

Research article

Aquaculture wastewater treatment through microalgal. Biomass potential applications on animal feed, agriculture, and energy

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ABSTRACT

The use of microalgae to remediate raw effluent from brown crab aquaculture was evaluated by performing batch mode growth tests using separately the microalgae *Chlorella vulgaris* (Cv), *Scenedesmus obliquus* (Sc), *Isochrysis galbana* (Ig), *Nannocloropsis salina* (Ns), and *Spirulina major* (Sp). Removal efficiencies in batch growth were 100% for total nitrogen and total phosphorus for all microalgae. Chemical oxygen demand (COD) remediations were all above 72%. Biomass productivity varied from 20.9 mg L⁻¹ day⁻¹ (*N. salina*) to 146.4 mg L⁻¹ day⁻¹ (*C. vulgaris*). The two best performing algae were *C. vulgaris* and *S. obliquus* and they were tested in semi-continuous growth, reaching productivities of 879.8 mg L⁻¹ day⁻¹ and 811.7 mg L⁻¹ day⁻¹, respectively. The bioremediation of the effluent was tested with a transfer system consisting of three independent containers and compared with the use of a single container. The single container had the same capacity and received weekly the same volume of effluent as the three containers together. The remediation capacity of the 3 containers was much higher than the single one. The supplementation with NaNO₃ was tested to improve the nutrient removal microalgae' capacity, with positive results. The removal efficiencies were 100% for total nitrogen and total phosphorus and higher than 96% for COD. The obtained *C. vulgaris* and *S. obliquus* biomass were composed of 31 and 35% proteins, 6 and 8% lipids, 39 and 30% carbohydrates, respectively. The composition of these biomass suggest that it can be used as novel and sustainable ingredients in aquaculture feeds. The algal biomass of Cv and Sc were used as biostimulants in the germination of wheat and watercress, and very promising results were attained, with increases in the germination index for Cv and Sc of 175% and 48% in watercress and 84% and 98% in wheat, respectively. The biomasses of Cv and Sc were also subjected to a torrefaction process with 72.5 ± 1.7% char yields. The obtained biochars were tested as biostimulants for germination seeds (wheat and watercress) and as bio-adsorbent of dye solutions.

1. Introduction

Intensive or semi-intensive aquaculture increases the concentration of nutrients in the aqueous medium. This accumulation is due to feed residues and excrements of the aquatic species produced, which stimulates the growth of several microorganisms, some of them pathogenic. In aquaculture, to prevent the development of diseases in animals, antibiotics and other antimicrobial agents are added. These agents of control and prevention of the proliferation of microorganisms may not be completely metabolized or excreted, thus bioaccumulating in the species produced and being transposed to the food chain could constitute a consumers' health risk (Rosa et al., 2020).

The regular discharge of nutrient-rich effluents into adjacent water bodies can also lead to eutrophication phenomena due to the uncontrolled proliferation of algae (micro and macro). This problem is particularly critical when these effluents with high organic and inorganic loads are discharged into aquatic environments with low dispersion rates, such as lakes or estuaries (Fonseca et al., 2021).

Thus the search for alternative and sustainable methods to control the excessive accumulation of nutrients and microorganisms in these growth mediums of aquatic species or in the corresponding effluents has been an area of growing interest (Lin et al., 2020; Paul et al., 2021).

Microalgae are eukaryotic microorganisms with a huge potential to remediate agroindustrial effluents due to their high photosynthetic

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efficiency and adaptability, to the few productive requirements, and metabolic plasticity. In the last decades microalgae have been studied with the purpose of remedying effluents concomitant with the production of biomass suitable for animal feed, soil fertilization and/or energy production. The use of microalgae may result in remediation processes with reduced emissions, energy savings and low costs when compared to conventional processes ((Apandi et al., 2019a; Nitsos et al., 2020).

Bioremediation of aquaculture effluents has been tested with several microalgae. The remediation rates of total nitrogen and total phosphorus from an aquaculture tank of *Penaeus vannamei* using *Chlorella vulgaris*, in continuous mode, were 86.1% and 82.7%, respectively (Gao et al., 2016). Nile tilapia effluent in batch mode using *S. obliquus* removed 88.7% and 100%, respectively and *C. sorokiniana*, 98.2% and 100%, respectively (Ansari et al., 2017). Batch mode *Mugil cephalus* effluent using *Tetraselmis suecica*, *Dunaliella tertiolecta* and *Isochrysis galbana* removed 94.4% and 96.0%, 95.4% and 91.2%, 66.0% and 91.9% for total nitrogen and total phosphorus, respectively (Andreotti et al., 2017). A wet market wastewater mainly from fish and seafood entrails (diluted 50% in deionized water) was remediated with *Scenedesmus* sp. achieving removal rates of 91% and 87% for total P and total N (Apandi et al., 2019b). Nevertheless, several of these works were performed with aquaculture effluent previously treated with ultraviolet light (Andreotti et al., 2017), filtered using 0.45 µm filter papers followed by autoclaving prior to microalgal inoculation (Ansari et al., 2017), settling overnight and filtered (Gao et al., 2016), or filtered through a membrane filter of 0.45 µm (Apandi et al., 2019b).

The algal biomass from aquaculture wastewater treatment can have different applications. The use of algal biomass for aquaculture feed is a common practice. However, there are few studies on the application of algae produced in wastewater as feed for aquaculture species. Li et al. (2019) used biomass of *Tetraselmis* sp. and *Phaeodactylum* sp. from aquaculture wastewater treatment in oysters feed with good results. The application of algal biomass as a biostimulant is understudied, though *Scenedesmus obliquus* biomass obtained from brewery wastewater treatment was tested as a biostimulant in seed germination with increased germination of 40% (Navarro-López et al., 2020). Other examples were done by Deepika and MubarakAli (2020) using *Chorococcum* sp. to promote growth in *Cucumis sativus*, *Solanum lycopersicum*, *Capsicum annuum*, and *Vigna radiata*, with good results. Grzesik et al. (2017) also studied the effect of applying a triple foliar spray of intact cells of *Chlorella* sp. and concluded it improved the growth of willow plants. Agwa et al. (2017) had positive results when using *C. vulgaris* for *Hibiscus esculentus* development and its role in enhancing soil fertility. Likewise, Marks et al. (2017) applied a liquid slurry to soil of live cells of *Chlorella* sp. from wastewater treatment concluding there is an enhancement of soil fertility.

Energy and material applications are additionally ways of using algal biomass. Thermochemical conversion process such as torrefaction can be used in order to produce chars for coal fuel and bio-adsorbent from algal biomass (Gan et al., 2018), to be used in soil amendment applications (Chu et al., 2020) or as biostimulant (Ennis et al., 2017).

The aim of this work was to propose a sustainable methodology for raw aquaculture effluent treatment, without the typical requirement use of filtration, UV sterilization or autoclaving, to decrease nitrogen, phosphorus, and COD concentrations. In the studied case, the aquaculture company receives brown crabs weekly and needs to carry out the treatment of the transport effluent of these animals, before releasing it. This effluent is highly charged with nitrogen and presents a remarkably high COD. The objective of the work was to optimize a semi-continuous remediation approach, that allows the effluent's remedy, considering the volumes received weekly by the company. A system consisting in bioreactor made up of three independent sequential containers were compared to a system using only a single container. In addition, the potential use of the produced microalgal biomass was evaluated theoretically as feed for aquatic species, and experimentally as biostimulant for seed germination, and in torrefaction process to produce char. The

obtained char was also tested as a fertilizer for seed germination and as an adsorbent of wastewater effluents with dyes in adsorption tests with methylene blue.

2. Material and methods

2.1. Raw materials

The aquaculture effluents were collected in March 2017 and October 2018 from Pesca Verde Lda., a seafood nursery company, located at Guincho (38°70'98.6" N, -9°48'45.3" W) – Cascais, Portugal. This effluent was produced by brown crabs (*Cancer pagurus*) that were transported in sea water by refrigerated truck from France to Portugal on a journey that lasts 24 h. The effluent was collected in 20 L plastic containers and stored at 4 °C, to minimize chemical and biological changes. Physico-chemical parameters of the effluent (as received) such as optical density (OD₅₄₀), pH, chemical oxygen demand (COD), biological oxygen demand (BOD₅), total nitrogen, nitrates, nitrites, total phosphorus and total and suspended solids were analyzed using the standard methodology from Standard Methods for the Examination of Water and Wastewater (Rice et al., 2017).

2.2. Microorganisms and culture conditions

Five microalgae species were selected for testing the aquaculture effluent remediation: *Chlorella vulgaris* (Cv), *Scenedesmus obliquus* (Sc), *Isochrysis galbana* (Ig), *Nannochloropsis salina* (Ns) and *Spirulina major*. The microalga *C. vulgaris* (INETI 58, LNEG_UBB, Portugal) (Cv) was isolated by LNEG, in Lisbon, Portugal. All the other microalgae cultures were purchased: *Scenedesmus obliquus* (ACOI 204/07, Coimbra University Algotec, Portugal) from Coimbra University Algotec in Portugal and *Isochrysis galbana* (CCAP 927/1, Scottish Marine Institute), *Nannochloropsis salina* (CCAP 849/2, Scottish Marine Institute) and *Spirulina major* (CCAP 1475/3, Scottish Marine Institute) to the Scottish Marine Institute in Scotland, UK.

Microalgae were inoculated to Erlenmeyers flasks sealed with hydrophobic cotton and agitated by an air flow (air pump Stellar 380 D, 25 L/h) to prevented cell sedimentation. The microalgae grew under artificial lighting (LED fluorescent lamps, 125 µE m⁻² s⁻¹, digital luxmeter ROLINE, model RO 1332A) with cycles of 12 h light/12 h dark. The inoculations were performed using 10 mL of the microalgae inoculum (with biomass concentrations between 4.9 and 5.15 g L⁻¹), nearly 1% of the medium, in order to have an initial optical density (at 540 nm) between 0.2 and 0.4 (Gouveia et al., 2016).

Three sets of experiments were performed: batch growth tests (1st set of experiments), semi-continuous growth tests with periodic transfer of 75 mL of raw effluent (2nd set of experiments) and semi-continuous growth tests with periodic transfer of 150 mL of raw effluent (3rd set of experiments).

The first set of experiments evaluated the batch growth of the five microalgae in the aquaculture effluent and the nutrient removal efficiency was evaluated. Each microalga was cultivated in 650 mL of raw aquaculture effluent, at 26 ± 1 °C, until reaching the stationary phase. The algal biomass was separated from the treated effluent that was characterized for critical parameters (total nitrogen, total phosphorus and COD).

The 2nd and the 3rd were performed at 15 ± 1 °C with the two best performing microalgae, *C. vulgaris* and *S. obliquus*, using a series of three reactors with 1.5 L each (configuration A) and a single reactor with 4.5 L (configuration B). The raw effluent transfer scheme for configurations A and B, in the 2nd and the 3rd trials is presented in Fig. 1.

Detailed raw effluent and culture medium transfer in 2nd and 3rd trials, for configurations A and B are described in Table 1. Hydraulic residence time in the 2nd trial was 20 days and in the 3rd trial was 10 days.

In the 3rd trial reactors 2 and 3 were also supplemented with aqueous

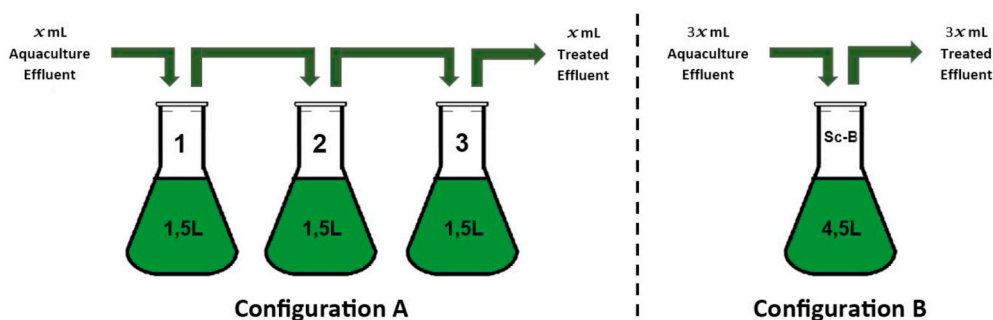


Fig. 1. Scheme of the transfer process for the 2nd and 3rd trials with configuration A and B. Configuration A – series of three reactors with 1.5 L with transfer of x mL every 48 h; Configuration B - single reactor with 4.5 L with weekly transfer of 3x mL; 2nd trial - Effluent transfer volume (x): 75 mL 3rd trial - Effluent transfer volume (x): 150 mL.

