Algal Research 60 (2021) 102548

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Microalgal photosynthetic inhibition and mixotrophic growth in Post Hydrothermal Liquefaction Wastewater (PHW)

Michael J. Stablein [a], Douglas H. Baracho [b], Jamison T. Watson [a], Jaqueline C. Silva [b], Yuanhui Zhang [a], Ana T. Lombardi [c,*]

[a] Agricultural and Biological Engineering Department, University of Illinois Urbana-Champaign, Urbana, IL, USA
[b] PPG ERN, Federal University of São Carlos, São Carlos, São Paulo, Brazil
[c] Department of Botany, Federal University of São Carlos, São Carlos, São Paulo, Brazil

ARTICLE INFO

Keywords:
Post hydrothermal liquefaction wastewater
Cell viability
Mixotrophy
Photosynthetic performance
Pigments
Biovolume

ABSTRACT

Hydrothermal Liquefaction converts wet organic biomass into renewable biocrude oil and simultaneously generates a toxic wastewater (PHW) that is, however, rich in nutrients and organic compounds. While inhibition of algae in diluted PHW has been reported, the underlying reasons are still unclear. The present research explores, for the first time, the effects of PHW on the growth, photoautotrophic functions, and biomass characteristics of four freshwater green microalgae. *Chlorolobion braunii, Chlorella sorokiniana, Chlorella vulgaris*, and *Scenedesmus quadricauda* were exposed to four PHW concentrations (0, 0.5, 1, and 2% *v*/v) and their physiological responses comprehensively investigated. Based in pulse amplitude fluorometry (PAM), a general inhibitory effect in photosynthesis was revealed, indicating diminished photoautotrophic activity. Rapid light curve (RLC) showed that different photosynthetic stages were affected and maximum quantum yield decreased, while Gompertz modeling of population growth highlighted differences in both growth rates and lag phases. Physiological adjustment is suggested by reduced cell viability, increased cell size, and modification in pigment profiles. *C. sorokiniana* stood out based on its limited inhibitory effects and biomass generation in PHW. The combination of diminished photosynthetic performance with increased growth rate affirmed mixotrophy. These findings add to a growing body of literature on the recycling of PHW and similar wastewaters by providing new insights into inhibition mechanisms considering algal photobiology and potential for enhanced biomass production through mixotrophic metabolism.

1. Introduction

The process of Hydrothermal Liquefaction (HTL), which principally uses high heat and pressure to convert wet organic wastes into renewable biocrude, generates a post HTL wastewater (PHW) byproduct that must be valorized to improve overall system viability [1]. Numerous researchers have studied PHW from different feedstocks to report the high concentrations of nutrients and complex matrix of organic compounds [2], attributing its toxicity to specific phenolic and heterocyclic nitrogen compounds [3,4]. The toxicity of PHW, depending on its source and composition, requires that it be diluted between 3 and 10 times for anaerobic digestion [4,5] and 50–200 times for algae culture [6,7]. Notably, such robust bacterial and algal remediation strategies for synergistic nutrient recovery and biomass production present an opportunity for researchers to simultaneously derive value from PHW through its treatment and recycle resources to HTL for further biocrude generation [5]. However, inhibition of these different PHW bioremediation strategies highlights a key bottleneck and requires further investigation in achieving these outcomes.

Algal growth on PHW has been prospected for the available nitrogen, phosphorus, and micronutrients requisite for algae, which has been widely evaluated across different species. Minowa and Sawayama [6] and Jena et al. [7] were among the first to compare *Chlorella* spp. growth on hydrothermal aqueous byproducts to standard media, demonstrating a significant need to dilute these wastewaters. Biller et al. [8] compared the pre- and post- nutrient concentrations and evaluated cell density, in vivo chlorophyll, and dry weight of *Spirulina, Chlorella,* and *Scenedesmus* across a range of algae PHW dilutions, reporting between 200 and 400

Abbreviations: PHW, Post Hydrothermal Liquefaction Wastewater; ETR, Electron Transport Rate; RLC, Rapid Light Curve; Chl, Chlorophyl; Car, Carotenoids.

* Corresponding author at: Rod. Washington Luís, km 235, São Carlos, São Paulo 13565, Brazil.
E-mail address: lombardi@ufscar.br (A.T. Lombardi).

https://doi.org/10.1016/j.algal.2021.102548
Received 12 July 2021; Received in revised form 14 October 2021; Accepted 22 October 2021
Available online 29 October 2021

times dilution as the ideal concentrations for mixotrophic biomass production. Alba et al. [9], highlighting the importance of nitrogen and carbon resources including acetic acid, expanded on the analysis of *Desmodesmus* spp. dry weight production by mixing algal media with PHW nutrients to achieved maximum growth, and nitrate and ammonia utilization within the first 30 h of experimentation. Most recently, Godwin et al. [10] and Hietala et al. [11] markedly improved maximum growth rates and reduced need for dilution by growing algal polycultures on PHW. In their discussion of cellular viability and biochemical composition, the authors also provided preliminary insights as to how PHW nutrients and stress influence algal functions and biomass production. While each of these studies have shed light on different species growth and PHW nutrient utilization in engineering contexts, there is still a knowledge gap in the direct inhibitory effects of PHW and how algal metabolism is affected in achieving maximum biomass production.

New methods and analysis for explaining hampered algal growth that bridge engineering and biological perspectives are paramount. Study of critical physiological functions and differences between species can provide insights on how to maximize biomass production in PHW. The objective of this study was to quantify algal inhibition through innovative analysis of photosynthetic performance and aspects of cell physiology in different species in order to identify optimal species for robust growth on PHW. To accomplish this goal, we focused on 3 processes: algal growth, photosynthesis and cellular physiology. Algal growth inhibition will be primarily examined through Gompertz modeling to quantify changes in growth rates and lag phases. Next, this study will profound the understanding of PHW inhibition through investigation of algal photosynthetic performance. These effects on photosystem II (PSII) will be described by maximum quantum yield (Φ_M) and rapid light curve (RLC) analysis, including light saturation (Ik) and electron transfer rate (ETR). Third, cellular physiology will be examined via cellular viability, biovolume, and pigment profiling. Finally, these collective parameters and maximum biomass production will be explained by photoautotrophic and mixotrophic metabolism in control media conditions and increasing PHW concentrations, as well as verifying differences through statistical significance.

2. Materials and methods

2.1. Properties of Post Hydrothermal Liquefaction Wastewater (PHW) used in the algae culture characterization

Food waste collected from a campus dining hall with volatile solid content of 94.6% and higher heating value of 24.6 MJ·kg^{-1} was used for the HTL feedstock. The food waste feedstock was pretreated by grinding (Weston/Pro#22) and then adding water and sieving (No. 4 Mesh 4750 Micron) to achieve a homogenous slurry, which presented a solid content of 13.3%. The HTL pilot reactor was operated at 300 °C and 1550 psi with a 60 min retention time, and the post-HTL wastewater (PHW) was collected after oil-aqueous-solids separation [12].

It is important to characterize the chemical properties of the PHW. The organic compounds were detected by Gas Chromatography Mass Spectroscopy (GC–MS), performed as follows. Analysis of the hydrothermal liquefaction aqueous phase product was performed with a 2 μL sample that was injected into a GC–MS system composed of an Agilent 6890 gas chromatograph, an Agilent 5973 mass selective detector, and an Agilent 7683B autosampler set up in split mode (10:1). A 60 m ZB-5MS column with a 0.32 mm nominal diameter and 0.25 μm film thickness, using an injection temperature of 250 °C and Mass Selective Detector transfer line at 250 °C was used for gas chromatography. The oven temperature was initially set to 70 °C with a hold time of 2 min, then increased at 5 °C·min^{-1} until reaching 300 °C, and held constant for 5 min. The source temperature was 230 °C, electron ionization was set at 70 eV, and spectra were scanned from 30 to 800 *m/z*. Individual peaks were identified by matching fragmentation patterns against a NIST (NIST08) database. Then, analysis of the output was performed to identify and categorize compounds resulting from the various HTL reactions. Table 1 presents the PHW organic compounds based on the relative peak area. Notably, the PHW was primarily comprised of acetic acid and a variety of heterocyclic nitrogen compounds. More detailed compounds classifications and descriptions have also been previously reported [3,13,14]. It is also important to quantify nutrients contents in accordance with APHA standards [15] prior to algae growth. Determined via Hach methodology 8000 and 8038, the Chemical oxygen demand (COD) and ammonia concentrations were measured at 75000 mg L^{-1} and 1500 mg L^{-1}, respectively.

2.2. Microalgae cultures

The freshwater microalgae used in a prescreening assay were *Chlorolobion braunii*, *Chlorella sorokiniana, Chlorella vulgaris, Muriella decolor, Scenedesmus quadricauda*, referred to as CB, CS, CV, MD, and SQ, respectively. Except for CS that was obtained from the Algal Biotechnology Laboratory (Federal University of São Carlos - UFSCar, Brazil), the other strains were obtained from the Freshwater Microalgae Culture Collection (CCMA) of the Botany Department (UFSCar, Brazil). This collection is registered in the World Data Center for Microorganisms under the number 835. These cultures were chosen based on their robust growth in wastewater or potential for biochemical recovery, including pigments and high-value chemicals [16].

Table 2 presents the classification, typical cell volume (autotrophic growth conditions), morphology, and notable characteristics for each of the species considered for growth on PHW for evaluation of growth characteristics and biomass production. These species represent a diverse group that have been tested in wastewater or modified media compositions in order to observe effects on photosynthetic and physiological characteristics, which can result from toxicity, osmolarity, contamination, and metabolic shifts [17]. Among several algae groups, *Chlorella* spp. and *Scenedesmus* spp. are among species that have most often been evaluated for growth in PHW [7,8,18].

Table 1
Chemical characterization of the Post Hydrothermal Liquefaction Wastewater (PHW) used in algae growth experiments (minimum relative abundance of 1% of total peak area from Gas Chromatography Mass Spectroscopy) ($n = 1$).

Compound Name	Classification	Retention Time (RT)	Relative Area (%)
Acetic acid	short chain acid	14.09	28.30
Ethanol	alcohol	3.51	5.86
3-Pyridinol, 6-methyl-	N-heterocyclic compounds	33.17	5.00
Isosorbide	oxygenate	30.55	3.99
2-Pyrrolidinone	N-heterocyclic compounds	26.70	3.91
Propanamide, 2-hydroxy-	amide	31.73	3.52
Acetamide	amide	21.11	2.95
Glycerin	oxygenate	31.72	2.83
3-Pyridinol	N-heterocyclic compounds	33.52	2.16
2-Cyclopenten-1-one, 2-methyl-	ketones and aldehydes	12.08	2.11
Propanoic acid	short chain acid	16.24	1.75
Pyrrolidine, 1-acetyl-	N-heterocyclic compounds	22.64	1.63
Pyrazine, methyl	N-heterocyclic compounds	9.60	1.52
Pyrazine, 2,5-dimethyl-	N-heterocyclic compounds	10.90	1.34
Pentanoic acid	short chain acid	19.22	1.33
Butanoic acid	short chain acid	18.30	1.14
Acetamide, *N*, *N*-dimethyl-	amide	18.29	1.10

Table 2
Description of algae species (Chlorophyta) used in prescreening assay of current study with BG-11 media and Post Hydrothermal Liquefaction Wastewater (PHW) dilutions.

