*Scenedesmus obliquus*' (1004), '*Acutodesmus obliquus*' (273), 'Article' (883) and 'Nonhuman' (712) were removed because they are clearly more frequently reported in this field of research or are not representative for this analysis. Keywords of the same meaning (*e.g.* diatoms and Bacillariophyta) and/or plurals had their values grouped. These documents mainly include terms related to the microalgae cultivations and their applications such as 'Biomass', 'Lipid', 'Wastewater' and related to ecotoxicological approach such as 'Toxicity', 'Bioaccumulation', '*Microcystis aeruginosa*' and '*Daphnia* spp.'. Nevertheless, others microalgae species also appear in the word cloud such as '*Chlorella vulgaris*', '*M. aeruginosa*', '*S. quadricauda*', '*Chlamydomonas reinhardtii*', '*Chlorella pyrenoidosa*' and '*Chlorella sorokiniana*'.

The keywords present in the documents of *T. obliquus* published in the last decade were analysed and it is also possible to observe a transition of interest (Fig. 6). In mid-2010, the documents were mostly studies related to photosynthesis (chlorophyll and carotenoids); years later, mid-2013, the interest was concern to ecotoxicology approach and

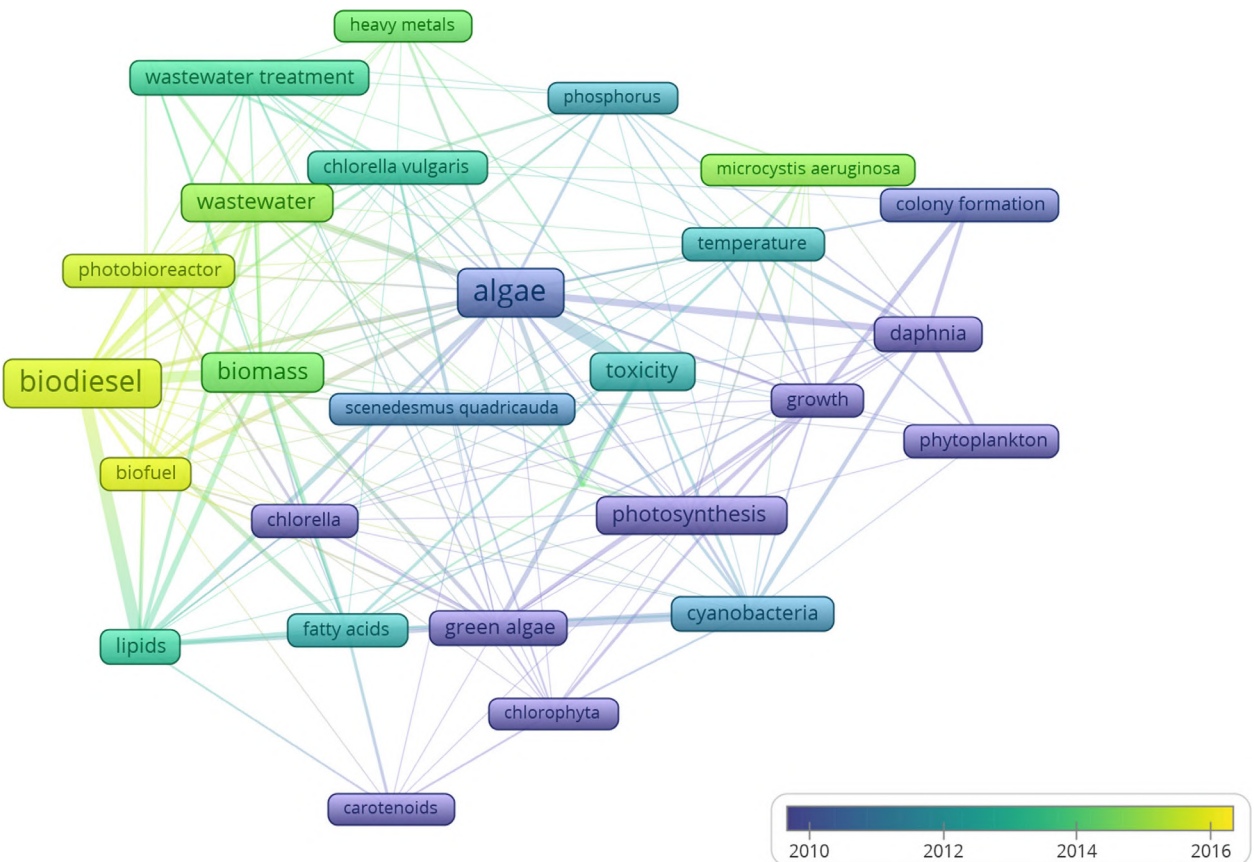

Figure 6 Keywords transition in documents on *Tetradesmus obliquus*.

interactions with other microalgae species (*Chlorella vulgaris* and *Microcystis aeruginosa*) and with zooplankton (*Daphnia*). Biofuels hotspot (biodiesel mainly) occurred in mid-2016.

Conclusions

This review reflects a broad image of current efforts to develop technological packages for production of biomass, biofuels and other emerging applications using the microalga *T. obliquus* (which may also be applicable to other microalgae species). It has also been proven that *T. obliquus* can be managed to develop a low cost (or no cost) nutritional regime complemented by highest growth rate, which provides high biomass productivity. The findings regarding the low-cost processing of biomass (flocculation and non-drying methods) that favour the biodiesel produced using *T. obliquus* also deserve to be highlighted. Nevertheless, biorefinery models can optimize processes involving the *T. obliquus* products.

It is necessary that the recent knowledge published is used in futuristic (or short-term) industrial applications so that *T. obliquus* products are highly competitive in different markets. It is clear that considerable investment in technological development and technical training is still needed, which needs to be carried out in a joint strategic planning of the political and economic powers.

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Declarations

Human and animal rights

The study did not involve human subjects or animal models.

Competing interests.

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contributions.

CYBO: Conceptualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. *CDLO*: Conceptualization, Formal analysis, Writing – review & editing. *RP*: Formal analysis, Writing – review & editing. *HCO*: Writing – review & editing. *ESA*: Writing – review & editing. *NS*: Writing – review & editing. *AOG*: Writing – review & editing.

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