Table 1

Transfer of raw effluent, culture medium and treated effluent used in Configuration A (three reactors with 1.5 L each) and B (single reactor with 4.5 L) for the 2nd and 3rd trials.

Set of Experiments	Transferred Volume (mL/48h)				Transferred Volume (mL/week)	
	Configuration A				Configuration B	
	Raw effluent addition to R1	R1 → R2	R2 → R3	Treated effluent removal from R3	Raw effluent addition	Treated effluent removal
2nd trial	75	75	75	75	225	225
3rd trial	150	150	150	150	450	450

NaNO₃ to a final concentration of 20 mgN L⁻¹ in the culture medium, every 48 h (Ansari et al., 2017).

2.3. Culture medium

The batch and semi-continuous growth trials were carried out using raw aquaculture effluent from brown crabs' transportation as a culture medium. Control trials were made for the batch growth experiments using synthetic culture medium adequate for each microalga. *C. vulgaris* grown in Chlorella medium (Vonshak and Maske, 1982), *Scenedesmus* sp. in Bristol medium, *Isochrysis* and *Nannochloropsis* in F/2 medium and *Spirulina* in ASW:BG11 medium (UTEX, 2020).

2.4. Microalgae growth

During the experiments, samples were collected every other day from the bioreactors to measure pH medium (pHTester PH-108) and microalgae concentration by measuring optical density at 540 nm (OD₅₄₀) using a spectrometer (Biocrome S4 Libra). Samples were also taken weekly to analyze total nitrogen, phosphorus and COD in the medium according to the methods described in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2017) and microalgae dry weight was performed by filtration the samples through a Whatman GF/C 47 mm filter, to evaluate algal growth.

For the 1st set of experiments when microalgae growth reached the stationary phase the experiment ended. The 2nd and 3rd experiments ended when COD value achieved the one that allows its discharge. At the end of the 2nd trial, the biomass was not collected, and the 3rd trial was followed in the same reactors after homogenization and partition by the 3 reactors. At the end of the 3rd trial, the culture was harvested by centrifugation at 7000 rpm for 5 min. The supernatant was evaluated for all the same parameters as at the beginning and the biomass was dewatering in a regular oven and dried at 45 °C for 48 h.

The ashes of the raw effluent and the microalgae obtained after

treatment were digested with concentrated HNO₃ and the resulting solutions were diluted to 100 mL and filtered with a Whatman No. 42 filter paper. The type of metals present in the effluents and their quantity were analyzed by atomic absorption spectrometry (ZEEnit 700 - WinAAS, Analytik Jena).

2.5. Microalgae biomass characterization

Before the biomass characterization, the microalgae biomass was ground in a coffee grinder. The total nitrogen present in the biomass was quantified by the modified Kjeldahl method (AOAC, 2006). The total protein was calculated by multiplying the total nitrogen by the conventional conversion factor of 6.25 (Jones, 1931).

Sugar content was determined by quantitative acid hydrolysis using the method of Miranda (Miranda et al., 2012) optimized for microalgae biomass carbohydrates extraction followed by the method of the phenol-sulfuric reagent (Dubois et al., 1956) for the total sugar content determination.

For the lipid content a soxhlet apparatus during 6 h with solvent n-hexane was used using about 1 g of algae biomass. The fatty acid methyl esters were prepared by adding equal parts of sample and methanolic KOH (2N) (Nagappan et al., 2019). The composition of the lipidic fraction in terms of fatty acids was determined by GC-MS analyzer - Gas Chromatography coupled with Mass Spectrometry (Focus GC, Polaris Q - Thermo), equipped with a InertCap 5MS/NP capillary column (30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness). The fatty acids were injected in splitless mode, at 250 °C and the GC temperature was programmed as follows: initial temperature of 40 °C, held for 1 min, increased to 150 °C at a rate of 10 °C/min, held for 15 min, afterwards, temperature was increased 250 °C at 5 °C/min and lastly increased 280 °C at 10 °C/min and held for 10 min. The transfer line and ion source temperatures were 250 °C and 230 °C, respectively. The fatty acids present in the hexane solvent were identified by comparing their mass spectra with those in NIST and WILEY databases and with the retention time and mass spectra of corresponding standards.

For the determination of total carotenoids, about 15 mg of ground biomass, 10 mL of acetone, and 0.7 mg of glass microspheres was used. Vortexing was carried out, followed by an ice bath and centrifugation at 3900 rpm (Hettich EBA 20), according to Gouveia et al. (1996). The total pigments were calculated by scanning spectrometry (Analytik Jena AG - Spekol 1500) between 380 and 700 nm. For the quantification of total pigments, the Beer Lambert equation (Equation (1)) with a value of 2150 was used for the specific optical coefficient and the maximum absorbance wavelength of the samples (Gouveia et al., 1996).

$$\text{Total Pigments (\%)} = \frac{A \times V \times f}{E_{1\%}^{1\text{cm}} \times m} \quad \text{Equation 1}$$

Where A is the absorbance (at the maximum absorption wavelength), V is the total volume of pigment extracted (mL), f is the dilution factor,

$E_{1\text{cm}}^{1\%}$ is the extinction coefficient and m the sample mass (g). It was used $E_{1\text{cm}}^{1\%} = 2150$ based on the average value of the carotenoids mostly found in microalgae, according to Gouveia and Empis (2003).

The moisture and ash content in the algal biomass was determined according to the method described in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2017).

2.6. Microalgal biomass as biostimulant

The germination index of *Triticum aestivum* (wheat) and *Nasturtium officinale* (watercress) was used to evaluate the biostimulant activity of microalgae, according to the methodology described by Zuconi et al. (1981). Biomass solutions of *Chlorella vulgaris* and *Scenedesmus obliquus* with an initial concentration of 5.7 g L^{-1} and 7.8 g L^{-1} , respectively, were diluted to 0.5 and 0.2 g L^{-1} to carry out bioassays to measure the germination index of the seeds in contact with each solution. 3 mL of biomass solutions (Cv-0.2; Cv-0.5; Sc-0.2 and Sc-0.5) or distilled water (control) were pipetted into 90 mm diameter Petri dishes, lined with sterile absorbent paper. 50 seeds were distributed to each box, with 3 replications per treatment and seed specie, which was sealed with parafilm and placed in an incubator at 28°C , without lighting for 5 days (Navarro-López et al., 2020). The germination percentage was recorded on the fifth day determined by Equation (2):

$$\text{Germination index (\%)} = \frac{G \times W}{G_c \times W_c} \times 100 \quad \text{Equation 2}$$

where G is the number of germinated seeds and W is the seedling dry weight (50°C) in the case of microalgae biomass. G_c and W_c are the same parameters but in the control (distilled water).

The mineral composition of the microalgae was evaluated through ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima).

2.7. Torrefaction process for biochar production

For the torrefaction trials, three types of biomasses were used, microalgal biomass and lignocellulosic (Lc) material. *Chlorella vulgaris* (Cv) and *Scenedesmus obliquus* (Sc) were the biomasses obtained from the aquaculture effluent bioremediation (3rd trial). The lignocellulosic material was an end-of-life pine material from the furniture industry which was crushed and dried.

The torrefaction trials were performed on a gas chromatography furnace (Thermo Finnigan Trace GC with FID), under oxygen-limited conditions. For each experiment, a total mass of 10 g was used, combining algal biomass and lignocellulosic biomass in a proportion of 0, 50 and 100% algal biomass (means Sc, Cv, Sc + Lc, Cv + Lc, and Lc). The trials were made in triplicate. The samples were placed in 250 mL glass flasks and heated up to 250°C ; the conditions were kept isothermal for 60 min. At the end, the furnace was cooled to 35°C , with a cooling rate (equal to the heating rate) of $14^\circ\text{C min}^{-1}$. The liquid phase was also collected and the mass yields of the solid and liquid were determined with an analytical balance (Mettler Toledo AB204-S). The gas phase was determined by difference. The triplicates of the obtained biochar were combined, milled, sieved with a $500 \mu\text{m}$ screen (Retsch) and analyzed for proximate and ultimate analysis. Ash content (Ash), volatile matter (VM) and moisture (M) were determined gravimetrically according to the methods described in ASTM 830-87, 897-88 and 949-88, respectively. Fixed carbon (FC) was established by difference, in dry weight. Ultimate analysis (carbon, hydrogen, nitrogen and sulfur contents) was performed using an elemental analyzer (Thermo Finnigan – CE Instruments Model Flash EA 112 CHNS series). Oxygen content was achieved by difference, in AFDW by subtracting from 100 the ash concentrations and the other elements determined.

High heating values (HHV) of the biomass chars were calculated using a correlation established by Parikh et al. (2005) based on

proximate composition data (Equation (3)):

$$\text{HHV (MJ Kg}^{-1}\text{, DW)} = 0.3536 [\text{FC}] + 0.1559 [\text{VM}] - 0.0078 [\text{Ash}] \quad \text{Equation 3}$$

Where FC, VM, and Ash are fixed carbon, volatile matter and ash content of the char, respectively, expressed in wt. %, DW.

2.8. Biochar as fertilizer

To determine the possible fertilizing effect of microalgae char, germination tests were carried out identical to those carried out for algal biomass solutions. Three chars were used for the germination test: *Scenedesmus* (Sc), *Scenedesmus* + Lignocellulosic Material (Sc + Lc) and Lignocellulosic Material (Lc), with two different concentrations (0.2 g L^{-1} and 0.5 g L^{-1}). The same plant seeds (*Triticum aestivum* and *Nasturtium officinale*) were used and the germination index determined by Equation (2).

The mineral composition of the biochars were evaluated through ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima).

2.9. Adsorption experiment

Methylene blue was used as the model dye for the char adsorption experiments, as it is a quite common contaminant of industrial wastewaters.

A quick adsorption test was developed to evaluate the adsorption ability of biomass biochar samples, (previously milled and sieved to a $500 \mu\text{m}$ diameter) using the cationic dye methylene blue (MB). A 5 mL of a MB aqueous solution (100 mg L^{-1}) and a mass sample of 25 mg were added to a test tube. The tube was shaken for 3 s (Heidolph top shaker) and then centrifuged at 4000 rpm for 5 min (Hettich EBA 20). The supernatant was transferred to another tube and the concentration of dye was determined by UV-vis spectrophotometry (Biochrom Libra S4) at 664 nm. The same procedure was carried out but leaving the char mass in contact with the MB aqueous solution for 48 h. The removal efficiency for both procedures was determined using Equation (4):

$$\text{Removal efficiency (\%)} = \left(\frac{C_i - C_f}{C_i} \right) \times 100 \quad \text{Equation 4}$$

where C_i and C_f are the initial and final concentrations (mg L^{-1}) of dye in the aqueous solution (Correia et al., 2017).

2.10. Statistical analyses

Experiments were performed in duplicate for microalgae growth and effluent treatment and in triplicate for all the biomass analysis, data were reported as mean \pm standard deviation (SD). The parameters such as removal efficiencies, productivities and germination index were compared using analysis of variance with one-way ANOVA, by IBM SPSS statistical 23 software. The mean values were compared using the Tukey HSD test and correlation was considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Effluent characterization and microalgae growth

The composition of aquaculture effluent used in the trials is presented in Table 2. For the 1st trial aquaculture effluent 1 was used and for the 2nd and 3rd ones, aquaculture effluent 2 was used.

Experiments demonstrated that microalgae could grow in these aquaculture effluents. The 1st trial was in batch mode, lasted for 33 days and ended when all nitrogen and phosphorus available were consumed and a decline in the optical density of the cultures was observed. The

Table 2Composition of aquaculture effluents (mean \pm SD, n = 3).