Species	Classification	Cell Volume (μm^3)	Morphology, Shape	Principal Characteristic	Reference
Chlorolobion braunii (CB)	*Chlorophyceae Sphaeropleales Selenastraceae*	3000	Prism on elliptic base	Photosynthetic resilience to environmental stressors	[19]
Chlorella sorokiniana (CS)	*Trebouxiophyceae Chlorellales Chlorellaceae*	150	Sphere	Grows well under mixotrophic conditions; Widely studied species	[20,21]
Chlorella vulgaris (CV)	*Trebouxiophyceae Chlorellales Chlorellaceae*	150	Sphere	Grows well in diverse substrates, including wastewater; Widely studied species	[22]
Muriella decolor (MD)	*Trebouxiophyceae Chlorellales Chlorellaceae*	100	Sphere	Lipid production, robust growth	[23]
Scenedesmus quadricauda (SQ)	*Chlorophyceae Sphaeropleales Scenedesmaceae*	3000	Prolate spheroid	Nutrient and metals recovery from wastewater	[24,25]

2.3. Screening experiment

In preparation for all screening and subsequent growth experiments, batch cultures in BG-11 medium [26] were kept under controlled laboratory conditions (25 ± 1 °C temperature, 12:12 h light:dark cycle, and 130 μmol photons m^2s^{-1} light intensity). Each culture was prepared for the following experiments through 3 cycles of growth and reinoculation in BG-11 media, demonstrating a maximum photosynthetic yield (Φ_M) of at least 0.7, to ensure the use of healthy photosynthetic inoculum cells for consideration of experimental results [27,28]. In order to determine ideal species for detailed study, a microplate assay was setup to determine the best species and concentrations of PHW for scaled up growth experiments. The growth of the 5 original strains from Table 2 was evaluated at PHW concentrations of 0 (control media), 0.5, 1.0, 2.0, 3.0, 5.0% (*v*/v), all inoculated at final cell density of 5×10^4 cells mL^{-1} and performed in triplicate. All plates were positioned relative to the light source to normalize light intensity despite coloration difference between the PHW concentrations. These plates were measured daily at an absorbance of 680 nm using an Epoch Biotek Plate Reader (Model, Vermont, USA), and the values were converted to natural log to highlight exponential algal growth.

2.4. Experimental design

The following experimental setup was performed in 60 mL culture volumes in order to collect daily samples for measurement of cell morphology and photosynthetic performance with species CB, CS, CV, and SQ. After filtration through a 0.45 μm (Sartorius Stedim Biotech; Goettingen, Germany) and autoclaving, PHW was added to each flask of 60 mL of sterile BG-11 media to achieve 0.5, 1.0, or 2.0% concentration (*v*/v), and the array of species and PHW concentrations are described in Table 3. The flasks were then inoculated with exponentially growing cells at an initial density of 5×10^4 cells·mL^{-1}. Experiments lasted 120 h, and all measurements were performed during the exponential phase (72 h), in addition to confirming achievement of the stationary phase. Three experimental replicates were done for each treatment.

Table 3
Description of experimental design for algae species grown in BG-11 media mixed with different concentrations of Post Hydrothermal Liquefaction Wastewater (PHW).

Species vs PHW %	0% PHW, only BG-11 media (Control - 0)	0.5%PHW + BG-11 media (Treatment 1)	1.0% PHW + BG-11 media (Treatment 2)	2.0%PHW + BG-11 media (Treatment 3)
Chlorolobion braunii (Species CB)	CB 0	CB 0.5	CB 1	CB 2
Chlorella sorokiniana (Species CS)	CS 0	CS 0.5	CS 1	CS 2
Chlorella vulgaris (Species CV)	CV 0	CV 0.5	CV 1	CV 2
Scenedesmus quadricauda (Species SQ)	SQ 0	SQ 0.5	SQ 1	SQ 2

2.5. Analytical methods

2.5.1. Cellular division, latency, and viability

The cultures were monitored daily for cell density (cells·mL^{-1}) and viability (%). Growth curves were plotted by taking the natural logarithm of cell density, and then, the curves were fit individually using the modified Gompertz equation (Eq. (1)) performed in Origin Pro software to determine the specific growth rate (μ) and latency as lag phase (λ) of each condition.

$$y(t) = a \cdot e^{-e^{(-\mu(\lambda - t))}} \quad (1)$$

where y is the cell density at time t (cells·mL^{-1}); a represents the maximum cell density (cells·mL^{-1}); μ is the specific growth rate ($hour^{-1}$); and λ is the lag phase (hours), specifying the latency period to achieve maximum growth rate (cells·mL^{-1}·$hour^{-1}$).

Cell viability, described as the assessment of whether cells are alive or not, is a key indicator for how cells maintain homeostasis and normal proliferation [29]. This parameter was determined daily for all treatments using a Muse Cell Analyzer cytometer (Merck Millipore, CA USA) that considers laser-based fluorescence detection of each cell event. The Muse cytometer is composed of a 532 nm green laser, a photodiode direct detector, and a photodiode type fluorescence detector (YLW 576/28, RED 680/30) to measure cells individually passing through the detector. With 1 mL of control sample in the exponential phase, a calibration curve was performed as a function of cell size and cell viability to establish normal percentages of health, dead, and debris for each species. The microcapillary line was washed between treatments for each of the daily measurements performed for triplicate flasks.

2.5.2. Photosynthesis and pigment analysis

For daily measurements of in vivo chlorophyll *a* concentration

($mg{\cdot}L^{-1}$), a calibration curve was constructed by plotting chlorophyll *a* concentration extracted from exponentially growing *Chlorella vulgaris* cells [30] against in vivo chlorophyll *a* fluorescence (Turner Designs, Trilogy, USA). The linear section of the calibration performed to calculate chlorophyll *a* concentration was constructed with *Chlorella vulgaris* extract and was fitted by means of linear regression using the Relative Fluorescence Units (RFU), obtaining an R^2 value of 0.9769 and generating Eq. (2).

$$[\text{Chlorophyll } a]\text{mg}{\cdot}\text{L}^{-1} = \frac{\text{Fluorescence RFU} + 8.07}{11369} \quad (2)$$

The default 4-channel excitation mode (wavelengths 470 nm, 520 nm, 645 nm, and 665 nm) of the PhytoPAM 101/103 fluorometer (Heinz-Walz, Germany) with a halogen EKE 150 W GX5.3, OSRAM light source was used to measure fluorescence. A 3 mL aliquot was sampled from the cultures and dark adapted (15 min) to allow for complete oxidation of photosystem II (PSII) reaction centers [27,28]. The maximum fluorescence for dark-adapted cells (F_M) was then obtained by applying a single saturating light flash (700 ms, 2000 μmol m^{-2} s^{-1}). After dark adapting again, multiple LED-pulses of increasing actinic light with an interval of 12 μsec start at low frequency (default setting 2 corresponding to approximately 25 Hz) and are used to determine light saturation through minimum fluorescence (F_0). The first pulse is relatively weak (1 μmol photon m^{-2} s^{-1}) and, in most cases, is sufficiently low to allow assessment of the dark-adapted fluorescence yield. For both measurements, each assay was run with a blank of only control BG-11 media or diluted PHW to account for background fluorescence, digitally suppressing unavoidable background signal with the automatic Zero-offset function, prior to all measurements with algae samples. Using these measurements, the maximum quantum yield (Φ_M) was determined daily through Eq. (3). where the maximum quantum yield of PSII (Φ_M) represents the difference between the maximum (F_M) and minimum (F_0) fluorescence yield divided by the maximum fluorescence, and F_V is given by the difference between F_M and F_0 [31]. This parameter indicates the maximum capacity to perform photosynthesis.

$$\Phi_M = \frac{(F_M - F_0)}{F_M} = \frac{F_V}{F_M} \quad (3)$$

During the exponential phase of growth (approximately 72 h), rapid light curves (RLC) were obtained by applying increasing intensities of Photosynthetic Active Radiation (PAR) ranging from 0 to 1730 μmol photons m^{-2} s^{-1} with light pulses at every 20s to determine the relative Electron Transport Rate (rETR). The plotted curve were adjusted according to Platt et al. [32] in determination of alpha (α), the initial slope of the RLC curve that represents the light utilization efficiency, ETR_{max} (maximum electron transport rate), and Ik (light saturation), according to Eqs. (4), (5), and (6), respectively. The Electron Transport Rate (ETR) is an approximation of the rate of electrons pumped through the photosynthetic chain [33], and the maximum value represents the photosystem capacity, oxidizing all of the PSII receptors to utilize absorbed light pulsated by PAM. The alpha (α) is a function of both light harvesting efficiency and photosynthetic conversion efficiency [34], which is the initial slope from the RLC when only light is limiting photosynthesis. Collectively, the RLC parameters determine the efficiency or inhibition of photoautotrophy in microalgae, while increased growth or biomass production can demonstrate use of organic resources, thus demonstrating mixotrophy. All mathematical calculations and data plotting were performed using the software Igor Pro (Wavemetrics, USA).

$$rETR = \Phi'_M \cdot PAR \quad (4)$$

$$rETR = rETR_m \cdot tanh\left(\frac{\alpha \cdot I}{rETR_m}\right) - R^B \quad (5)$$

$$Ik = \frac{rETR_m}{\alpha} \quad (6)$$

Samples were taken at the 72 h mark (exponential phase) from each PHW concentration and species in order to measure pigments. Samples for pigments ($\mu g{\cdot}L^{-1}$) determination were filtered through preweighed 0.45 μm filters (Sartorius Stedim Biotech; Goettingen, Germany) and allowed to dry for 24 h. Then, the filters were dissolved in dimethyl sulfoxide (DMSO) and absorbance (Abs) was measured at 665, 649, and 480 nm for calculation of chlorophylls *a* and *b*, and carotenoids. Eqs. (7), (8), and (9) were used for calculation chlorophyll *a, b,* and carotenoids, respectively [35].

$$\text{Chl } a = 12.47(Abs665) - 3.62(Abs649) \quad (7)$$

$$\text{Chl } b = 25.06(Abs649) - 6.5(Abs665) \quad (8)$$

$$\text{Carotenoids} = \frac{(1000(Abs480) - 1.29(\text{Chl } a) - 53.78(\text{Chl } b))}{220} \quad (9)$$

2.5.3. Dry weight and biovolume

Samples were taken at the 72 h mark (exponential phase) from each PHW concentration and species in order to measure biomass yield. Samples for total dry weight accumulation ($mg{\cdot}L^{-1}$) were filtered through glass fiber filters previously muffled at 400 °C for 8 h. The filters containing the algal biomass were allowed to dry for 48 h at 40 °C, transferred to a desiccator to let cool before measuring mass in determination of dry weight. Dry weight was measured using an analytical balance with 1 μg precision and 1.5 μg repeatability (Mettler Toledo AG, Switzerland).