	Total nitrogen (mg N L ⁻¹)	Total phosphorus (mg P L ⁻¹)	COD (g O ₂ L ⁻¹)	BOD ₅ (g O ₂ L ⁻¹)	Total solids content (g L ⁻¹)	Total ashes content (g L ⁻¹)	Optical density (540 nm)
Aquaculture effluent 1	168.3 \pm 1.3	32.9 \pm 1.0	1.95 \pm 0.07	0.98 \pm 0.18	46.0 \pm 0.7	34.1 \pm 0.2	0.212 \pm 0.2
Aquaculture effluent 2	737.8 \pm 4.7	22.1 \pm 1.0	5.20 \pm 0.14	3.05 \pm 0.21	40.9 \pm 0.7	33.7 \pm 0.4	1.871 \pm 4.2

objective of this experiment was to determine which two algae species had the best performance in terms of remediation and biomass production. Microalgae *C. vulgaris* and *S. obliquus* managed to achieve the remediation after 22 days, in contrast to the other microalgae that only reached remediation after 33 days. Biomass concentration reached after 22 days was significantly higher for the microalga *Chlorella vulgaris* grown in aquaculture effluent (3.22 ± 0.04 g L⁻¹) when compared with the one grown in the control medium (1.36 ± 0.39 g L⁻¹) (Fig. 2). The same situation occurred for *Scenedesmus obliquus*, 2.20 ± 0.21 g L⁻¹ in aquaculture effluent and 1.60 ± 0.03 g L⁻¹ in synthetic medium.

I. galbana had similar growth for control and aquaculture effluent: 1.09 ± 0.02 g L⁻¹ and 1.03 ± 0.03 g L⁻¹, respectively after 33 days. A lower growth was found in aquaculture effluent when using *N. salina* than in control medium: 0.69 ± 0.06 g L⁻¹ and 1.47 ± 0.13 g L⁻¹, respectively.

The 2nd and 3rd trials were in semi-continued mode and lasted 36 days and 34 days, respectively. The two microalgae selected were *Chlorella vulgaris* and *Scenedesmus obliquus* because they were the ones who, in addition to being able to remedy the initial effluent in less time, achieved higher biomass concentration. *S. obliquus* was also chosen for the test with the configuration B (reactor with triple effluent volume: Sc-B) because in preliminary tests it has demonstrated a better ability to remedy these aquaculture effluents, especially COD.

The microalga *S. obliquus* stood out during the 2nd trial, but *C. vulgaris* performed better in the 3rd trial, as shown by Fig. 3, which explains the evolution of productivity in the Cv3-A, Sc3-A and Sc-B reactors, over the weeks for the two trials.

The yield on the last day of 2nd and 3rd trials for the various reactors is shown in Fig. 4, where are an evident trend towards higher productivity in the final reactors, Cv3-A and Sc3-A. In the 2nd trial *S. obliquus* was the microalga with the best performance with a productivity of 427.7 mg L⁻¹ day⁻¹. Although, in the 3rd trial *C. vulgaris* had a productivity of 879.8 mg L⁻¹ day⁻¹ significantly higher than *S. obliquus* in the two configurations: A - 3 reactors of 1500 mL with 811.7 mg L⁻¹ day⁻¹ (Sc3-A); B - one reactor of 4.5 L (Sc-B) with 731.0 mg L⁻¹ day⁻¹.

Gao et al. (2016) grew *C. vulgaris* in shrimp aquaculture wastewater with lower concentration of nutrients (6.8 mg L⁻¹ for total nitrogen and 0.5 mg L⁻¹ for total phosphorus) in a membrane photobioreactor and attained a biomass yield of 7.3 and 42.6 mg L⁻¹ day⁻¹ for batch mode and continuous mode, respectively, values significantly lower of those

obtained in the present study (146.4 mg L⁻¹ day⁻¹ in batch mode and 540.6 mg L⁻¹ day⁻¹ in continuous mode for Cv). In another study, with aquaculture wastewater containing lower nutrients concentrations (60 mg L⁻¹ for TN, 6.8 mg L⁻¹ for TP and 112 mgO₂ L⁻¹ for COD), the microalga *Chlorella* sp. reached biomass productivity of 243 mg L⁻¹ day⁻¹ (Kuo et al., 2016). Apandi et al. (2019b) grew *Scenedesmus* sp. in a wet market wastewater mostly from fish and seafood entrails (with 480 mg L⁻¹ for TN, 87 mg L⁻¹ for TP and 1754 mgO₂ L⁻¹ for COD) achieving a maximum productivity of 98.54 mg L⁻¹ day⁻¹. Higher productivity of microalgae was expected in the present study since the effluent used was richer in nutrients.

3.2. Bioremediation evaluation

The remediation rate in the aquaculture effluent for total nitrogen and total phosphorus was 100% for all selected microalgae (Table 3). Regarding COD, the remediation rate was higher than 72% for all algae and higher than 52% for BOD₅. Therefore, there was no microalgae that stood out significantly from the rest.

Concerning the remediation efficiency of the two microalgae selected for 2nd and 3rd trials, it became evident that in the 2nd trial the available nitrogen and phosphorus were also consumed, but the COD in the last reactor did not reach the concentration needed to be released: lower than 150 mg O₂ L⁻¹ (Portuguese Ministry of the Environment, 1998). In the 3rd trial, it was decided to add a nitrogen supplementation of 20 mg N L⁻¹ every other day in the 2nd and 3rd reactors so that the culture could lower COD levels. In these reactors, the nitrogen levels were too low to allow the culture to grow, a situation that has already been verified by Bona et al. (2014) and Markou et al. (2016).

The remediation rate in the aquaculture effluent for total nitrogen and total phosphorus was also 100% for the two trials and two microalgae (Table 4). Regarding the COD, the remediation rate was higher than 78% in the 2nd trial and it was verified that with the nitrogen supplementation in the 3rd trial, the microalgae were able to remedy more than 90% (Fig. 5). The BOD₅ was remediated in the 2nd trial between 91 and 96%, but when nitrogen supplementation was added, the results approached the total removal both for Cv and Sc.

Comparable studies with aquaculture effluents from Nile Tilapia obtained remediations of 99.8% for nitrate and 99.7% for phosphate for the microalga *Chlorella vulgaris* (Tejido-Núñez et al., 2019). The

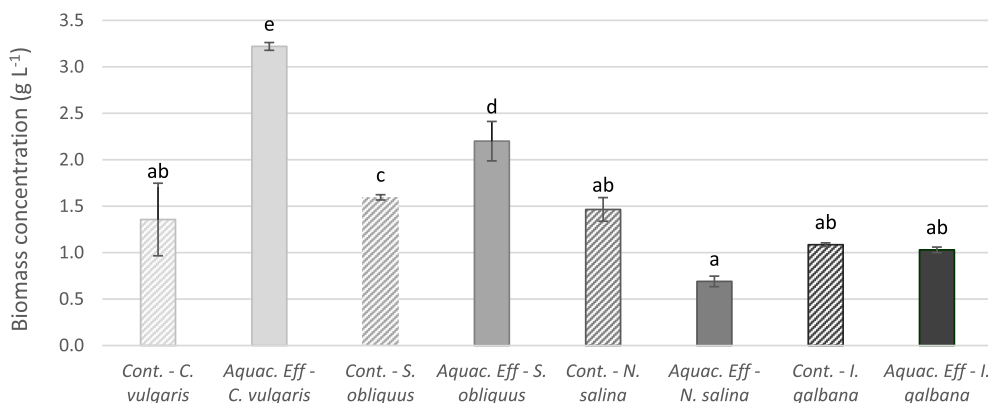


Fig. 2. Biomass concentration of the microalgae in aquaculture effluent (Aquac. Eff.) and control (Cont.) in the 1st trial (mean \pm SD, n = 3) after 22 days (for Aquac. Eff. - *C. vulgaris* and Aquac. Eff. - *S. obliquus*) and after 33 days for the remaining reactors of trial.

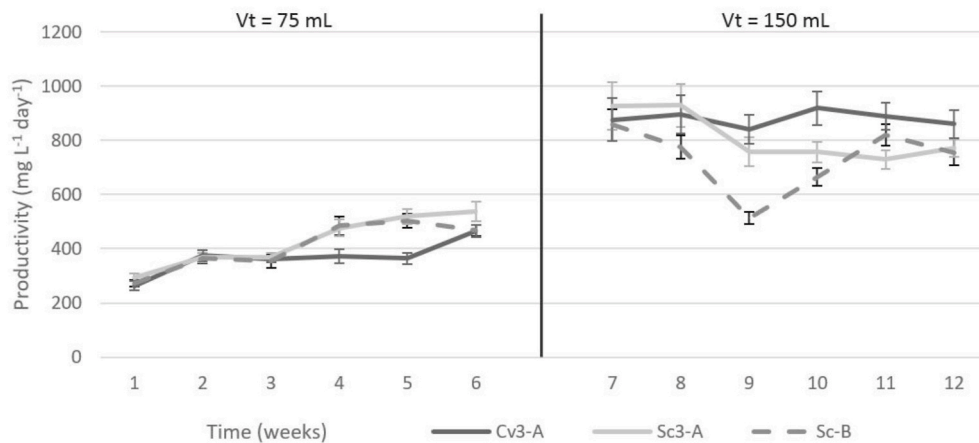


Fig. 3. Progress of biomass productivity in aquaculture effluent for the 2nd and 3rd trials (mean \pm SD, n = 3). Vt – transference volume; Cv – *Chlorella vulgaris*; Sc – *Scenedesmus obliquus*; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L).

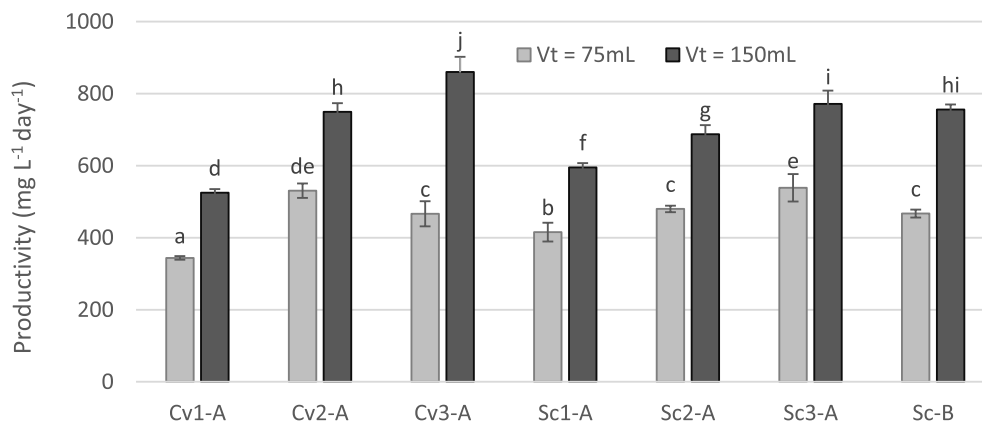


Fig. 4. Biomass productivity in the 2nd and 3rd trials for all reactors (Vt – transference volume; Cv – *Chlorella vulgaris*; Sc – *Scenedesmus obliquus*; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L)).

Table 3

Remediation rates of aquaculture effluent by microalgae in the 1st trial (mean \pm SD, n = 3).

		Total Nitrogen (%)	Total Phosphorus (%)	COD (%)	BOD ₅ %
1 st trial	<i>C. vulgaris</i>	100 \pm 0.0	100 \pm 0.0	86.6 \pm 3.1 ^{bc}	72.2 \pm 5.2 ^{ab}
	<i>S. obliquus</i>	100 \pm 0.0	100 \pm 0.0	90.6 \pm 1.6 ^{bc}	52.0 \pm 3.1 ^a
	<i>S. major</i>	100 \pm 0.0	100 \pm 0.0	94.0 \pm 4.0 ^c	68.0 \pm 2.4 ^{ab}
	<i>N. salina</i>	100 \pm 0.0	100 \pm 0.0	71.8 \pm 4.7 ^a	77.7 \pm 4.9 ^b
	<i>I. galbana</i>	100 \pm 0.0	100 \pm 0.0	83.9 \pm 0.1 ^b	68.2 \pm 1.0 ^{ab}

Table 4

Remediation rates for aquaculture effluent for *C. vulgaris* and *S. obliquus* in the 2nd and 3rd trials (mean \pm SD, n = 3).

		Total Nitrogen (%)	Total Phosphorus (%)	COD (%)	BOD ₅ %
2 nd trial	<i>C. vulgaris</i>	100 \pm 0.0	100 \pm 0.0 ^c	91.5 \pm 1.1 ^b	95.3 \pm 0.3 ^b
	<i>S. obliquus</i>	100 \pm 0.0	100 \pm 0.0 ^c	90.3 \pm 0.2 ^{ab}	96.3 \pm 0.4 ^c
	<i>S. obliquus</i> (Sc-B)	100 \pm 0.0	100 \pm 0.0 ^c	88.4 \pm 0.3 ^a	91.3 \pm 0.1 ^a
3 rd trial	<i>C. vulgaris</i>	100 \pm 0.0	96.5 \pm 0.1 ^a	96.2 \pm 0.0 ^c	99.7 \pm 0.0 ^d
	<i>S. obliquus</i>	100 \pm 0.0	98.6 \pm 0.0 ^b	97.7 \pm 0.6 ^c	99.7 \pm 0.0 ^d
	<i>S. obliquus</i> (Sc-B)	100 \pm 0.0	99.3 \pm 0.1 ^c	90.2 \pm 0.3 ^{ab}	99.4 \pm 0.0 ^d

microalgae *S. obliquus* and *C. sorokiniana* were able to remediate 88.7% and 100% for total nitrogen and 98.2% and 100% for total phosphorus respectively, concerning COD, *S. obliquus* and *C. sorokiniana* removed respectively 42 and 69% (Ansari et al., 2017). In another study with

aquaculture wastewater from *Mugil cephalus*, the microalga *Isochrysis galbana* was able to reduce 66% of the total nitrogen and 80% of the total phosphorus in the effluent (Andreotti et al., 2017). The best COD remediation in the present study occurred in the case of microalgae that

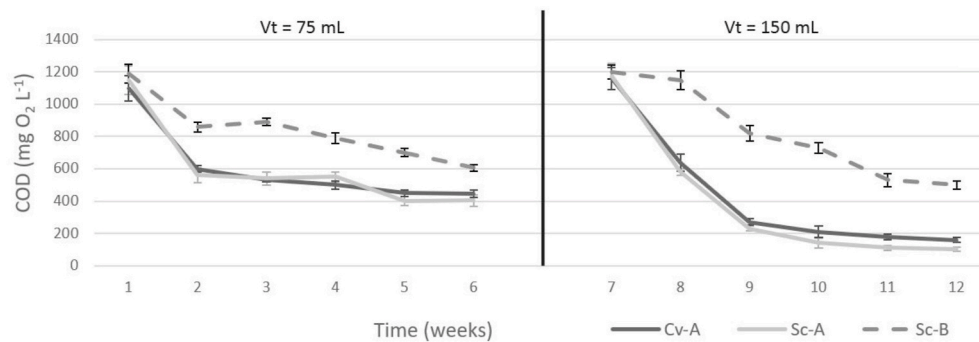


Fig. 5. Progress of COD in aquaculture effluent for the 2nd and 3rd trials. Between 2nd and 3rd trials, 1 week passed, for the culture to rebalance. (Vt – transference volume; Cv – *Chlorella vulgaris*; Sc – *Scenedesmus obliquus*; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L)).

did not immediately consume all the existing nitrogen.

3.3. Characterization of microalgal biomass

The algal biomass produced was evaluated by quantifying the protein, carbohydrate, lipid, and ash contents at the end of the 1st and 3rd experiments (Fig. 6). In the 1st, the higher protein content was achieved in synthetic medium ($37.5 \pm 0.4\%$ for Cv and $21.0 \pm 0.2\%$ for Sc). The algae grown in the effluent had $11.8 \pm 3.5\%$ (1st trial) and $31.4 \pm 3.4\%$ (3rd trial) of protein content. In terms of carbohydrates, the Sc grown in control medium had $48.0 \pm 3.5\%$ and in the effluent $23.6 \pm 0.8\%$, while the other algae grown in the effluent had $35.4 \pm 7.4\%$ (1st trial) and $34.3 \pm 4.7\%$ (3rd trial) of carbohydrates. Related to lipids the microalga that stood out was Ns grown in control medium with 32.6% while Cv and Sc grown in the effluent present 18.6% and 12.8% , respectively. The ash content in the microalgae is significantly higher for Sc and Ns grown in the effluent, although a high value was found for *I. galbana* and *N. salina* grown in synthetic medium because F/2 is essentially salt water. A study with *Scenedesmus* sp. grew in wet market wastewater

found a composition of algal biomass with higher protein content (48.7%), even compared to the control, and a lipid content also superior (27.1%) in relation to that obtained in the present study. The major fatty acid compound was oleic (C18:1) with 34.6% (Apandi et al., 2019b), as in the current study.

In the 3rd trial, the composition of the microalgae was very homogeneous among the three of them (Cv-A, Sc-A and Sc-B), although the microalga with the highest protein content was Sc-A with $35.2 \pm 2.3\%$ followed by Cv-A ($30.6 \pm 2.1\%$). Cv-A produced more carbohydrates ($39.4 \pm 0.3\%$) while Sc-A produced more lipids ($7.9 \pm 0.5\%$). The ash content in the microalgae was, again, significantly higher because of the dissolved salts in the aquaculture effluents of sea water, $34.2 \pm 0.5\%$ for Sc-B followed by Sc-A with $26.8 \pm 0.7\%$ and Cv-A ($24.2 \pm 0.4\%$).

Concerning the fatty acid composition of the microalgae biomass, in this study there was a predominance mixture of unsaturated fatty acids including palmitoleic (C16:1), (C16:2), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and conjugated linoleic acid (CLA), as well as saturated fatty acids including palmitic (C16:0), stearic (C18:0), behenic (C22:0) and lignoceric (C24:0). Table 4 presents the variations of fatty acids in

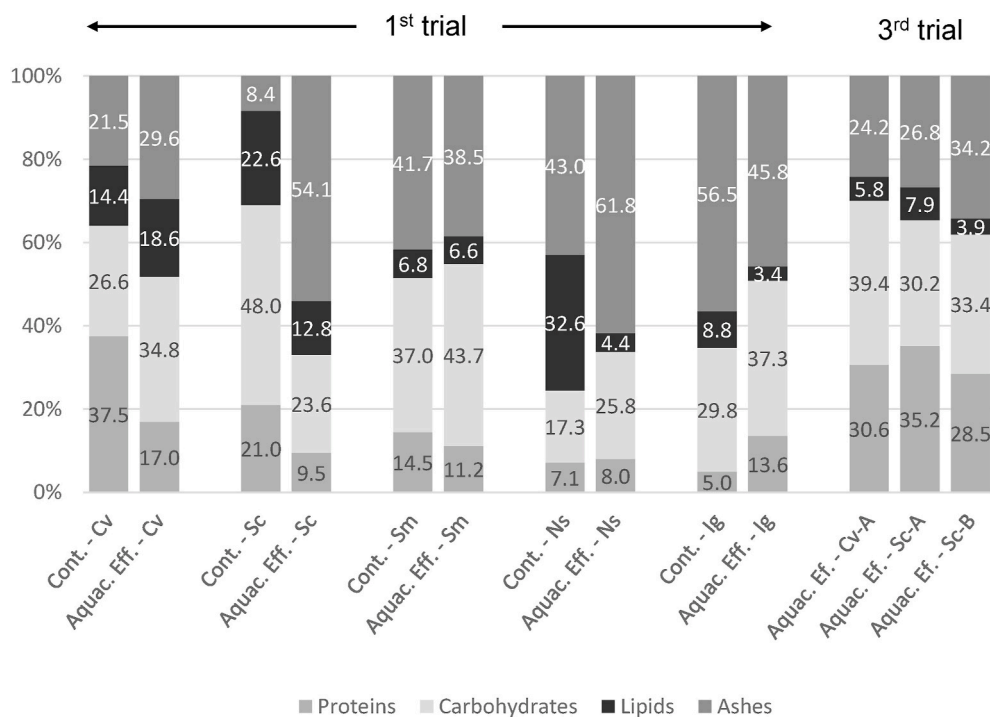


Fig. 6. Biomass composition (% DW) for the five microalgae in the 1st trial, and for the two microalgae in two configurations (A and B) in the 3rd trial (mean, n = 3). Cv – *Chlorella vulgaris*; Sc – *Scenedesmus obliquus*; Sm – *Spirulina major*; Ns – *Nannocloropsis salina*; Ig – *Isochrysis galbana*; Cont. – control; Aquac. Eff. – Aquaculture effluent; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L).

the microalgae grown in aquaculture effluent used. The fatty acid with the greatest expression in all microalgae is oleic acid followed by palmitic acid, except for *Nannochloropsis salina*, which is palmitic followed by palmitoleic acid. For growth in semi-continuous mode (3rd trial), a larger number of peaks was detected in the chromatograms (some unsaturated fatty acids), but with less concentration of each one, as C10:0, C12:0, C15:1, C16:4, C16:3, C18:4, C18:3 (Table 5). Zhang and collaborators produced *Chlorella sorokiniana* in mariculture wastewater (filtering through 0.22 µm membranes) and obtained biomass composition with 43% proteins and 34–43% lipids, composed predominantly of C16:0 and C18:2 fatty acids (Zhang et al., 2019). The quality of the fatty acids in the studied algal biomass showed an interesting application in animal feed. It is known that the increase in polyunsaturated fatty acids in fish feed is beneficial for their growth, development, as well as intrinsic quality of the product (Han et al., 2019).

Regarding the total pigments, the microalga *Chlorella vulgaris* (Cv-A) was the one with the highest amount, $0.84 \pm 0.04\%$ (in DW), followed by Sc-A with $0.66 \pm 0.01\%$ and finally Sc-B with $0.56 \pm 0.02\%$. Hodalifa (2010) detected a much lower pigment content, around 0.17% in the *Scenedesmus obliquus* microalgae grown in diluted olive mill wastewater. Another author reached a lower total pigment content of 0.41% for *C. vulgaris* grown in treated wastewater (Fernández-Linares et al., 2017). Nevertheless, according to Milledge, the average concentration of carotenoids in microalgae varies between 0.1 and 2% (Milledge, 2011), reaching, for some specific microalgae species, 8–14% and if the production process was optimized accordingly (Kalra et al., 2020).

For feeding applications of aquatic animals, the high protein content is crucial to have high economic value. The aquatic animals need about 40% protein in their feed to have a normal and balanced growth (Zhang

et al., 2019). However, other researchers focus their attention to the amount of PUPA's, antioxidants and immunosuppressants (Ayyat et al., 2018; Dotta et al., 2018; Lazo et al., 2020). In other studies, with *Chlorella vulgaris* in aquaculture effluents, biomasses were obtained with similar protein levels, about 44–46%, compared to those obtained in the present study of 40% and 48% (AFDW) for Cv-A and Sc-A, respectively. Though the levels of carbohydrates were lower, around 18% (Daneshvar et al., 2018), while in the present case they were 52 and 41% for Cv-A and Sc-A, respectively. Regarding the amount of lipids and profile, in general the microalgae have a balanced fatty acid composition for animal feed, as they have a high content of monounsaturated and polyunsaturated fatty acids (Zhang et al., 2019). It is noted that there is a greater incidence of unsaturated fatty acids in the biomass of microalgae in semi-continuous growth, compared to those in batch growth, especially for *S. obliquus*. Daneshvar et al. (2018) e Kuo et al. (2016) obtained higher amounts of lipids in the microalgae obtained in aquaculture effluents (8–23%), still with an identical proportion between saturated and unsaturated fatty acids compared to the present study.

The analysis of the mineral composition revealed the presence of sodium, magnesium, calcium, potassium, and iron in similar proportions to those detected for saltwater and for raw aquaculture effluent. Reduced amounts of zinc ($0.40 \pm 0.02 \text{ mg L}^{-1}$), cadmium ($0.07 \pm 0.0 \text{ mg L}^{-1}$) and aluminum ($1.18 \pm 0.1 \text{ mg L}^{-1}$) were detected in the effluents treated by the microalgae, yet smaller than those of the original effluents, $0.5 \pm 0.0 \text{ mg L}^{-1}$, $0.10 \pm 0.0 \text{ mg L}^{-1}$ and $1.67 \pm 0.05 \text{ mg L}^{-1}$, for zinc, cadmium, and aluminum, respectively. These leads to assume a certain absorption of these elements by the microalgae. However, very small amounts of these metals are involved. No traces of lead, chromium, copper, and manganese were detected. Given these data, the use

Table 5

Fatty acids in the microalgae grown in the aquaculture effluent for 1st and 3rd trial (Cv - *C. vulgaris*, Sc - *S. obliquus*, Sm - *S. major*, Ns - *N. salina*, Ig - *I. galbana*, A - configuration A (three reactors with 1.5 L each), B - configuration B (single reactor with 4.5 L)).