For biovolumes, cells were preserved in acid lugol solution before counting using a Fuchs-Rosenthal cell counting chamber under an optical microscope (Nikon Eclipse model E200, Japan). Thirty cells were measured per replicate of each treatment and used to calculate cell biovolume ($\mu m^3{\cdot}cell^{-1}$), based on the methodology of Hillebrand et al. [36], and pictures were taken at 400 times magnification.

2.5.4. Statistical Analyses

Individual data sets compared the controls with treatments using ANOVA and with varying levels of significance (10%, 5%, and 1%). All *p*-values comparing the control and the treatment for different response variables at 72 h are presented in the supplementary materials (Table A2).

3. Results and discussion

3.1. Screening experiment

An array of different algae phylogenies (Table 2), including previously unreported species, were compared for the potential of growing on several concentrations of PHW. Growth curves constructed from the natural log of measured microplate absorbance values in the screening experiment are presented as Fig. 1. This also allowed for analysis of linear segments in consideration of maximum growth rate, lag phase, and determining the optimal PHW concentrations to proceed on more detailed study on PHW effects in the growth and photobiology of the organisms.

Among all species tested, it is evident that *CS* presented the best growth at all PHW concentrations, being able to proceed with cell division at a maximum of 3% (Fig. 1) and growing better than in the control (Figs. 1 and 2). *MD* stood out as the only species in the screening test that did not present growth on any concentration of PHW during the studied interval. Therefore, it was not considered outside of the initial screening of algae species. All other species tolerated up to 0.5% PHW with the exception of *CB*, which presented increased absorbance at 1% PHW after a notable lag phase. The screening experiment allowed for

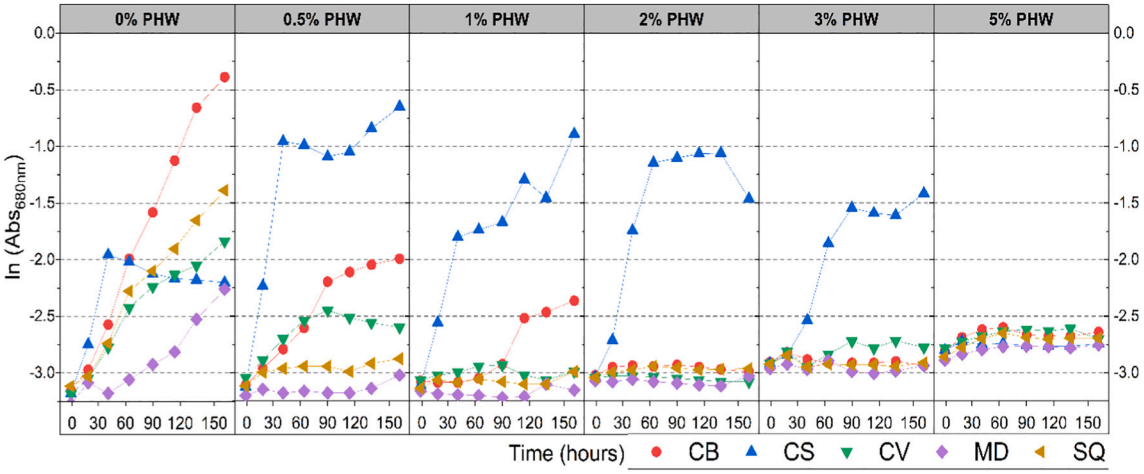

Fig. 1. Growth curves normalized by natural log (+/− SD, $n = 3$) for selected algae species grown in a Post Hydrothermal Liquefaction Wastewater (PHW) gradient ranging from 0 to 5%. The respective species are denoted by the letter assignment: *Chlorolobion braunii (CB), Chlorella sorokiniana (CS), Chlorella vulgaris (CV), Muriella decolor (MD), Scenedesmus quadricauda (SQ).*

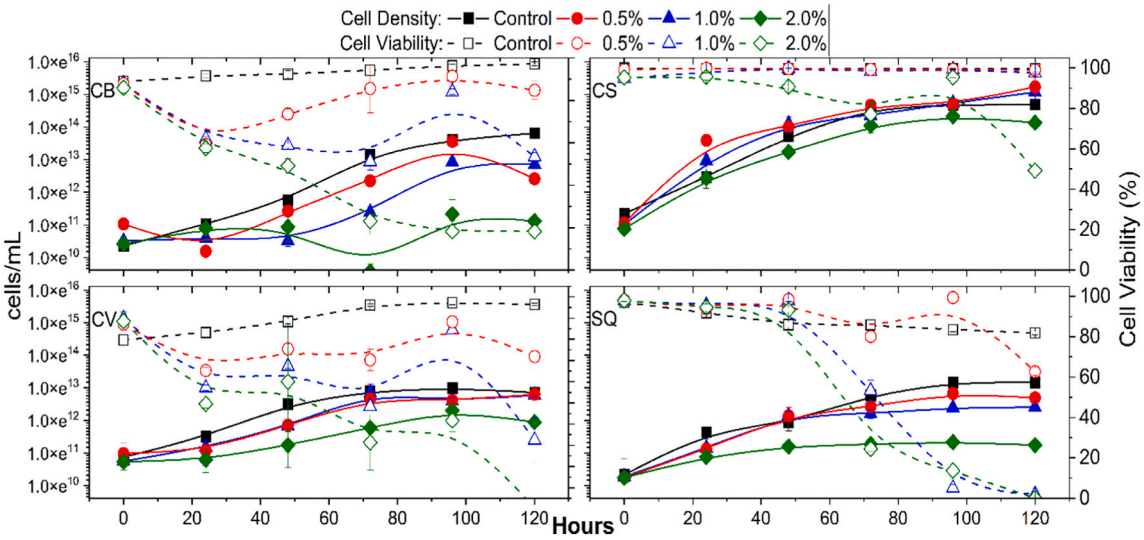

Fig. 2. Cell density and viability (+/− SD, $n = 3$) for 4 species *Chlorolobion braunii (CB)*, *Chlorella sorokiniana (CS), Chlorella vulgaris (CV),* and *Scenedesmus quadricauda (SQ)* in 0–2% Post Hydrothermal Liquefaction Wastewater (PHW) concentrations.

observing that the maximum growth rate and lag phase generally occurred between 24 and 96 h for all species. Given the most notable inhibitory effects occurred between the range of 0–2% PHW, these concentrations and time intervals were chosen for more rigorous study of growth inhibition, photosynthetic performance, and the previously specified physiological characteristics.

3.2. Cellular division, latency, and viability

Given the results from the screening evaluation, the inhibitory effects of PHW on cell density (cells·mL^{-1}) and viability (%) were measured in different concentrations when mixed with BG-11 growth media. In these experiments, cell viability is used as a key indicator for inhibition because it reflects how the cells physiologically maintain homeostasis and normal proliferation, coupled with cell density [29]. After reconstruction of these targeted growth curves, the modified Gompertz equation was used to compare maximum exponential growth rate and lag phase (latency) from these results, presented as Fig. 2 and Table 4.

All experiments were started with healthy inoculum cells in the exponential phase from autotrophic growth conditions (cell viability >90% and maximum photosynthetic yield ~0.7) [29]. Noting that *CV* presents viability <90%, this strain was isolated originally from wastewater and, in related previous studies, has shown a preference and improved capacity to use organic nutrients. In the present study, both the inoculum prior to experimentation and treatment conditions with PHW at time 0 all presented >90% viability, and this noticeable difference is attributed to CV being exposed to the BG-11 inorganic nutrients at the onset of experimentation. All other control conditions were almost constant in viability, while the increasing PHW concentrations (0.5, 1.0, and 2.0%) caused cellular viability to decrease between 0 and 30% for 0.5% PHW and as much as 50–100% at 2%PHW concentrations over the total measured growth period (120h) for all species. These inhibitory effects were notable through decreased values beginning no later than 8 h after inoculation, as well as during visual inspection in daily cell counts. Presenting a lag phase 15% greater than the control (Table 4), *CB* showed some growth up to 1% PHW, and its cell viability recovered after this lag phase period, which can be considered a physiological adjustment [37]. Notably, these CB cells also demonstrated significant increases of as much as 274% in biovolume, and this suggested that the cells were growing but unable to divide because of

Table 4
Maximum growth rates and lag phase (λ) determined via fitting of growth curves ($n = 3$) by modified Gompertz equation for algae species *Chlorolobion braunii (CB)*, *Chlorella sorokiniana (CS)*, *Chlorella vulgaris (CV)*, and *Scenedesmus quadricauda (SQ)* grown in 0–2% Post Hydrothermal Liquefaction Wastewater (PHW) gradient.

Species	PHW%	Lag phase (λ, hrs)	Max growth rate (cells·mL^{-1}·hr^{-1})
Chlorolobion braunii (CB)	Control	63.85	$1.68{\cdot}10^4$
	0.5	73.10	$1.07{\cdot}10^4$
	1.0	55.44**	$4.34{\cdot}10^3$
	2.0	2419.78**	*
Chlorella sorokiniana (CS)	Control	57.88	$3.0{\cdot}10^4$
	0.5	26.03	$4.3{\cdot}10^4$
	1.0	49.66	$3.4{\cdot}10^4$
	2.0	47.07**	$1.5{\cdot}10^4$
Chlorella vulgaris (CV)	Control	32.63	$6.1{\cdot}10^3$
	0.5	48.78	$4.3{\cdot}10^3$
	1.0	94.99	$3.1{\cdot}10^3$
	2.0	27.99**	$1.2{\cdot}10^3$
Scenedesmus quadricauda (SQ)	Control	42.56	$6.6{\cdot}10^3$
	0.5	49.04	$3.8{\cdot}10^3$
	1.0	23.88**	$3.2{\cdot}10^3$
	2.0	0.74**	$1.4{\cdot}10^3$

* Values with an asterisk had Gompertz model fits that did not yield a valid growth rate within the studied growth period.

** Values with a double asterisk were not fit appropriately by the modified Gompertz model within the studied growth period, and thus the observed lag phase are nonrepresentative of trends.

physiological constraints, discussed in further detail in Section 3.4. On the other hand, CS was the exception to these decreased viability values and apparent inhibition, as its cell viability was similar to the control in up to 1% PHW but then decreased to 50% in 2% PHW.

Cellular viability varied among species and PHW concentrations, and some spikes that ranged from 5 to 20% were present in the 72-96 h culture interval. A common trend in cell viability was its decrease of between 10 and 40% (p-value <.01) after the 96-h mark, which occurred for 3 out of the 4 species studied at varying PHW concentrations (CB >1%, CV >1%, and SQ > 0.5% PHW). These results are shown in Fig. 2. Such behavior corresponds to conditions where the cells did not present any growth in the screening test and later demonstrate significantly diminished autotrophic function. Decreased cell viability of algal cells exposed to stressful conditions has been previously reported upon their exposure to specific toxicants, as metals ions [38,39] and organic wastewater compounds [40,41]. Physiologically, these can be attributed to biochemical reorganization such as decreased chlorophyll synthesis, discussed later in Section 3.3 (and Fig. A3 in supplemental material). The reorganization is needed for cell division and/or to protect the photosynthetic apparatus [42].