	1 st trial					3 rd trial		
	Cv	Sc	Sm	Ns	Ig	Cv-A	Sc-A	Sc-B
C10:0	n.d.	n.d.	n.d.	n.d.	n.d.	0.16	n.d.	n.d.
C12:0	n.d.	n.d.	n.d.	n.d.	n.d.	0.04	n.d.	n.d.
C14:0	0.35	0.57	0.48	2.60	0.57	0.73	0.85	0.86
C15:1	n.d.	n.d.	n.d.	n.d.	n.d.	0.09	n.d.	n.d.
C15:0	0.11	0.18	0.18	0.56	n.d.	0.31	0.31	0.51
C16:4	n.d.	n.d.	n.d.	n.d.	n.d.	2.53	n.d.	n.d.
C16:3	n.d.	n.d.	n.d.	n.d.	n.d.	1.20	2.19	4.34
C16:2	1.36	n.d.	4.69	n.d.	0.67	2.22	1.85	3.47
C16:1	5.92	1.86	n.d.	29.07	7.00	5.18	9.05	13.33
C16:0	28.26	32.16	27.14	46.36	30.84	16.01	18.08	11.99
C17:1	n.d.	n.d.	n.d.	n.d.	n.d.	1.02	n.d.	n.d.
C17:0	0.29	2.22	1.19	0.66	n.d.	0.58	0.41	0.36
C18:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17
C18:3	n.d.	n.d.	n.d.	n.d.	n.d.	0.53	1.18	1.44
C18:2	2.56	n.d.	n.d.	1.05	2.89	9.21	7.88	6.42
C18:1	51.36	45.07	53.52	15.47	51.83	39.38	46.49	37.46
(CLA)	n.d.	n.d.	n.d.	n.d.	2.95	2.92	4.74	14.26
C18:0	8.20	15.77	9.55	3.30	3.26	4.48	2.76	1.88
C20:2	n.d.	n.d.	n.d.	n.d.	n.d.	0.22	0.49	0.39
C20:1	n.d.	n.d.	n.d.	n.d.	n.d.	2.52	1.54	0.41
C20:0	0.41	0.61	0.95	0.29	n.d.	0.93	0.33	0.33
C22:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.34	n.d.
C22:1	n.d.	n.d.	n.d.	n.d.	n.d.	1.42	0.23	n.d.
C22:0	0.24	0.36	0.33	0.08	n.d.	1.09	0.61	0.47
C23:0	0.21	0.30	0.25	n.d.	n.d.	n.d.	n.d.	n.d.
C24:0	0.31	0.61	0.59	0.16	n.d.	0.35	0.67	0.71
C25:0	0.10	0.19	0.27	n.d.	n.d.	n.d.	n.d.	n.d.
C26:2	n.d.	n.d.	n.d.	n.d.	n.d.	2.88	n.d.	n.d.
C26:1	n.d.	n.d.	n.d.	n.d.	n.d.	2.68	n.d.	n.d.
C26:0	0.08	0.10	0.15	0.07	n.d.	1.31	n.d.	0.24
C28:0	0.24	n.d.	0.72	0.33	n.d.	n.d.	n.d.	0.99
Saturated	38.49	52.57	41.27	54.42	34.67	26.00	24.01	18.33
Unsaturated	61.20	46.93	58.21	45.58	65.33	74.00	75.99	81.67

n.d. – not detected.

of microalgal biomass would not be limited by the presence of heavy metals.

3.4. Application of microalgal biomass as biostimulant

Phytohormones are known to influence plant growth and development. These phytohormones can be found in microalgae extracts and include gibberellins, cytokinins, auxins, ethylene and abscisic acid (Morais Junior et al., 2020). To assess the microalgae's biostimulation capacity in seed germination, a complete chemical analysis of its composition could be performed, including the content of amino-acids and the profile of phytohormones. Nevertheless, this process is complex and time consuming, so alternatively, germination tests could be conducted in seeds watering with microalgae cultures, to assess its direct effect on germination and growth.

To evaluate the potential biostimulant effect, it was determined the germination index (GI) of the control, with distilled water (100%), and the ones obtained when used microalgae (if higher than 100% it was considering the presence of biostimulant activity).

Fig. 7 shows the results of the tested microalgae cultures on the germination of the two species of seeds (wheat and watercress).

All microalgae had a positive effect on seed germination, except *Scenedesmus obliquus* with a concentration of 0.5 g L^{-1} (Sc-A - 0.5 g L^{-1}) for watercress seeds with $72.0 \pm 3.4\%$, probably a toxicity effect due to the high concentration. The microalga *S. obliquus* has a higher amount of proteins and lipids in its composition, a factor that can lead to an inhibitory effect when in higher concentrations (Puglisi et al., 2020). This situation is no longer registered when using the 0.2 g L^{-1} concentration. This effect of toxicity with reduced germination index had already been noticed for concentrations below 1 g L^{-1} by Navarro-López et al. (2020). Although the number of germinated seeds is lower, the average weight of each seedling is greater than that of the control. Despite the few published works on the effect of algae on seed germination, it is known that bioactive compounds are necessary in very small amounts (Chhaya et al., 2021). The highest GI for watercress was reached for the extract of Cv-A - 0.5 g L^{-1} with $275.2 \pm 10.4\%$, meaning a huge increase of 175% when compared with the control (distilled water). The highest GI for wheat was achieved for the extract of Sc-A - 0.2 g L^{-1} with $197.7 \pm 1.3\%$, which corresponds to almost 100% more than the control. The results demonstrated the potential of microalgae resulting from the treatment of aquaculture effluent to be used as stimulants to a more sustainable agriculture practices. The biomass of both microalgae had some important macro and micronutrients from the point of view of plant nutrition, namely potassium, phosphorus, calcium, magnesium, and sodium, although it also as aluminum (Table 6).

Promising results had already been achieved by Navarro-López et al. (2020) with the microalga *S. obliquus* produced in brewery effluent.

However the results attained were 40% higher than the control. The use of microalgae as biostimulants is a way to replace the use of synthetic fertilizers, contributing to a more efficient and sustainable use of resources.

3.5. Torrefaction process as a stabilization process for the algal biomass

The torrefaction process produced similar char yield regardless of its feedstock biomass, which varied between 70% and 74%. The remaining products (condensate and gas) present some variation depending on the type of origin biomass, but the most significant differences were in terms of produced gas but with a non-regular variation (Fig. 8).

Chars obtained from the co-torrefaction of sewage sludge and lignocellulosic material have similar properties to char obtained in present study. This is due to the characteristics of the feedstocks, which are similar, with high levels of ash and water (Barskov et al., 2019). In these cases, the incorporation of lignocellulosic material resulted in an increase in the content of volatile matter and fixed carbon and a reduction in ash content. The HHV of lignocellulosic material increases significantly with the torrefaction process. Therefore, its addition to other materials, namely sewage sludge or microalgae, leads to an increase in the HHV of the mixture (Li et al., 2020; Zheng et al., 2020).

Table 7 presents the proximate and elemental analysis, and the high heating value (HHV) of the obtained biochars.

The torrefied lignocellulosic material produced a char with higher heating value than microalgae chars or mixture (microalgae + lignocellulosic material) chars because it has less ash, more volatile matter and a higher fixed carbon. The microalgae grown in aquaculture effluents have higher ash content due the salt water but even freshwater microalgae have more ash than lignocellulosic material (Barskov et al., 2019; Fakayode et al., 2020).

The process of torrefaction reduced the O/C and H/C ratios of the raw materials (0.69 and 1.90 for algae and 0.64 and 1.49 for lignocellulosic material, respectively), producing a char with an elemental composition better than a peat and close to lignite, indicating the upgrading process in the obtained biochars. The lower HHV obtained from microalgae chars compared to lignocellulosic material can be attributed to the lower carbon content and higher ash content in algal biomass (Yu et al., 2017a).

The process of torrefaction increases the hydrophobicity of the torrefied material, the energy density and reduces grinding energy requirement of biomass (Cahyanti et al., 2020), inducing an upgrading process in the obtained biochar. The production of char from microalgae has the advantages of greater stability and density of the final material, which also converts into lower transportation costs (Fakayode et al., 2020).

Since the calorific value of the obtained chars is relatively low, their

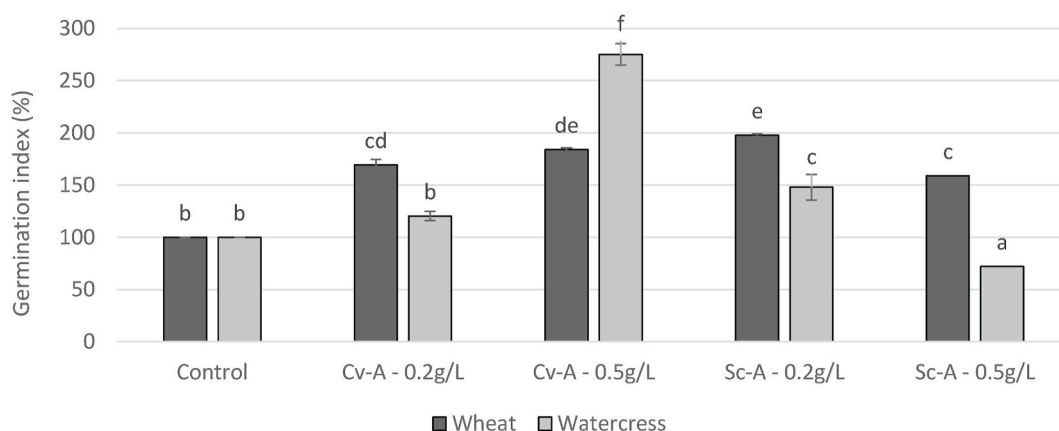


Fig. 7. Germination Index (% GI) for the control and the two microalgae cultures (Cv – *Chlorella vulgaris* and Sc – *Scenedesmus obliquus*) in two concentrations (0.2 and 0.5 g L^{-1}) as potential biostimulant activity (mean \pm SD, n = 3). A – configuration A (three reactors with 1.5 L each).

Table 6

Chemical characteristics of microalgae biomass used in biostimulant germination tests, presented in g/Kg.

	Al	B	Ba	Ca	Cu	Fe	K	Mg	Mn	Na	P	Si	Sr	Zn
<i>C. vulgaris</i>	1.64	0.04	0.10	13.97	0.04	0.25	4.24	7.26	0.01	5.27	1.13	0.16	0.12	0.13
<i>S. obliquus</i>	0.31	0.04	0.01	11.61	0.09	0.40	4.90	6.53	0.01	5.32	0.00	0.15	0.10	0.09

Note: The presence of Ag, As, Bi, Cd, Co, Hg, Mo, Sb, Sn was not detected in the microalgae biomass. The elements Cr, Li, Pb, Se, Ti, Tl, W and Zr were only detected vestigially.

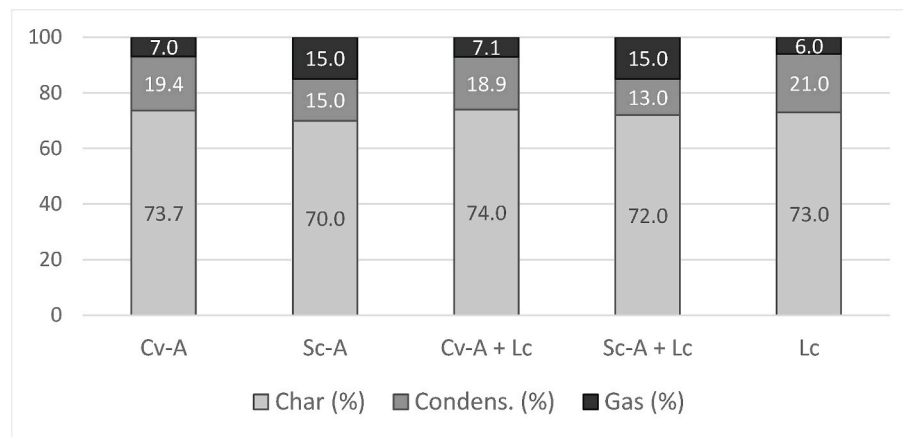


Fig. 8. Torrefied process with the yields on char, condensate and gas (Sc-A: 10g of *Scenedesmus obliquus*; Cv-A: 10g of *Chlorella vulgaris*; Sc-A + Lc: 5 g of *S. obliquus* + 5 g of lignocellulosic material; Cv-A + Lc: 5 g of *C. vulgaris* + 5 g of lignocellulosic material; Lc: 10g of lignocellulosic material).

Table 7Proximate and elemental analyses of the biochars on a dry basis. (Sc-A: 10g of *Scenedesmus obliquus*; Cv-A: 10g of *Chlorella vulgaris*; Sc-A + Lc: 5 g of *S. obliquus* + 5 g of lignocellulosic material; Cv-A + Lc: 5 g of *C. vulgaris* + 5 g of lignocellulosic material; Lc: 10g of lignocellulosic material).

	Ash	Volatile Matter	Fix Carbon	N	C	H	S	O	O/C ratio	H/C ratio	HHV
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)			(MJ/Kg)
Cv-A	33.5 ± 0.8	56.8 ± 1.1	9.6 ± 1.4	6.0 ± 0.1	39.3 ± 0.5	4.2 ± 0.0	1.1 ± 0.0	15.8 ± 0.1	0.30	1.28	12.0
Sc-A	38.4 ± 0.2	54.7 ± 0.3	6.9 ± 0.2	6.0 ± 0.0	38.1 ± 0.0	4.1 ± 0.0	1.2 ± 0.0	12.3 ± 0.0	0.24	1.30	10.7
Cv-A + Lc	17.3 ± 0.8	66.1 ± 0.6	16.6 ± 0.3	3.4 ± 0.3	46.3 ± 0.1	5.2 ± 0.1	0.6 ± 0.0	27.2 ± 0.1	0.44	1.34	16.0
Sc-A + Lc	24.6 ± 0.9	67.8 ± 1.5	7.6 ± 0.6	4.0 ± 0.0	43.9 ± 0.2	4.8 ± 0.0	0.7 ± 0.0	22.0 ± 0.3	0.38	1.32	13.1
Lc	3.8 ± 0.3	81.2 ± 1.1	15.1 ± 0.9	1.0 ± 0.0	50.9 ± 0.3	5.4 ± 0.1	0.1 ± 0.0	38.9 ± 0.2	0.57	1.27	18.0

use for energy purposes would not be the most profitable, so it was decided to test their use for material purposes: as a fertilizer and as an adsorbent.

3.6. Biochar as fertilizer

The use of microalgae char as a fertilizer for the germination of

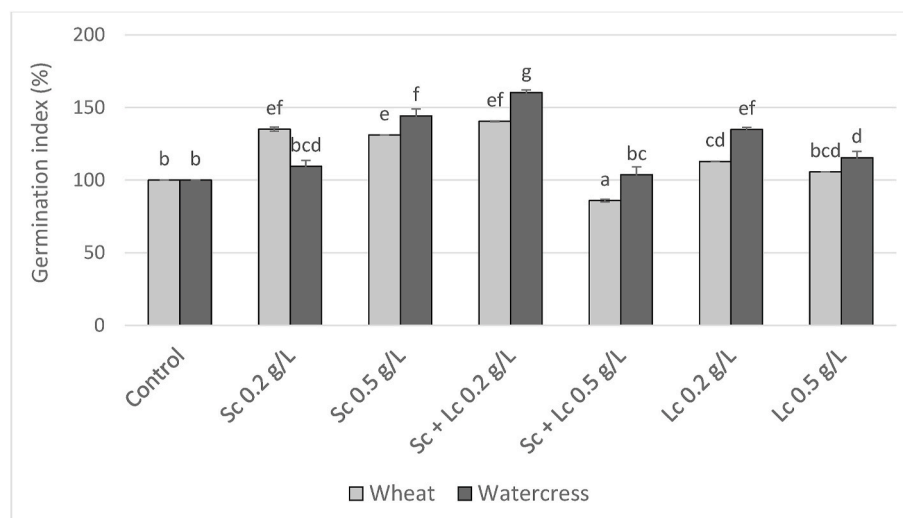


Fig. 9. Germination Index (% GI) for the three tested biochars (Sc – *Scenedesmus*; Sc + Lc – *Scenedesmus* + Lignocellulosic material; Lc - Lignocellulosic material) with two concentrations (0.2 and 0.5 g L⁻¹) as fertilizer (mean ± SD, n = 3).

wheat and watercress seeds was tested and demonstrated a positive effect compared to the control with distilled water. Fig. 9 shows the results of the tested biochar on the germination of the same two species of seeds (wheat and watercress).

All biochar had a positive effect on seed germination, except for the mixture of *Scenedesmus* and lignocellulosic material with 0.5 g L^{-1} (Sc + Lc 0.5 g L^{-1}) in wheat ($85.9 \pm 0.9\%$), probability due to the high concentration it led to a toxicity effect. The highest GI in both seeds was reached for Sc + Lc 0.2 g L^{-1} , followed by Sc 0.5 g L^{-1} for watercress and Sc 0.2 g L^{-1} for wheat. It should be highlighted that the increase in the germination index was above 60% in watercress and 40% in wheat. Which constitutes a significant increase in germination and initial growth of seedlings. (Chu et al. (2020) used *C. vulgaris* biomass char produced in poultry farm wastewater to fertilize wheat and obtained gains of 14.5% in wheat yield grain. In another study, on tomato fertilization with char from wastewater treatment microalgae biomass, a 52% increment in plant height was attained on the 10th day and 42% on the 20th day (Arun et al., 2020).

Table 8 indicates the mineral constitution of char's ashes used in the germination tests.

The tested biochars had a high amount of calcium, mainly the char from microalgae, but also magnesium and potassium. These three minerals are important in the germination and general development of plants. The char from the *S. obliquus* microalga also had a small amount of phosphorus as well as other micronutrients such as boron, copper, iron, manganese, sodium and zinc, which denotes the fertilizing component of these chars (Maw et al., 2020). The proximate analysis of the chars also revealed the presence of nitrogen (6% for Sc, 4% for Sc + Lc and 1% for Lc) and sulfur (1.2% for Sc, 0.7% for Sc + Lc and 0.1% for Lc). Reinforcing that the microalgae char was the most nutritionally rich, although also had more aluminum.

3.7. Biochar adsorption capacity

The obtained chars have a relatively low calorific value, so it was decided to test these biomaterials as low-cost adsorbents in the remediation of aqueous effluents contaminated with heavy metals or cationic dyes.

In the adsorption tests with the cationic dye methylene blue, the removal efficiency of an activated commercial char is close to 100% (in the present study was 97.8% for 3 s); nevertheless the production of these chars presents several negative factors, namely the irreversibility of the adsorption process, the activation of the char is expensive and its production generates other contaminated effluents (Jahandar Lashaki et al., 2016).

Regarding the adsorption tests of the biochars it can be concluded that microalgae chars had greater adsorption capacity than lignocellulosic ones. The biochar from *Scenedesmus* biomass was the one that had the greatest adsorption capacity of the dye, with 39.2% and 62.9% adsorption for 3 s and 48 h, respectively. The adsorption capacity of Cv and Sc + Lc were similar, with 35.7% and 34.7% for 3 s and 56.5% and 58.3% for 48 h, respectively. The mixture of the two materials produced biochars with adsorption capacity near to the microalgae chars, though Cv + Lc was somewhat inferior with 32.8% for 3 s and 52.3% for 48 h. Nevertheless, lignocellulosic material char had a much lower dye adsorption capacity, 16.4% for 3s and 36.8% for 48 h. The adsorption

achieved is not particularly high; the chars obtained had not undergone any pretreatment and its attainment does not require high energy expenses. In addition, the biomass that originated the chars had already treated an aquaculture effluent.

The surface area of algal biomass chars can be analyzed for porosity and pore volume. Some studies have shown that, unlike the lignocellulosic biomass that maintains its structure, the algal biomass subject to torrefaction presents a very different structure from the original, becoming compact and irregular (Binda et al., 2020), which will tend to increase its adsorption capacity. A measure of the ability of microalgae biochar to adsorb cation nutrients is the cation exchange capacity of biochar. Therefore, a biochar with high cation exchange capacity has a useful effect by avoiding nutrient leaching in the soil. Although not all nutrients are retained, these chars are able to preserve a high exchange capacity for other cations such as Ca, K, Mg and Na (Roberts et al., 2015). As cation exchange capacity is correlated with the ash content, it was proposed that the alkali and earth alkali metals in microalgae biomass favored the formation of oxygen-containing surface functional groups in the resultant biochar (Yu et al., 2017b). The increase in the absorption capacity and removal of the methylene blue dye is attributed to the increase of oxygen groups on the surface, as well as pore channels and in-situ generated carboxyl groups by hydrogen bonding and ion exchange. Electrostatic interaction between the biochar and the dye molecules that occur in the process of dye removal is also crucial (Liu et al., 2019).

3.8. Analysis of algal biomass integration in aquaculture species feed

The microalgae biomass produced could also be use as supplementation for feeding aquaculture species, because microalgae had a good quantity of protein and have a certain carotenoid content which contributes to the attractive colors of aquaculture animals (Zhang et al., 2019).

According to the assessments developed in the laboratory and the initial situation of the company that has to treat about 300 L of aquaculture transportation effluent every week, it would be possible to use the pre-existing tanks in the company to receive and remedy the weekly effluent, applying valves of passage between three tanks and reproducing the transfer systems in an automated way. The tanks already have a water oxygenation system which also allows the water agitation. Starting from three contiguous tanks with 4 m^3 each, used with only 0.25 m of water height (to allow light to enter the water column and obtain a stable microalgae culture and consequently reducing the capacity of the tanks to 25%), it is possible to receive the 300 L weekly effluent and guarantee its treatment through transfers between tanks every other day, during one week. At the end of the week, 300 L of treated effluent with 1.1 kg of microalgal biomass could be released directly into a tank with small shrimp (krill type) that serve as feed for aquaculture animals produced by the company, such as brown crab, lobsters and spinous spider crab. The algal biomass produced per week would be approximately 1.1 kg (DW) for *Chlorella vulgaris* and 0.82 kg for *Scenedesmus obliquus* (Fig. 10).

Another option would be to concentrate the biomass by decantation (releasing the treated water) and process it into pellets serving as feed for aquaculture fish (Dineshbabu et al., 2019; Milhazes-Cunha and Otero, 2017). In this case, a 4 m^3 tank would have a capacity for about

Table 8

Characterization of the mineral constitution g/Kg by inductively coupled plasma mass spectrometry (ICP-MS) of the biochars used in germination tests. (Sc – *Scenedesmus*; Sc + Lc – *Scenedesmus* + Lignocellulosic material; Lc - Lignocellulosic material).

	Al	B	Ba	Ca	Cu	Fe	K	Li	Mg	Mn	Na	P	Si	Sr	Ti	Zn	Zr
Sc	2.56	0.08	0.28	54.34	0.45	3.31	7.57	0.01	14.14	0.09	5.18	0.57	0.63	0.21	0.04	0.83	0.07
Sc + Lc	1.63	0.06	0.04	42.04	0.33	2.70	5.16	0.01	13.15	0.05	3.10	0.00	1.56	0.20	0.03	0.52	0.01
Lc	1.92	0.02	0.12	23.88	0.05	0.84	1.07	0.04	1.64	0.06	0.25	0.00	2.11	0.09	0.05	0.20	0.00

Note: The presence of Ag, As, Bi, Cd, Co, Hg, Mo, Sb, Sn and Tl was not detected in the analyzed chars. The elements Cr, Ni, Pb, Se and W were only detected vestigially.