The particular PHW tolerance threshold was different for each species. There was a decreasing trend in cell density as PHW concentration increased when comparing to the control, as exemplified by CB and CV dropping from $1x10e^{13}$ to $1x10e^{11}$ (Fig. 2). CS exclusively demonstrated this effect just at the 2% PHW concentration, decreasing from $1x10e^{15}$ to $1x10e^{14}$. Based on the Gompertz model, as described in Eq. (1), it was possible to compare the exponential growth rates and lag growth phases among treatments that are provided in Table 4. Analyzing microalgae growth curves in residual waters can be complex because the data may not follow the traditional growth kinetics. Irregular growth patterns can be obtained in non-ideal conditions, such as lag phases during the exponential growth. Such irregular patterns can be understood as different ways the organism find to survive the adverse condition and may vary according to the composition of the residual water and species. Even though the Gompertz equation is one of the most complete models that considers the whole growth curve, some data points failed to be fit, and these discrepancies were denoted (*, **) in Table 4. The Gompertz curves for each condition are provided in the supplementary materials (Fig. A1).

Based on Gompertz equation, lag phases (λ) indicate the amount of time that the modelled curve took to achieve the maximum growth rate, measured as cells·mL^{-1}·hr^{-1} and, because the healthy status of inocula was guaranteed, the length of lag phases can truly be interpreted as a time the cells needed to become better suited to the new environment. Any length of time that is different, either longer or shorter, than that observed for the control is thus a response to the PHW. Lag phases in limited algal cultures are a poorly understood growth stage, even though it is rather common. In general, they are considered a period of restoration of enzyme and substrate concentrations to the levels necessary before active growth can begin [37].

It can be noted that all species except for CS demonstrated inhibitory effects at 0.5% PHW, observed as both decreased maximum growth rates (1.68 to $1.07{\cdot}10^4$, 6.1 to 4.3 $\cdot 10^3$, 6.6 to $3.8{\cdot}10^3$ for CV, CV, and SQ, respectively) and increased lag phases (63.9 to 73.1, 32.6 to 48.8, and 42.6 to 49.0 for CV, CV, and SQ, respectively) that were proportional to the PHW concentrations (Table 4). Both artifacts quantitatively describe the inhibitory properties of the PHW augmented growth media. Despite the optimal nutrient mixture from BG-11, the PHW matrix limited algal growth. In the case of CB exposed to 0.5% PHW, active growth was recovered after the lag phase. However, the data in higher PHW concentrations was not fit well the by the Gompertz model (marked by * or ** in Table 4), and no growth was obtained. A similar effect has also been noted for algae physiology adaptation when using wastewater nutrients and organics in previous studies with *Chlorella and Scenedesmus* [43]. Independent of the lag phase length, increased maximum growth rates suggest that cells successfully leveraged supplementary PHW nutrients [44], as noted for CB at 0.5% and CS at the 0.5% and 1% PHW conditions. Considering CB, it can be attributed to a preservation of autotrophic condition supported by chlorophyll *a* levels similar to the control (Fig. A3).

The observed positive changes in growth rates and lag phases in CS are attributed to combined normal autotrophic growth on inorganic nutrients in control BG-11 media and additional utilization of PHW organics nutrients, described as mixotrophic growth [45]. Such autotrophic, heterotrophic, and mixotrophic conditions have been compared using model compounds like glucose in some algae species, including CV [46] and CS [47]. The authors similarly reported increased maximum growth rate and biomass production upon utilization of organic carbon, similar to the present findings at up to 1% PHW. On the one hand, some significant PHW constituents, such as short chain acids, amines, amides, etc. [2], have been reported to stimulate algal growth through heterotrophic pathways [49]. Acetic acid, notably measured in abundance in the PHW feedstock (Table 1), is a usable carbon source in algal growth [8,51] and can explain the presently observed accelerated growth and increased cell density during mixotrophic growth of CS [52]. On the other hand, other organic compounds in PHW (listed in Table 1), such as the heterocyclic nitrogen compounds pyrrolidinone and pyridinol, have been reported to present inhibitory effects[3,4]. Pham et al. [76] specified that the methylation of such cyclic compounds conferred toxicity to Chinese hamster ovary cells. Among the potential deleterious organic compounds in the PHW matrix, some of the inhibitory and metabolic mechanisms have been specified for other microorganisms, including inhibiting enzymes, cell membrane disruption, and inducing DNA damage, among others [77]. While some articles focused on PHW have reported inhibition of algae and speculated the causal PHW constituents [78-81], there is no conclusive evidence and the specific inhibitory mechanisms have not been clearly elucidated. Notably, *CS* presented approximately 100% viability, increased cell density, and improved growth rate models at all PHW concentrations up to 1%. These growth qualities in the present PHW nutrient mixture highlight the CS species as an ideal candidate for overcoming inhibitory effects, leveraging organic wastewater nutrients, and algae biomass production.

3.3. Photosynthesis and pigments analysis

Algal strains that are not hindered by photoinhibition are the best candidates to be used in mixotrophic cultivation, according to Castro et al. [53], and justified further analysis of mixotrophy and photoinhibition in the present study. In order to demonstrate mixotrophic growth in PHW conditions, pulse amplitude modulated fluorescence parameters were associated with growth performance for the algae. Chlorophyll *a* concentrations and maximum photosynthetic yield (Φ_M) were measured up to the end of the exponential phase (96 h), presented as Fig. 3. Previously unreported for algal PHW cultivation, maximum photosynthetic yield (Φ_M) serves as a measurement for the potential of autotrophic function, normally with a maximum value between 0.7 and 0.8 in healthy cells [27,29]. While chlorophyll can be a proxy for algal growth, these in vivo measurements of photosynthetic metabolites and maximum quantum yield upon exposure to PHW clarify the diminished autotrophic function. The presented results underscore that high concentrations, greater than 2% PHW (Fig. 1), inhibit photosynthetic performance and provide new insights to explain diminished algal growth. Overall, the inhibitory effects of high PHW concentrations were observed at 2% for all species, but the potency of these effects on photosynthesis and the tolerance levels were variable for each species, as discussed in further detail below.

Maximum photosynthetic yield was close to control values for most species up to 0.5% PHW, ranging between 0.6 and 0.8. At higher PHW concentrations, CB, CV and SQ had significantly lower photosynthetic yields (*p*-value <.01), reaching as low as 0.1–0.2 by the end of the growth period. While CV presented the most significant autotrophic inhibition to PHW toxicity at all studied concentrations, SQ nearly maintained a maximum yield of 0.6 during the exponential phase, similar to results reported by Candido & Lombardi [16] in the use of vinasse for algal growth of different species. The diminished autotrophic functions on these two species were apparent early on during its growth phase (<0.6 at 24 h) and are similarly connected to the viability results (0–30% at 120 h, Fig. 2) and decreased chlorophyll *a* concentration (less than control at 72 h, Fig. 3a), suggesting that some essential cell functions were interrupted.

By contrast, *CS* did not present any decreases in maximum yield for the studied PHW concentrations and growth period. The chlorophyll *a* production was reduced by 26% only at 2% PHW, which is in accordance with decreased cellular density (dropping from $1x10e^{13}$ to $1x10e^{11}$) and viability (<50%) (p-value <.01). Considering that this species belongs taxonomically to same Class as CV and MD (Trebouxiophyceae, see Table 2), this difference highlights the particular adaptive capacity of each species to PHW, which is influenced by both the organism tolerance for organic matter [54] and balance between autotrophic and heterotrophic metabolism [55]. Many studies evaluating algae growth on PHW have targeted *Chlorella vulgaris* and *Scenedesmus* spp. [7,8]; however, *CS* proves to be the most adapted [10,11] and presented the least inhibitory effects in increased PHW concentrations.

At increasing PHW concentrations, the matching trends between species growth and photosynthesis can elucidate PHW inhibition mechanisms, based on different algal responses and mixotrophic capacities. It is clear that some autotrophic cell functions were hampered at increasing concentrations of PHW over the total growth period (96 h). Detailed analysis of how growth and photosynthesis are affected in mixotrophic conditions is critical to explain these inhibitory effects from PHW, noting that these relative maximized parameters are applicable to optimization of algal metabolism and biomass productivity [56]. In further analysis of autotrophic functions, Rapid Light Curves (RLC) obtained by Pulse Amplitude Modulated (PAM) fluorometry describe PSII electron transport during the exponential growth phase (Fig. 4). These results include the relative Electron Transport Rate (ETR) with respect to Photosynthetic Active Radiation (PAR) and the Ik (light saturation), maximum ETR, and alpha (α) values [57,58].

The PAM and RLC results corroborate the lower maximum photosynthetic yield (<0.6 after 24 h) and chlorophyll production (less than control after 48 h), as CV and SQ showed decreased ETR (40–70% at 0.5% PHW) at increasing PHW concentrations (*p*-value <.01). The high sensitivity and inhibition of algae photosynthesis due to wastewater ammonium and organic constituents from different sources using PAM methods was also studied by Misaki et al. [59]. The authors reported as much as 40% photosynthetic inhibition and deactivation mechanisms for PSII, in addition to suggesting inhibition of chlorophylls and carotenoids biosynthesis, fatty acid elongation, and cell membrane biosynthesis, among others. Previous studies on algal mixotrophy have concluded that organic carbon, such as acetate, is consumed more readily and can also lead to reduced photosynthetic activity, due to shifts in such metabolic resources for carbon assimilation [51].

In general, Fig. 4 shows that in comparison to controls, Ik decreased and stabilized at a lower value (>33% reduction) as PHW concentration increased for the two *Chlorella* (CS and CV) and SQ. The light saturation parameter (Ik) relates to light acclimation processes in algae, and according to Yentsch and Lee (1966) [60], Ik will shift towards lower values if light independent reactions are affected to a higher degree than the light reactions. Hence, the maximum rate of photosynthesis is achieved at lower light intensities, resulting in lower ETR_{max}. In fact, this

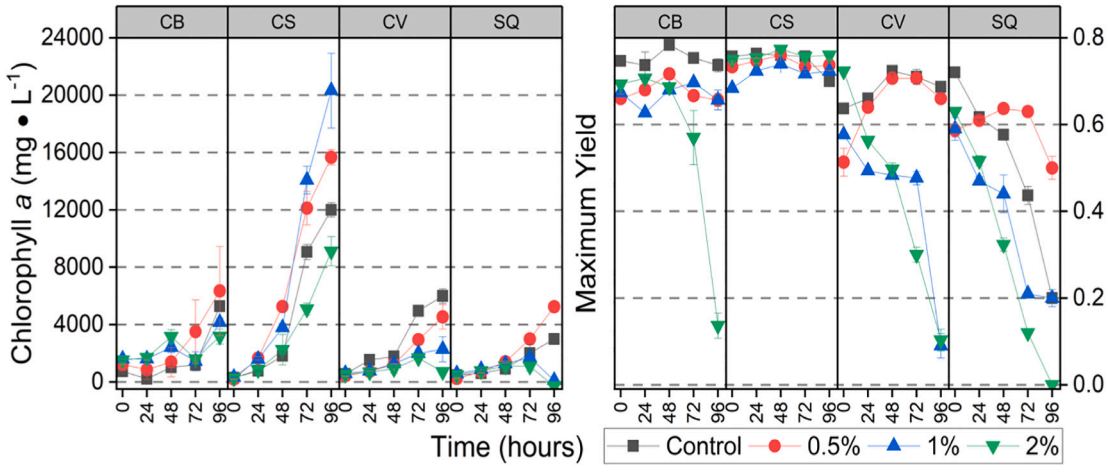

Fig. 3. (a) In vivo Chlorophyll *a* production (+/− SD, $n = 3$) and (b) Maximum quantum yield (+/− SD, n = 3) for 4 species *Chlorolobion braunii (CB)*, *Chlorella sorokiniana (CS)*, *Chlorella vulgaris (CV)*, and *Scenedesmus quadricauda (SQ)* during exponential growth across a 0–2% Post Hydrothermal Liquefaction Wastewater (PHW) gradient.