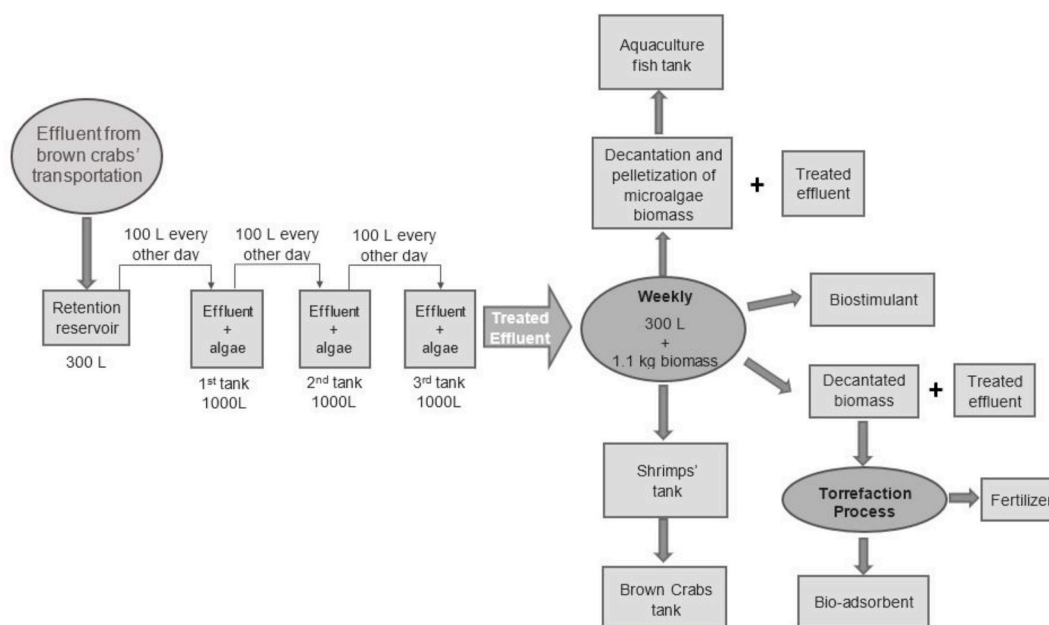


Fig. 10. Graphical representation of the aquaculture effluent treatment process with weekly quantity flows. The treated effluent with the microalgae biomass could be direct send to shrimps' tank or decanted and sent to aquaculture fish tank, use as biostimulant or use as bio-adsorbent or fertilizer after a torrefaction process.

200 fish (with a 0.5 kg harvest weight), since the ratio is 25 kg m^{-3} (Craig and Helfrich, 2017). Aquaculture fish are typically fed 1–5% of their body weight per day (Opiyo et al., 2018). According to the algal biomass obtained from *C. vulgaris*, it would be possible to provide 3–16% of the weekly feed of the fish growing in this tank with the biomass obtained in the effluent treatment. According to Shields and Lupatsch (2012), one of the reasons for not including more microalgae in aquaculture animals feed is due to the high cost it represents. From a nutritional and digestibility point of view, there would be no disadvantage, however the microalgae species to be included in the diet of aquaculture animals would have to be studied due to the lower palatability of algal meal. Dineshbabu et al. (2019) also state that a food based on microalgae can be used in aquaculture alone or combined with a regular feed. According to Oostlander et al. (2020) the main source of nutrients for larval and juvenile fish stages and for all bivalve filter-feeder stages are microalgae.

The existing alternatives for remediation and biomass production in these cases often involve the existence of membrane reactors, with the frequent problems of complexity, bridging and cost (Kumar et al., 2020). Although the need to become wastewater treatment processes more sustainable has made microalgae-based WWT a promising alternative to conventional bacterial-based processes. The development of a circular economy approach, with WWT and subsequent use of algal biomass for material purposes makes the whole process appealing from an environmental and economic point of view.

4. Conclusions

Seafood production effluent from crabs is suitable to growth the tested microalgae, *Chlorella vulgaris* and *Scenedesmus obliquus*, with excellent rates of bioremediation for total nitrogen, total phosphorus, COD, and BOD₅ (100%, 96.5%, 96.2% and 99.7% for Cv and 100%, 98.6%, 97.7% and 99.7% for Sc, respectively). The results allow the authors to conclude a better bioremediation performance on semi-continuous mode with three reservoirs, instead of one with the same volume, ensuring that the volume of transfers of raw aquaculture effluent does not exceed 10% of each reservoir.

In addition, the composition of microalgae obtained from the treatment kept a high level of protein and carbohydrates and could therefore

be used as a supplement for animal feed. However, further testing would be necessary to determine the optimal inclusion of microalgae in the different aquaculture species diets.

Alternatively, the use of the produced algal biomass showed very promising results as a biostimulant in the germination index with increments in the germination of wheat and watercress seeds of 175% (Cv) and 98% (Sc), respectively. The results of the torrefied biomass were also promising as an energy vector (72% biochar), as well as stimulant in seeds' germination (60% in watercress and 40% in wheat). Besides, the produced char could also be used as an adsorbent in effluents contaminated with dyes, reaching 63% of adsorption without any additional treatment.

Credit author statement

Catarina Viegas: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing; Luísa Gouveia: Writing – review & editing, Funding acquisition; Margarida Gonçalves: Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition.

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.

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References

- Agwa, O.K., Ogugbue, C.J., Williams, E.E., 2017. Field evidence of *Chlorella vulgaris* potentials as a biofertilizer for *Hibiscus esculentus*. *Int. J. Agric. Res.* 12, 181–189. <https://doi.org/10.3923/ijar.2017.181.189>.
- Andreotti, V., Chindris, A., Brundu, G., Vallanc, D., Francavilla, M., García, J., 2017. Bioremediation of aquaculture wastewater from *Mugil cephalus* (Linnaeus, 1758) with different microalgae species. *Chem. Ecol.* 33, 750–761. <https://doi.org/10.1080/02757540.2017.1378351>.
- Ansari, F.A., Singh, P., Guldhe, A., Bux, F., 2017. Microalgal cultivation using aquaculture wastewater: integrated biomass generation and nutrient remediation. *Algal Res* 21, 169–177. <https://doi.org/10.1016/j.algal.2016.11.015>.
- AOAC (Association of Official Analytical Chemists), 2006. Official Methods of Analysis of AOAC International, 18th Editi. Association of Official Analytical Chemists.
- Apandi, Najeeha, Mohamed, R., Al-Gheethi, A., Kassim, A., 2019a. Microalgal biomass production through phycoremediation of fresh market wastewater and potential applications as aquaculture feeds. *Environ. Sci. Pollut. Res.* 26, 3226–3242. <https://doi.org/10.1007/s11356-018-3937-3>.
- Apandi, N., Mohamed, R.M.S.R., Al-Gheethi, A., Gani, P., Ibrahim, A., Kassim, A.H.M., 2019b. *Scenedesmus* biomass productivity and nutrient removal from wet market wastewater, A bio-kinetic study. *Waste and Biomass Valorization* 10, 2783–2800. <https://doi.org/10.1007/s12649-018-0313-y>.
- Arun, J., Gopinath, K.P., Vigneshwar, S.S., Swetha, A., 2020. Sustainable and eco-friendly approach for phosphorus recovery from wastewater by hydrothermally carbonized microalgae: study on spent bio-char as fertilizer. *J. Water Process Eng.* 38, 101567. <https://doi.org/10.1016/j.jwpe.2020.101567>.
- Ayyat, M.S., Ayyat, A.M.N., Al-Sagheer, A.A., El-Hais, A.E.A.M., 2018. Effect of some safe feed additives on growth performance, blood biochemistry, and bioaccumulation of aflatoxin residues of Nile tilapia fed aflatoxin-B1 contaminated diet. *Aquaculture* 495, 27–34. <https://doi.org/10.1016/j.aquaculture.2018.05.030>.
- Barskov, S., Zappi, M., Buchireddy, P., Dufreche, S., Guillory, J., Gang, D., Hernandez, R., Bajpai, R., Baudier, J., Cooper, R., Sharp, R., 2019. Torrefaction of biomass: a review of production methods for biochar from cultured and waste lignocellulosic feedstocks. *Renew. Energy* 142, 624–642. <https://doi.org/10.1016/j.renene.2019.04.068>.
- Binda, G., Spanu, D., Bettinetti, R., Magagnin, L., Pozzi, A., Dossi, C., 2020. Comprehensive comparison of microalgae-derived biochar from different feedstocks: a prospective study for future environmental applications. *Algal Res* 52, 102103. <https://doi.org/10.1016/j.algal.2020.102103>.
- Bona, F., Capuzzo, A., Franchino, M., Emilio, M., 2014. Semicontinuous nitrogen limitation as convenient operation strategy to maximize fatty acid production in *Neochloris oleoabundans*. *Algal Res* 5, 1–6. <https://doi.org/10.1016/j.algal.2014.03.007>.
- Cahyanti, M.N., Doddapaneni, T.R.K.C., Kikas, T., 2020. Biomass torrefaction: an overview on process parameters, economic and environmental aspects and recent advancements. *Bioresour. Technol.* 301, 122737. <https://doi.org/10.1016/j.biortech.2020.122737>.
- Chhaya Yadav, B., Jogawat, A., Gnanasekaran, P., Kumari, P., Lakra, N., Lal, S.K., Pawar, J., Narayan, O.P., 2021. An overview of recent advancement in phytohormones-mediated stress management and drought tolerance in crop plants. *Plant Gene* 25, 100264. <https://doi.org/10.1016/j.plgene.2020.100264>.
- Chu, Q., Lyu, T., Xue, L., Yang, L., Feng, Y., Sha, Z., Yue, B., Mortimer, R., Cooper, M., Pan, G., 2020. Hydrothermal carbonization of microalgae for phosphorus recycling from wastewater to crop-soil systems as slow-release fertilizers. *J. Clean. Prod.* 283, 124627. <https://doi.org/10.1016/j.jclepro.2020.124627>.
- Correia, R., Gonçalves, M., Nobre, C., Mendes, B., 2017. Impact of torrefaction and low-temperature carbonization on the properties of biomass wastes from *Arundo donax* L. and *Phoenix canariensis*. *Bioresour. Technol.* 223, 210–218. <https://doi.org/10.1016/j.biortech.2016.10.046>.
- Craig, S., Helfrich, L., 2017. Understanding fish nutrition, feeds, and feeding. *Virginia Coop. Ext.* 420, 1–6.
- Daneshvar, E., Antikainen, L., Koutra, E., Kornaros, M., Bhatnagar, A., 2018. Investigation on the feasibility of *Chlorella vulgaris* cultivation in a mixture of pulp and aquaculture effluents: treatment of wastewater and lipid extraction. *Bioresour. Technol.* 255, 104–110. <https://doi.org/10.1016/j.biortech.2018.01.101>.
- Deepika, P., MubarakAli, D., 2020. Production and assessment of microalgal liquid fertilizer for the enhanced growth of four crop plants. *Biocatal. Agric. Biotechnol.* 28, 101701. <https://doi.org/10.1016/j.bcab.2020.101701>.
- Dineshbabu, G., Goswami, G., Kumar, R., Sinha, A., Das, D., 2019. Microalgae–nutritious, sustainable aqua- and animal feed source. *J. Funct. Foods* 62, 103545. <https://doi.org/10.1016/j.jff.2019.103545>.
- Dotta, G., de Andrade, J.I.A., Garcia, P., Alves Jesus, G.F., Mourinho, J.L.P., Mattos, J.J., Dias Bainy, A.C., Martins, M.L., 2018. Antioxidant enzymes, hematology and histology of spleen in Nile tilapia fed supplemented diet with natural extracts challenged with *Aeromonas hydrophila*. *Fish Shellfish Immunol.* 79, 175–180. <https://doi.org/10.1016/j.fsi.2018.05.024>.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356. <https://doi.org/10.1021/ac60111a017>.
- Ennis, C.J., Clarke, J., Neate, K., Cerejeira, J., Tull, L., 2017. Hydrothermal extraction of microalgae fatty acid Influences hydrochar phytotoxicity. *Front. Environ. Sci.* 5, 1–8. <https://doi.org/10.3389/fenvs.2017.00047>.
- Fakayode, O.A., Aboagari, E.A.A., Zhou, C., Ma, H., 2020. Co-pyrolysis of lignocellulosic and macroalgae biomasses for the production of biochar – a review. *Bioresour. Technol.* 297, 122408. <https://doi.org/10.1016/j.biortech.2019.122408>.
- Fernández-Linares, L.C., Guerrero Barajas, C., Durán Páramo, E., Badillo Corona, J.A., 2017. Assessment of *Chlorella vulgaris* and indigenous microalgae biomass with treated wastewater as growth culture medium. *Bioresour. Technol.* 244, 400–406. <https://doi.org/10.1016/j.biortech.2017.07.141>.
- Fonseca, A.L., Newton, A., Cabral, A., 2021. Local and meso-scale pressures in the eutrophication process of a coastal subtropical system: challenges for effective management. *Estuar. Coast Shelf Sci.* 250, 107109. <https://doi.org/10.1016/j.ecss.2020.107109>.
- Gan, Y.Y., Ong, H.C., Show, P.L., Ling, T.C., Chen, W.H., Yu, K.L., Abdullah, R., 2018. Torrefaction of microalgal biochar as potential coal fuel and application as bio-adsorbent. *Energy Convers. Manag.* 165, 152–162. <https://doi.org/10.1016/j.enconman.2018.03.046>.
- Gao, F., Li, C., Yang, Z.H., Zeng, G.M., Feng, L.J., Liu, J.zhi, Liu, M., Cai, H.wen, 2016. Continuous microalgae cultivation in aquaculture wastewater by a membrane photobioreactor for biomass production and nutrients removal. *Ecol. Eng.* 92, 55–61. <https://doi.org/10.1016/j.ecoleng.2016.03.046>.
- Gouveia, L., Empis, J., 2003. Relative stabilities of microalgal carotenoids in microalgal extracts, biomass and fish feed: effect of storage conditions. *Innovat. Food Sci. Emerg. Technol.* 4, 227–233. [https://doi.org/10.1016/S1466-8564\(03\)00002-X](https://doi.org/10.1016/S1466-8564(03)00002-X).
- Gouveia, L., Graça, S., Sousa, C., Ambrosano, L., Ribeiro, B., Botrel, E.P., Neto, P.C., Ferreira, A.F., Silva, C.M., 2016. Microalgae biomass production using wastewater: treatment and costs. Scale-up considerations. *Algal Res* 16, 167–176. <https://doi.org/10.1016/j.algal.2016.03.010>.
- Gouveia, L., Veloso, V., Reis, A., Fernandes, H., Novais, J., Empis, J., 1996. Evolution of pigment composition in *Chlorella vulgaris*. *Bioresour. Technol.* 57, 157–163. [https://doi.org/10.1016/0960-8524\(96\)00058-2](https://doi.org/10.1016/0960-8524(96)00058-2).
- Grzesik, M., Romanowska-Duda, Z., Kalaji, H.M., 2017. Effectiveness of cyanobacteria and green algae in enhancing the photosynthetic performance and growth of willow (*Salix viminalis* L.) plants under limited synthetic fertilizers application. *Photosynthetica* 55, 510–521. <https://doi.org/10.1007/s11099-017-0716-1>.
- Han, P., Lu, Q., Fan, L., Zhou, W., 2019. A review on the use of microalgae for sustainable aquaculture. *Appl. Sci.* 9. <https://doi.org/10.3390/app9112377>.
- Hodaifa, G., Martínez, M.E., Sánchez, S., 2010. Influence of temperature on growth of *Scenedesmus obliquus* in diluted olive mill wastewater as culture medium. *Eng. Life Sci.* 10, 257–264. <https://doi.org/10.1002/elsc.201000005>.
- Jahandar Lashaki, M., Atkinson, J.D., Hashisho, Z., Phillips, J.H., Anderson, J.E., Nichols, M., 2016. The role of beaded activated carbon's pore size distribution on heel formation during cyclic adsorption/desorption of organic vapors. *J. Hazard Mater.* 315, 42–51. <https://doi.org/10.1016/j.jhazmat.2016.04.071>.
- Jones, D.B., 1931. Factors for converting percentages of nitrogen in food and feed into percentages of proteins. United States Dep. Agric. Circular 1–22. <https://doi.org/10.1163/q3.SIM.00374>.
- Kalra, R., Gaur, S., Goel, M., 2020. Microalgae bioremediation: a perspective towards wastewater treatment along with industrial carotenoids production. *J. Water Process Eng.* 101794. <https://doi.org/10.1016/j.jwpe.2020.101794>.
- Kumar, R., Ghosh, A.K., Pal, P., 2020. Synergy of biofuel production with waste remediation along with value-added co-products recovery through microalgae cultivation: a review of membrane-integrated green approach. *Sci. Total Environ.* 698, 134169. <https://doi.org/10.1016/j.scitotenv.2019.134169>.
- Kuo, C.M., Jian, J.F., Lin, T.H., Chang, Y. Bin, Wan, X.H., Lai, J.T., Chang, J.S., Lin, C.S., 2016. Simultaneous microalgal biomass production and CO₂ fixation by cultivating *Chlorella* sp. GD with aquaculture wastewater and boiler flue gas. *Bioresour. Technol.* 221, 241–250. <https://doi.org/10.1016/j.biortech.2016.09.014>.
- Lazo, J.P., Fuentes-Quesada, J.P., Villareal-Rodarte, G., Viana, M.T., Baron-Sevilla, B., 2020. The effect of dietary n-3 LC-PUFA levels on growth, survival, and feed utilization in juvenile *Totoaba macdonaldi*. *Aquaculture* 525, 735350. <https://doi.org/10.1016/j.aquaculture.2020.735350>.
- Li, M., Callier, M.D., Blancheton, J.P., Galès, A., Nahon, S., Triplet, S., Geoffroy, T., Menniti, C., Fouilland, E., Roque d'orbecastel, E., 2019. Bioremediation of fishpond effluent and production of microalgae for an oyster farm in an innovative recirculating integrated multi-trophic aquaculture system. *Aquaculture* 504, 314–325. <https://doi.org/10.1016/j.aquaculture.2019.02.013>.
- Li, M., Wang, H., Huang, Z., Yuan, X., Tan, M., Jiang, L., Wu, Z., Qin, X., Li, H., 2020. Comparison of atmospheric pressure and gas-pressurized torrefaction of municipal sewage sludge: properties of solid products. *Energy Convers. Manag.* 213, 112793. <https://doi.org/10.1016/j.enconman.2020.112793>.
- Lin, G., Li, K., Liang, S., Li, Y., Su, Y., Wang, X., 2020. Compound eutrophication index: an integrated approach for assessing ecological risk and identifying the critical element controlling harmful algal blooms in coastal seas. *Mar. Pollut. Bull.* 150, 110585. <https://doi.org/10.1016/j.marpolbul.2019.110585>.
- Liu, S., Li, J., Xu, S., Wang, M., Zhang, Y., Xue, X., 2019. A modified method for enhancing adsorption capability of banana pseudostem biochar towards methylene blue at low temperature. *Bioresour. Technol.* 282, 48–55. <https://doi.org/10.1016/j.biortech.2019.02.092>.
- Markou, G., Ionomou, D., Muylaert, K., 2016. Applying raw poultry litter leachate for the cultivation of *Arthrospira platensis* and *Chlorella vulgaris*. *Algal Res* 13, 79–84. <https://doi.org/10.1016/j.algal.2015.11.018>.
- Marks, E.A.N., Miñón, J., Pascual, A., Montero, O., Navas, L.M., Rad, C., 2017. Application of a microalgal slurry to soil stimulates heterotrophic activity and promotes bacterial growth. *Sci. Total Environ.* 605–606, 610–617. <https://doi.org/10.1016/j.scitotenv.2017.06.169>.