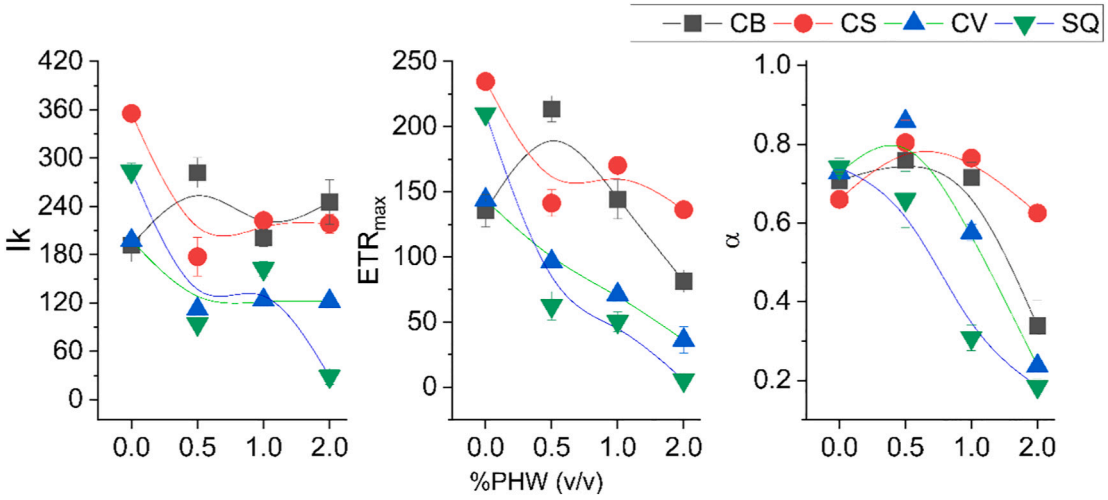

Fig. 4. Ik, maximum electron transport rates, and alpha (α) values (+/− SD, $n = 3$) for 4 species *Chlorolobion braunii (CB)*, *Chlorella sorokiniana (CS)*, *Chlorella vulgaris (CV)*, and *Scenedesmus quadricauda (SQ)* through exponential growth (72 h) across a 0–2% Post Hydrothermal Liquefaction Wastewater (PHW) gradient.

was a common trend for 3 algae in the present research (>40% reduction compared to controls for CS, CV, SQ), but CB was a notable exception, increasing by 40% in 0.5% PHW.

CB showed a uniquely increased Ik and ETR in the 0.5% PHW concentration (p-value <.01), even though there was no change in the light use efficiency (α) at up to 1% PHW. Interestingly, CB was the only algae whose chl *a* concentration did not decrease to values lower than the control (Fig. A3), even though the ratio of chl *a:b* gradually decreased as PHW increased (Fig. 5), meaning that chl *b* increased with PHW. Candido and Lombardi [16] also showed that some species can present accelerated ETR for increasing PAR upon growth in sugarcane vinasse. To this effect, it is noteworthy that CB presented the greatest biovolume increase (Table 5) and that the cells did not proliferate as efficiently for increase in population density (Fig. 2). The collective present findings with respect to PHW demonstrate stress that resulted on algal cells inhibition [29], as similarly reported for other species by Marchello et al. [52]. Explaining this exception, it was furthered by two important responses, the ratio of total chlorophyll/carotenoids (Fig. 5) in CB that was unaffected and the shift of Ik towards higher light intensity. Together,

Table 5
Table organizing the respective increases in cell biovolume (+/− SD, $n = 30$) for algal species *Chlorolobion braunii (CB)*, *Chlorella sorokiniana (CS)*, *Chlorella vulgaris (CV)*, and *Scenedesmus quadricauda (SQ)* at 72 h for direct comparison of control and 2% Post Hydrothermal Liquefaction Wastewater (PHW).

Species	Morphology, Shape	Biovolume (μm^3) Control (72 h)	2%PHW (72 h)	% Increase
Chlorolobion braunii (CB)	Prism on elliptic base	2790 ± 1620	10,400 ± 4340	274%
Chlorella sorokiniana (CS)	Sphere	1640 ± 825	2210 ± 1270	35%
Chlorella vulgaris (CV)	Sphere	4190 ± 1680	5630 ± 2160	34%
Scenedesmus quadricauda (SQ)	Prolate spheroid	864 ± 414	1300 ± 628	50%

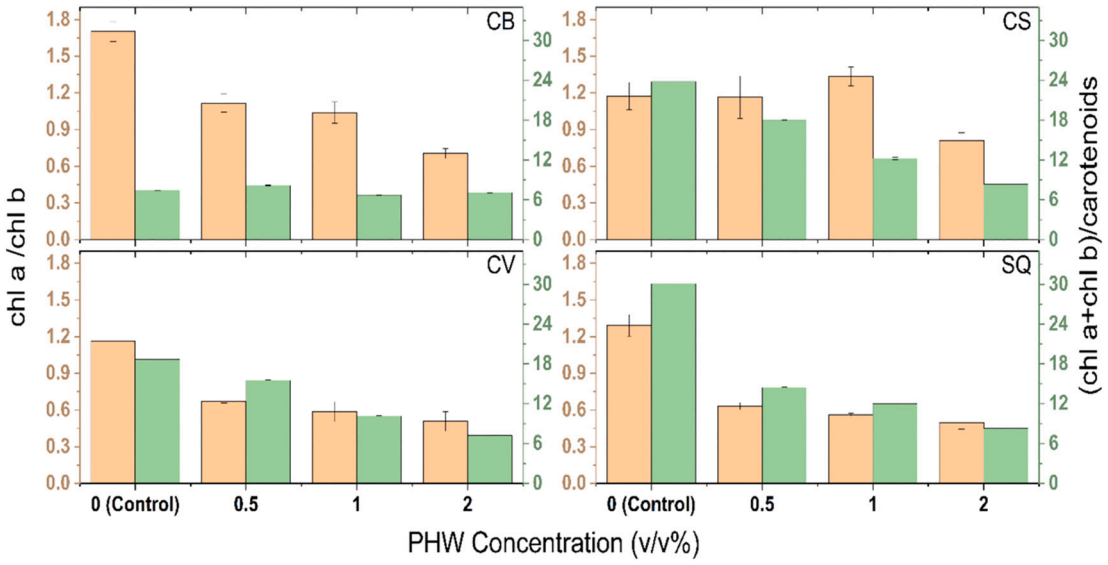

Fig. 5. Ratio of Chlorophyll *a:b* (orange bar, left axis) and Total (chl a + chl b) Chlorophyll:Carotenoids (green bar, right axis) (+/− SD, $n = 3$) for 4 species *Chlorolobion braunii (CB)*, *Chlorella sorokiniana (CS)*, *Chlorella vulgaris (CV)*, and *Scenedesmus quadricauda (SQ)* at 72 h across a 0–2% Post Hydrothermal Liquefaction Wastewater (PHW) concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

these suggest that light reactions were more affected than the light independent reactions of photosynthesis in PHW exposed CB.

Carotenoids played important role in the survival of the Chlorophyta in the presence of PHW. Both CB and CS, the two algae that maintained cell dividing ability in PHW (Fig. 3), accumulated carotenoids equal to or higher than the controls in PHW concentrations lower than 2% (Fig. A5), whereas the opposite behavior was observed in CV and SQ. For CV and SQ, carotenoids production decreased 7× and 5× in 0.5% PHW, respectively. It is known that carotenoids help the cell deal with excess energy by its dissipation through the xanthophyll cycle [61], thus protecting and preserving PSII. As discussed in Skashaug et al. [62], a decreased photosynthesis rate can occur by two main processes, either a decrease in chlorophyll *a* cross section and/or by decrease in the minimum turnover time for photons in PS II, and this results in lower ETR. In this research, decreased ETR was a common trend for all PHW concentrations and algae.

The RLC results for CS, linked to its better growth in 0.5 and 1% PHW than in the control, is evidence of its capacity to perform mixotrophy. The maintenance or increase of efficiency of light use (α) in 0.5% and 1% PHW is notable and is supported by the increase in chlorophyll *a* content and persistence of the ratio chl *a:b*. Based on the decrease in Ik and ETR, we confirm the lower rate of photosynthesis and demonstrate the interference of PHW on CS metabolism. The dark reactions were more affected than the light reactions of photosynthesis in CS, since Ik shifted towards lower values [51,54]. By decreasing the dark reactions, the light dependent photosynthetic process diminished as well. From these results, we suggest that photosynthetic machinery in PSII was not affected by up to 1% PHW. Instead, PSII may have been protected by activation of the xanthophyll cycle supported by the carotenoids, which helped in energy dissipation [61]. The increased carotenoids production in CS is confirmed by data presented in Fig. A4. Conclusively, the evidenced decrease in photosynthesis rate and overall improved growth using PHW organic carbon substrates confirms CS mixotrophic growth.

While the RLC parameters demonstrate changes in algal photoautotrophic performance, pigment profiling can provide complementary information to explain physiological adaptation to environments. As a result of unfavorable conditions and environmental stressors, cells generate complementary light harvesting and oxidative stress reducing compounds [20,63], acting as reductants to facilitate regeneration of PSII components [64]. While chlorophyll *a* acts as the predominant light harvesting compound, carotenoids are produced to dissipate energy from chlorophyll to quench triplet states, prevent photodamage, and stabilize the membrane, as well as to neutralize free radicals and reactive oxygen species [65]. Increased carotenoid pigment content has been associated with acclimation of microalgae to wastewater stress, increasing their tolerance [66]. Furthermore, the authors explained that carotenoid accumulation in wastewater-grown algae can provide tolerance against reactive oxygen species, as shown presently for CS and CB (Fig. A4). On the other hand, chlorophyll *b* has a role in transference of energy to chl *a* and in controlling antenna size [67,68]. These pigment production and ratio responses to environmental conditions such as PHW organics and metals, although particular to each species, can further describe physiological adaptation and changes towards mixotrophic metabolism, as reported for growing algae on polluted environments [66,69]. For the 4 individual species across the PHW gradient, Fig. 5 presents both the ratio of chlorophyll *a:b* and total chlorophyll: carotenoids during exponential growth.

The notable decreases in pigment ratios compared to the control across species attest to the global effects of PHW inhibition on photosynthesis. All species presented a decrease of between 40 and 70% in the chlorophyll *a:b* ratio at increasing concentrations of PHW, achieving a final ratio of approximately 0.6. A chl *a:b* ratio greater than 1 means that there were enough chl *a* for photosynthesis to occur in a still preserved photosynthetic apparatus, noting that chl *a:b* is usually lower than 1 in PHW treatments. It can be observed that the chl *a:b* ratios were lower than 1 for CB in the 1 and 2% PHW, thus photosynthesis was hampered. For CV and SQ, the chl *a:b* was higher than 1 only for the controls. CS was the only organism where the chl *a:b* ratio was lower than 1 and photosynthesis had been impaired at just the 2% PHW condition, notably where chl *a* was also lower than the control (p-value $<.05$). Among the presently tested Chlorophyta, the chlorophyll *a:b* ratio for the controls varied within the expected values (1.2–1.6) for this Phylum of algae according to Eggink et al. [70]. These authors showed that, despite possible variation depending on species, the value is usually around 1.4.

Two main pigments, chlorophyll *a* and chlorophyll *b*, are involved in photosynthesis of green algae. Decreased ability of the cells to produce chlorophyll *a* (or its degradation) will cause an imbalance in the ratio chl *a:b* and, thus, decreased photosynthesis. This is what happened in 2% PHW for all 4 species investigated, thus limiting primary production. Notably, CS was the unique species that had chl *a:b* ratio ~ 1.2–1.3 in control conditions and up to 1% PHW, while CB had chl *a:b* ratio 1.6 in control and 1 in 0.5% PHW. For all other algae and treatments, the chl *a:b* ratio varied between ~0.6–0.9, denoting more chl *b* than *a*. The literature shows that in addition to its role in energy transfer to chlorophyll *a*, chlorophyll *b* is also responsible for the organization and size of the optical cross section of the light harvesting antenna complex [67,68]. According to Negi et al. [68], high chl *b* led to increased antenna size and less efficient photosynthesis.

For the total Chl/Car ratio, there was an approximate decrease of 75% in the species CS, CV, and SQ for 2% PHW concentrations. While these species present Chl/Car ratios of between 18 and 30 in the control conditions, it is worth noting that all species presented nearly the same final ratio of 8 total chlorophyll:carotenoids at 2% PHW. *CB* stood out from this decreasing trend for all PHW concentrations evaluated, presenting a uniquely consistent pigment ratio of around 6 across all control and PHW treatments. This distinct pigment profile and high chlorophyll *a* content corroborates the increase in ETR at 0.5% PHW (Fig. 4). Previous studies have also noted some of the unique photosynthetic properties of this species upon exposure to metals [19,71], but further study could explain these responses with respect to specific nutrients and organic compounds in PHW.

Overall, the notable trends and unique physiologies, organized by species, can be linked to differential algal capacities for mixotrophy and responses to PHW toxicity. These previously unreported data will help in determining critical limitations for microbial inhibition in PHW. However, the overarching similarities in algal growth characteristics and biomass production are more important for determining how these organics benefit biomass production, which is a primary goal of PHW nutrient recycling from HTL processes [48,50,72].

3.4. Dry weight and biovolume

Fig. 6 presents the changes in dry weight for each species across the PHW gradient, measured at 72 h during the exponential phase. All species presented greater dry weights when growth media was augmented with PHW, increasing from approximately 40–64 mg•L^{-1} in the control to 125–150 mg•L^{-1} in the optimal condition (2%PHW). While CB at 0.5 and 1% were the exceptions the various species doubled or tripled biomass upon provision of PHW organic compounds and nutrients. Previously, it was demonstrated that CV and SQ presented diminished cell functions at lower concentrations (0.5 and 1% PHW), being unable to perform cell division (Fig. 2) or photosynthesis (Fig. 3) normally.

The size regulation of the light-harvesting antenna by decreased chlorophyll *b* levels and increased photosynthetic efficiency has been shown to result in about 40% biomass increase in green algae [68,73], but this cannot be exploited to explain the present results. First, the light environment inside cultures were initially the same, independent of PHW concentration, and second, chl *b* increased in all cultures at 2% PHW in simultaneous increase of dry biomass (Fig. A4). The exception was CS, whose chlorophyll *b* content was the same for control and

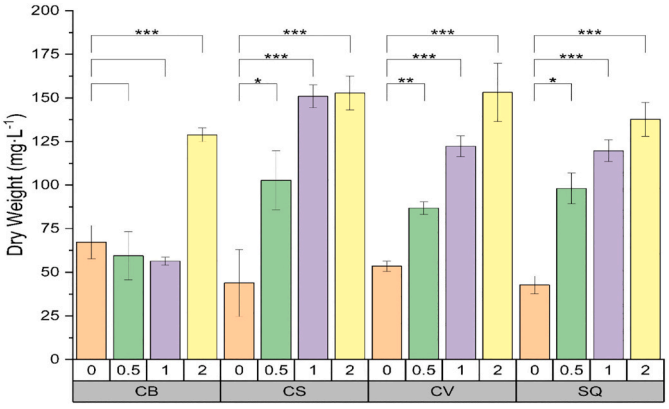

Fig. 6. Dry weight (+/− SD, $n = 3$) for 4 species *Chlorolobion braunii (CB), Chlorella sorokiniana (CS), Chlorella vulgaris (CV),* and *Scenedesmus quadricauda (SQ)* at 72 h across a 0–2% Post Hydrothermal Liquefaction Wastewater (PHW) gradient. Statistical difference between the control and treatments was conducted via analysis of variance (ANOVA). *Denotes *p*-value < .10, **denotes p-value < .05, ***denotes p-value < .01, and no symbol denotes a lack of statistical significance between the treatment and control ($p > .1$).

treatments and biomass increased as well. However, it is a remarkable coincidence that 3 out of 4 species had biomass increase of around 35% in 2% PHW.

The observed increases in biomass production indicate that the cells were metabolically active but unable to proliferate, thus increasing in size. This is confirmed by biovolume results presented in Table 5. Given that all species presented reduced cell densities at 2% PHW, however, the cells must have been increasing in biovolume to achieve the increased biomass production. Measurements were also taken to estimate biovolume based on cell shape [36], presented in Table 5, and optical microscope photographs are included to verify the increased cell size (Fig. A5). It is evident that the cells accumulate mass at higher PHW concentrations, but they are not necessarily dividing. This is attributed to the mitigated autotrophic function or redistribution of metabolic resources under mixotrophic growth conditions.

Increased cell density, biomass, and biovolume have all been demonstrated in autotrophic and mixotrophic growth with organic carbon for various algal species [56,74,75], agreeing with the increasing dry weight harvested during exponential growth for increasing PHW concentrations. The first notable species in this study, CB, presented the most significant increase in cell size (274%) at the 2% PHW concentration. Referring to Fig. 2, this species presented a decrease in cell density, and a unique photosynthetic and pigments profiles at increasing concentrations of PHW (Fig. 5). Baracho et al. [19] also demonstrated the resilience of CB to varying copper conditions with respect to different photosynthetic parameters, and the authors similarly reported both reduced cell density and minimal changes in pigment yields.

In rationalizing the comparatively performance of *CS,* literature provided some explanations as to how this species leverage PHW nutrients. Marchello et al. [52] described the biochemical changes and photosynthetic limitations comparing between photoautotrophic and mixotrophic conditions. Wan et al. [75] additionally used increasing concentrations of glucose to demonstrate a biomass production maximum between 10 and 15 $g{\cdot}L^{-1}$ of glucose that can also affect gene expression. Both authors exemplified metabolic shifts upon provision of organic carbon, and their results corroborate the present findings that PHW caused shifts towards mixotrophic metabolism. The physiological and photosynthetic results of this study demonstrate that *CS* is a promising species for mixotrophic growth and enhanced biomass production in PHW·In the present study, the 2% PHW condition resulted in the greatest increase in dry weight (*p*-value <.01), ranging between 125 and 160 $mg{\cdot}L^{-1}$, and biovolume reaching 34–274% of the control cells, despite the cell densities decreasing (p-value <.01) in increasing PHW concentrations (Fig. 2) and vital functions as autotrophy being hampered. These quantifiable physiological and morphological changes allow for identification of trends between species and concentrations of PHW, corresponding to characteristic cellular functions and metabolic shifts.

4. Conclusions

Photosynthetic performance and mixotrophic growth of the freshwater Chlorophyta *Chlorolobion braunii, Chlorella sorokiniana, Chlorella vulgaris,* and *Scenedesmus quadricauda* were investigated in increasing concentrations (up to 2%) of Post Hydrothermal Liquefaction Wastewater (PHW) diluted in BG11 culture medium. *CS* showed the best adaptation with growth stimulated by up to 1% PHW and ~35% increase in biomass yield, despite its lower photosynthetic rate, indicating mixotrophy. *CB* presented the most significant increase in cell size and doubled its biomass in PHW. *CV* and *SQ* showed the most severely inhibited growth and photosynthetic functions in PHW, with both chlorophyll *a* and *b* lower than the respective controls. Based on the present results *CS* is suggested as an ideal candidate species for further investigation to study utilization of PHW nutrients via mixotrophy. The comprehensive research presented here contributes to the understanding of photobiology related mechanisms by which microalgae deal with the physiological limitations imposed by PHW and provides new insights on algae growth in the wastewater.

CRediT authorship contribution statement

Michael J. Stablein: Conceptualization, Data curation, Investigation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Douglas H. Baracho:** Investigation, Methodology, Formal analysis. **Jamison T. Watson:** Formal analysis, Visualization, Writing – review & editing. **Jaqueline C. Silva:** Formal analysis, Investigation. **Yuanhui Zhang:** Project administration, Supervision. **Ana T. Lombardi:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Project administration, Supervision.

Uncited references

[48,50]

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.

Acknowledgements

The authors acknowledge the support from the University of Illinois Urbana-Champaign Student Sustainability Committee, Lemann Institute for Brazilian Studies, and the Sao Paulo Research Foundation (FAPESP) grant for Sao Paulo Researchers in International Collaboration (SPRINT). ATL and JCS acknowledge the Sao Paulo Research Foundation (FAPESP) (2018/07988-5; 2019/07579-0) and CNPq (304280/2019-4) for financial support. DBS acknowledges CNPq (168889/2018-8). Dr. Giovana Tommaso provided input on Gompertz Modeling for microbial growth in Post-Hydrothermal Liquefaction Wastewater (PHW).

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102548.

References

[1] J. Watson, T. Wang, B. Si, W.T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, Prog. Energy Combust. Sci. 77 (2020), https://doi.org/10.1016/j.pecs.2019.100819.

[2] M.J. Stablein, A. Aierzhati, J. Watson, B. Si, Y. Zhang, Characterization and bioremediation potential of byproducts from hydrothermal liquefaction of food wastes, Bioresour. Technol. Rep. 12 (2020), 100555, https://doi.org/10.1016/j.biteb.2020.100555.

[3] M. Pham, L. Schideman, J. Scott, N. Rajagopalan, M.J. Plewa, Chemical and biological characterization of wastewater generated from hydrothermal liquefaction of spirulina, Environ. Sci. Technol. 47 (2013) 2131–2138, https://doi.org/10.1021/es304532c.

[4] L. Leng, J. Li, Z. Wen, W. Zhou, Use of microalgae to recycle nutrients in aqueous phase derived from hydrothermal liquefaction process, Bioresour. Technol. 256 (2018) 529–542, https://doi.org/10.1016/J.BIORTECH.2018.01.121.

[5] Y. Zhou, L. Schideman, G. Yu, Y. Zhang, A synergistic combination of algal wastewater treatment and hydrothermal biofuel production maximized by nutrient and carbon recycling, Energy Environ. Sci. 6 (2013) 3765–3779, https://doi.org/10.1039/c3ee24241b.

[6] T. Minowa, S. Sawayama, Novel microalgal system for energy production with nitrogen cycling, Fuel 78 (1999) 1213–1215, https://doi.org/10.1016/S0016-2361(99)00047-2.

[7] U. Jena, N. Vaidyanathan, S. Chinnasamy, K.C. Das, Evaluation of microalgae cultivation using recovered aqueous co-product from thermochemical liquefaction of algal biomass, Bioresour. Technol. 102 (2011) 3380–3387, https://doi.org/10.1016/J.BIORTECH.2010.09.111.

[8] P. Biller, A.B. Ross, S.C. Skill, A. Lea-Langton, B. Balasundaram, C. Hall, R. Riley, C. A. Llewellyn, Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process, Algal Res. 1 (2012) 70–76, https://doi.org/10.1016/j.algal.2012.02.002.

[9] L.Garcia Alba, C. Torri, D. Fabbri, S.R.A. Kersten, D.W.F.Wim Brilman, Microalgae growth on the aqueous phase from Hydrothermal Liquefaction of the same microalgae, Chem. Eng. J. 228 (2013) 214–223, https://doi.org/10.1016/j.cej.2013.04.097.

[10] C.M. Godwin, D.C. Hietala, A.R. Lashaway, A. Narwani, P.E. Savage, B. J. Cardinale, Algal polycultures enhance coproduct recycling from hydrothermal liquefaction, Bioresour. Technol. 224 (2017) 630–638, https://doi.org/10.1016/J.BIORTECH.2016.11.105.

[11] D.C. Hietala, C.K. Koss, A. Narwani, A.R. Lashaway, C.M. Godwin, B.J. Cardinale, P.E. Savage, Influence of biodiversity, biochemical composition, and species identity on the quality of biomass and biocrude oil produced via hydrothermal liquefaction, Algal Res. 26 (2017) 203–214, https://doi.org/10.1016/J.ALGAL.2017.07.020.

[12] A. Aierzhati, J. Watson, B. Si, M. Stablein, T. Wang, Y. Zhang, Development of a mobile, pilot scale hydrothermal liquefaction reactor: food waste conversion product analysis and techno-economic assessment, Energy Convers. Manag. X. (2021), 100076, https://doi.org/10.1016/j.ecmx.2021.100076.

[13] J. Watson, T. Wang, B. Si, W.-T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, Prog. Energy Combust. Sci. 77 (2020), 100819, https://doi.org/10.1016/J.PECS.2019.100819.

[14] G. Tommaso, W.-T. Chen, P. Li, L. Schideman, Y. Zhang, Chemical characterization and anaerobic biodegradability of hydrothermal liquefaction aqueous products from mixed-culture wastewater algae, Bioresour. Technol. 178 (2015) 139–146, https://doi.org/10.1016/J.BIORTECH.2014.10.011.

[15] A.W.W.A (AWWA), W.E.F. (WEF), American Public Health Association (APHA), Standard methods for the examination of water and wastewater, 22nd ed., 2012. Washington DC.

[16] C. Candido, A.T. Lombardi, Mixotrophy in green microalgae grown on an organic and nutrient rich waste, World J. Microbiol. Biotechnol. 36 (2020) 20, https://doi.org/10.1007/s11274-020-2802-y.

[17] M. Bilal, T. Rasheed, J. Sosa-Hernández, A. Raza, F. Nabeel, H. Iqbal, Biosorption: an interplay between marine algae and potentially toxic Elements—A review, Mar. Drugs. 16 (2018) 65, https://doi.org/10.3390/md16020065.

[18] P.J. McGinn, K.C. Park, G. Robertson, L. Scoles, W. Ma, D. Singh, Strategies for recovery and recycling of nutrients from municipal sewage treatment effluent and hydrothermal liquefaction wastewaters for the growth of the microalga Scenedesmus sp. AMDD, Algal Res. 38 (2019), https://doi.org/10.1016/j.algal.2019.101418.

[19] D.H. Baracho, J.C. Silva, A.T. Lombardi, The effects of copper on photosynthesis and biomolecules yield in Chlorolobion braunii, J. Phycol. 55 (2019) 1335–1347, https://doi.org/10.1111/jpy.12914.

[20] S. Kim, J. Eun Park, Y.B. Cho, S.J. Hwang, Growth rate, organic carbon and nutrient removal rates of Chlorella sorokiniana in autotrophic, heterotrophic and mixotrophic conditions, Bioresour. Technol. 144 (2013) 8–13, https://doi.org/10.1016/j.biortech.2013.06.068.

[21] M.A. Chia, A.T. Lombardi, M.da G.G. Melão, C.C. Parrish, Phosphorus levels determine changes in growth and biochemical composition of Chlorella vulgaris during cadmium stress, J. Appl. Phycol. 29 (2017) 1883–1891, https://doi.org/10.1007/s10811-017-1111-9.

[22] C. Candido, A.T. Lombardi, The physiology of Chlorella vulgaris grown in conventional and biodigested treated vinasses, Algal Res. 30 (2018) 79–85, https://doi.org/10.1016/j.algal.2018.01.005.

[23] N. da Silva Nonato, E.L. Nottar Escobar, D.M. Kochepka, R. Bianchini Derner, M. G. Montes D'Oca, M.L. Corazza, L. Pereira Ramos, Extraction of muriella decolor lipids using conventional and pressurized solvents and characterization of their fatty acid profile for biodiesel applications, J. Supercrit. Fluids 158 (2020), 104750, https://doi.org/10.1016/j.supflu.2020.104750.

[24] H. Navitha, K.R. Kousar, A comparative study on the efficiency of Chlorella vulgaris and Scenedesmus quadricauda in reduction of phosphate from coffee processing wastewater, Environ. Ecol. 37 (4) (2019) 1183–1185.

[25] B. Kizilkaya, G. Türker, R. Akgül, F. Doğan, Comparative study of biosorption of heavy metals using living green algae Scenedesmus quadricauda and neochloris pseudoalveolaris: equilibrium and kinetics, J. Dispers. Sci. Technol. 33 (2012) 410–419, https://doi.org/10.1080/01932691.2011.567181.

[26] E. Rippka, J. Deruelles, N.B. Waterbury, Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria, 1979.

[27] P. Juneau, P.J. Harrison, Comparison by PAM fluorometry of photosynthetic activity of nine marine phytoplankton grown under identical conditions, Photochem. Photobiol. 81 (2005) 649, https://doi.org/10.1562/2005-01-13-ra-414.1. ¶.

[28] A.T. Lombardi, M.T. Maldonado, The effects of copper on the photosynthetic response of Phaeocystis cordata, Photosynth. Res. 108 (2011) 77–87, https://doi.org/10.1007/s11120-011-9655-z.

[29] B. Elisabeth, F. Rayen, T. Behnam, Microalgae culture quality indicators: a review, Crit. Rev. Biotechnol. 41 (2021) 457–473, https://doi.org/10.1080/07388551.2020.1854672.

[30] W.T. Shoaf, B.W. Lium, Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide, Limnol. Oceanogr. 21 (1976) 926–928, https://doi.org/10.4319/lo.1976.21.6.0926.

[31] U. Schreiber, Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview, in: Chlorophyll a Fluoresc, Springer, Netherlands, 2007, pp. 279–319, https://doi.org/10.1007/978-1-4020-3218-9_11.

[32] T. Platt, C. Gallegos, W. Harrison, Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton, 1980. Undefined.

[33] P.J. Ralph, R. Gademann, Rapid light curves: a powerful tool to assess photosynthetic activity, Aquat. Bot. 82 (2005) 222–237, https://doi.org/10.1016/j.aquabot.2005.02.006.

[34] S. Jodowska, A. Lata, Mechanisms of photoacclimation on photosynthesis level in cyanobacteria, in: Adv. Photosynth. - Fundam. Asp, InTech, 2012, https://doi.org/10.5772/26154.

[35] A.R. Wellburn, The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution, J. Plant Physiol. 144 (1994) 307–313, https://doi.org/10.1016/S0176-1617(11)81192-2.

[36] H. Hillebrand, C.D. Dürselen, D. Kirschtel, U. Pollingher, T. Zohary, Biovolume calculation for pelagic and benthic microalgae, J. Phycol. 35 (1999) 403–424, https://doi.org/10.1046/j.1529-8817.1999.3520403.x.

[37] G.E. Fogg, B. Thake. , 1987. Algae Cultur (accessed June 24, 2021) https://books.google.com/books?hl=en&lr=&id=KGzvVU71tPkC&oi=fnd&pg=PR7&dq=Fogg,+G.E.+and+Thake,+B.+(1987)+Algae+Cultures+and+Phytoplankton+Ecology.&ots=VgYg94YYVv&sig=mzmqtYPOhA61P3GlfnwNSaX3Kxc#v=onepage&q=Fogg%2C G.E. and Thake%2C B.

[38] T.Y. Suman, S.R. Radhika Rajasree, R. Kirubagaran, Evaluation of zinc oxide nanoparticles toxicity on marine algae Chlorella vulgaris through flow cytometric, cytotoxicity and oxidative stress analysis, Ecotoxicol. Environ. Saf. 113 (2015) 23–30, https://doi.org/10.1016/j.ecoenv.2014.11.015.

[39] L. Li, C. Shao, T.F. Lin, J. Shen, S. Yu, R. Shang, D. Yin, K. Zhang, N. Gao, Kinetics of cell inactivation, toxin release, and degradation during permanganation of Microcystis aeruginosa, Environ. Sci. Technol. 48 (2014) 2885–2892, https://doi.org/10.1021/es405014g.

[40] U. Žvab, D.S. Kukulin, M. Fanetti, M. Valant, Bioremediation of copper polluted wastewater-like nutrient media and simultaneous synthesis of stable copper nanoparticles by a viable green alga, J. Water Process Eng. 42 (2021), 102123, https://doi.org/10.1016/j.jwpe.2021.102123.

[41] J. Lee, E.J. Choi, K. Rhie, Validation of algal viability treated with total residual oxidant and organic matter by flow cytometry, Mar. Pollut. Bull. 97 (2015) 95–104, https://doi.org/10.1016/j.marpolbul.2015.06.029.

[42] C.M. Duarte, Time lags in algal growth: generality, causes and consequences, J. Plankton Res. 12 (1990) 873–883, https://doi.org/10.1093/plankt/12.4.873.

[43] S.K. Gupta, F.A. Ansari, A. Shriwastav, N.K. Sahoo, I. Rawat, F. Bux, Dual role of Chlorella sorokiniana and Scenedesmus obliquus for comprehensive wastewater treatment and biomass production for bio-fuels, J. Clean. Prod. 115 (2016) 255–264, https://doi.org/10.1016/j.jclepro.2015.12.040.

[44] S. Kim, J. Eun Park, Y.B. Cho, S.J. Hwang, Growth rate, organic carbon and nutrient removal rates of Chlorella sorokiniana in autotrophic, heterotrophic and mixotrophic conditions, Bioresour. Technol. 144 (2013) 8–13, https://doi.org/10.1016/j.biortech.2013.06.068.

[45] J.A. Borowitzka, A. Michael, Beardall, John Raven, The Physiology of Microalgae, Springer International Publishing, 2016, https://doi.org/10.1007/978-3-319-24945-2.

[46] D. Mitra, J.(Hans) van Leeuwen, B. Lamsal, Heterotrophic/mixotrophic cultivation of oleaginous Chlorella vulgaris on industrial co-products, Algal Res. 1 (2012) 40–48, https://doi.org/10.1016/j.algal.2012.03.002.
[47] H. Wang, H. Xiong, Z. Hui, X. Zeng, Mixotrophic cultivation of Chlorella pyrenoidosa with diluted primary piggery wastewater to produce lipids, Bioresour. Technol. 104 (2012) 215–220, https://doi.org/10.1016/j.biortech.2011.11.020.
[48] M. Erkelens, A.S. Ball, D.M. Lewis, The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic tetraselmis sp, Bioresour. Technol. 182 (2015) 378–382.
[49] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic cultures of microalgae: metabolism and potential products, Water Res. 45 (2011) 11–36, https://doi.org/10.1016/j.watres.2010.08.037.
[50] S. Wang, X. Shi, B. Palenik, Characterization of Picochlorum sp. Use of wastewater generated from hydrothermal liquefaction as a nitrogen source, Algal Res. 13 (2016) 311–317, https://doi.org/10.1016/J.ALGAL.2015.11.015.
[51] P.B. Heifetz, B. Förster, C.B. Osmond, L.J. Giles, J.E. Boynton, Effects of acetate on facultative autotrophy in Chlamydomonas reinhardtii assessed by photosynthetic measurements and stable isotope analyses, Plant Physiol. 122 (2000) 1439–1445, https://doi.org/10.1104/pp.122.4.1439.
[52] A.E. Marchello, A.C. dos Santos, A.T. Lombardi, C.W.O. de Souza, G.C. Montanhim, Physiological and ecological aspects of Chlorella sorokiniana (Trebouxiophyceae) under photoautotrophic and mixotrophic conditions, Microb. Ecol. 76 (2018) 791–800, https://doi.org/10.1007/s00248-018-1170-8.
[53] Y. Castro, Handbook of Microalgal Culture, (n.d.). https://www.academia.edu/35862840/Handbook_of_Microalgal_Culture (accessed November 30, 2020).
[54] T.A. Karasyova, E.O. Klose, R. Menzel, C.E.W. Steinberg, Natural organic matter differently modulates growth of two closely related coccal green algal species, Environ. Sci. Pollut. Res. 14 (2007) 88–93, https://doi.org/10.1065/espr2006.06.317.
[55] C. Yang, Q. Hua, K. Shimizu, Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions, Biochem. Eng. J. 6 (2000) 87–102, https://doi.org/10.1016/S1369-703X(00)00080-2.
[56] A.E. Marchello, A.C. dos Santos, A.T. Lombardi, C.W.O. de Souza, G.C. Montanhim, Physiological and ecological aspects of Chlorella sorokiniana (Trebouxiophyceae) under photoautotrophic and mixotrophic conditions, Microb. Ecol. 76 (2018) 791–800, https://doi.org/10.1007/s00248-018-1170-8.
[57] J. Serôdio, S. Vieira, S. Cruz, H. Coelho, Rapid light-response curves of chlorophyll fluorescence in microalgae: relationship to steady-state light curves and non-photochemical quenching in benthic diatom-dominated assemblages, Photosynth. Res. 90 (2006) 29–43, https://doi.org/10.1007/s11120-006-9105-5.
[58] F.L. Figueroa, C.G. Jerez, N. Korbee, Use of in vivo chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems, Lat. Am. J. Aquat. Res. 41 (2013) 801–819, https://doi.org/10.3856/vol41-issue5-fulltext-1.
[59] K. Misaki, Y. Morita, K. Kobayashi, Y. Sugawara, Y. Shimizu, T. Kusakabe, Evaluation of algal photosynthesis inhibition activity for dissolved organic matter with the consideration of inorganic and coloring constituents, Chemosphere 224 (2019) 333–342, https://doi.org/10.1016/j.chemosphere.2019.02.106.
[60] C. Yentsch, R.W. Leez, A Study of Photosynthetic Light Reactions, and A New Interpretation of Sun and Shade Phytoplankton, 1966. Undefined.
[61] K.J.M. Mulders, P.P. Lamers, D.E. Martens, R.H. Wijffels, Phototrophic pigment production with microalgae: biological constraints and opportunities, J. Phycol. 50 (2014) 229–242, https://doi.org/10.1111/jpy.12173.
[62] E. Sakshaug, A. Bricaud, Y. Dandonneau, P.G. Falkowski, D.A. Kiefer, L. Legendre, A. Morel, J. Parslow, M. Takahashi, Erratum: parameters of photosynthesis: definitions, theory and interpretation of results (Journal of plankton research (1997) 19 (1637–1670)), J. Plankton Res. 20 (1998) 603, https://doi.org/10.1093/plankt/20.3.603.
[63] O. Osundeko, H. Davies, J.K. Pittman, Oxidative stress-tolerant microalgae strains are highly efficient for biofuel feedstock production onwastewater, Biomass Bioenergy 56 (2013) 284–294.
[64] N.K. Choudhury, R.K. Behera, Photoinhibition of photosynthesis: role of carotenoids in photoprotection of chloroplast constituents, Photosynthetica 39 (2001) 481–488, https://doi.org/10.1023/A:1015647708360.
[65] L. Novoveská, M.E. Ross, M.S. Stanley, R. Pradelles, V. Wasiolek, J.F. Sassi, Microalgal carotenoids: a review of production, current markets, regulations, and future direction, Mar. Drugs. 17 (2019), https://doi.org/10.3390/md17110640.
[66] O. Osundeko, A.P. Dean, H. Davies, J.K. Pittman, Acclimation of microalgae to wastewater environments involves increased oxidative stress tolerance activity, Plant Cell Physiol. 55 (2014) 1848–1857, https://doi.org/10.1093/pcp/pcu113.
[67] N. Friedland, S. Negi, T. Vinogradova-Shah, G. Wu, L. Ma, S. Flynn, T. Kumssa, C. H. Lee, R.T. Sayre, Fine-tuning the photosynthetic light harvesting apparatus for improved photosynthetic efficiency and biomass yield, Sci. Rep. 9 (2019) 1–12, https://doi.org/10.1038/s41598-019-49545-8.
[68] S. Negi, Z. Perrine, N. Friedland, A. Kumar, R. Tokutsu, J. Minagawa, H. Berg, A. N. Barry, G. Govindjee, R. Sayre, Plant J. 103 (2020) 584–603, https://doi.org/10.1111/tpj.14751. †.
[69] A.S. Ayangbenro, O.O. Babalola, A new strategy for heavy metal polluted environments: a review of microbial biosorbents, Int. J. Environ. Res. Public Health 14 (2017) 94, https://doi.org/10.3390/ijerph14010094.
[70] L.L. Eggink, H. Park, J.K. Hoober, The role of chlorophyll b in photosynthesis: hypothesis, BMC Plant Biol. 1 (2001) 1–7, https://doi.org/10.1186/1471-2229-1-2.
[71] G.S. Rocha, C.C. Parrish, E.L.G. Espíndola, Effects of copper on photosynthetic and physiological parameters of a freshwater microalga (Chlorophyceae), Algal Res. 54 (2021), 102223, https://doi.org/10.1016/j.algal.2021.102223.
[72] Y. Zhou, L. Schideman, G. Yu, Y. Zhang, A synergistic combination of algal wastewater treatment and hydrothermal biofuel production maximized by nutrient and carbon recycling, Energy Environ. Sci. 6 (2013) 3765–3779.
[73] R.T. Sayre, S. Negi, G. Govindjee, Light regulation of photosynthetic light harvesting doubles the biomass yield in the green alga chlamydomonas, Photosynthetica 58 (2020) 974–975, https://doi.org/10.32615/ps.2020.049.
[74] X. Liu, S. Duan, A. Li, N. Xu, Z. Cai, Z. Hu, Effects of organic carbon sources on growth, photosynthesis, and respiration of Phaeodactylum tricornutum, J. Appl. Phycol. 21 (2009) 239–246, https://doi.org/10.1007/s10811-008-9355-z.
[75] M. Wan, P. Liu, J. Xia, J.N. Rosenberg, G.A. Oyler, M.J. Betenbaugh, Z. Nie, G. Qiu, The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in Chlorella sorokiniana, Appl. Microbiol. Biotechnol. 91 (2011) 835–844, https://doi.org/10.1007/s00253-011-3399-8.
[76] M. Pham, L. Schideman, J. Scott, N. Rajagopalan, M.J. Plewa, Chemical and biological characterization of wastewater generated from hydrothermal liquefaction of spirulina, Environ. Sci. Technol. (2013) 2131–2138.
[77] F. Monlau, C. Sambusiti, A. Barakat, M. Quéméneur, E. Trably, J.P. Steyer, H. Carrère, Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review, Biotechnol. Adv. 32 (5) (2014) 934–951. https://doi.org/10.1016/J.BIOTECHADV.2014.04.007.
[78] P. Biller, A.B. Ross, S.C. Skill, A. Lea-Langton, B. Balasundaram, C. Hall, R. Riley, C. A. Llewellyn, Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process, Algal Res. (2012) 70–76.
[79] M. Erkelens, A.S. Ball, D.M. Lewis, The application of activated carbon for the treatment and reuse of the aqueous phase derived from the hydrothermal liquefaction of a halophytic Tetraselmis sp, Bioresour. Technol. 182 (2015) 378–382. https://doi.org/10.1016/J.BIORTECH.2015.01.129.
[80] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic cultures of microalgae: metabolism and potential products, Water Res. (2011) 11–36.
[81] S. Wang, X. Shi, B. Palenik, Characterization of Picochlorum sp. Use of wastewater generated from hydrothermal liquefaction as a nitrogen source, Algal Res. (2016) 311–317.