- Maw, M.J.W., Houx, J.H., Fritschi, F.B., 2020. Nitrogen fertilization of high biomass sorghum affects macro- and micronutrient accumulation and tissue concentrations. *Ind. Crop. Prod.* 156, 112819. <https://doi.org/10.1016/j.indcrop.2020.112819>.
- Milhazes-Cunha, H., Otero, A., 2017. Valorisation of aquaculture effluents with microalgae: the Integrated Multi-Trophic Aquaculture concept. *Algal Res* 24, 416–424. <https://doi.org/10.1016/j.algal.2016.12.011>.
- Milledge, J.J., 2011. Commercial application of microalgae other than as biofuels: a brief review. *Rev. Environ. Sci. Bio/Technology* 10, 31–41. <https://doi.org/10.1007/s11157-010-9214-7>.
- Miranda, J.R., Passarinho, P.C., Gouveia, L., 2012. Pre-treatment optimization of *Scenedesmus obliquus* microalga for bioethanol production. *Bioresour. Technol.* 104, 342–348. <https://doi.org/10.1016/j.biortech.2011.10.059>.
- Morais Junior, W.G., Gorgich, M., Corrêa, P.S., Martins, A.A., Mata, T.M., Caetano, N.S., 2020. Microalgae for biotechnological applications: cultivation, harvesting and biomass processing. *Aquaculture* 528, 735562. <https://doi.org/10.1016/j.aquaculture.2020.735562>.
- Nagappan, S., Kumar, R.R., Balaji, J.R., Singh, S., Verma, S.K., 2019. Direct saponification of wet microalgae by methanolic potassium hydroxide using acetone as co-solvent. *Bioresour. Technol. Reports* 5, 351–354. <https://doi.org/10.1016/j.biteb.2018.05.010>.
- Navarro-López, E., Rufz-Nieto, A., Ferreira, A., Gabriel Acién, F., Gouveia, L., 2020. Biostimulant potential of *Scenedesmus obliquus* grown in brewery wastewater. *Molecules* 25, 1–16. <https://doi.org/10.3390/molecules25030664>.
- Nitsos, C., Filali, R., Taidi, B., Lemaire, J., 2020. Current and novel approaches to downstream processing of microalgae: a review. *Biotechnol. Adv.* 45, 107650. <https://doi.org/10.1016/j.biotechadv.2020.107650>.
- Oostlander, P.C., van Houcke, J., Wijffels, R.H., Barbosa, M.J., 2020. Microalgae production cost in aquaculture hatcheries. *Aquaculture* 525, 735310. <https://doi.org/10.1016/j.aquaculture.2020.735310>.
- Opiyo, M.A., Marijani, E., Muendo, P., Odede, R., Leschen, W., Charo-Karisa, H., 2018. A review of aquaculture production and health management practices of farmed fish in Kenya. *Int. J. Vet. Sci. Med.* 6, 141–148. <https://doi.org/10.1016/j.ijvsm.2018.07.001>.
- Parikh, J., Channiwala, S.A., Ghosal, G.K., 2005. A correlation for calculating HHV from proximate analysis of solid fuels. *Fuel* 84, 487–494. <https://doi.org/10.1016/j.fuel.2004.10.010>.
- Paul, B., Bhattacharya, S.S., Gogoi, N., 2021. Primacy of ecological engineering tools for combating eutrophication: an ecohydrological assessment pathway. *Sci. Total Environ.* 762, 143171. <https://doi.org/10.1016/j.scitotenv.2020.143171>.
- Portuguese Ministry of the Environment, 1998. Decree-Law No. 236/98, 1998, of the Portuguese Ministry of the Environment of 1 August Establishing Water Quality Standards. *Diário da República - Série I*.
- Puglisi, I., Barone, V., Fragalà, F., Stevanato, P., Baglieri, A., Vitale, A., 2020. Effect of microalgal extracts from *Chlorella vulgaris* and *Scenedesmus quadricauda* on germination of *Beta vulgaris* seeds. *Plants* 9, 1–14. <https://doi.org/10.3390/plants9060675>.
- Rice, E.W., Baird, R.B., Eaton, A.D. (Eds.), 2017. *Standard Methods for the Examination of Water and Wastewater*, 23th ed. American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC.
- Roberts, D.A., Paul, N.A., Dworjanyn, S.A., Bird, M.I., De Nys, R., 2015. Biochar from commercially cultivated seaweed for soil amelioration. *Sci. Rep.* 5, 1–6. <https://doi.org/10.1038/srep09665>.
- Rosa, J., Lemos, M.F.L., Crespo, D., Nunes, M., Freitas, A., Ramos, F., Pardal, M.Á., Leston, S., 2020. Integrated multitrophic aquaculture systems – potential risks for food safety. *Trends Food Sci. Technol.* 96, 79–90. <https://doi.org/10.1016/j.tifs.2019.12.008>.
- Shields, R., Lupatsch, I., 2012. Algae for aquaculture and animal feeds. In: Posten C, W.C. (Ed.), *Microalgal Biotechnology: Integration and Economy*. De Gruyter. <https://doi.org/10.1515/9783110298321.79>.
- Tejido-Núñez, Y., Aymerich, E., Sancho, L., Refardt, D., 2019. Treatment of aquaculture effluent with *Chlorella vulgaris* and *Tetrademus obliquus*: the effect of pretreatment on microalgae growth and nutrient removal efficiency. *Ecol. Eng.* 136, 1–9. <https://doi.org/10.1016/j.ecoleng.2019.05.021>.
- UTEX, 2020. Algal Culture Medium Recipes [WWW Document]. UTEX Cult. Collect. Algae. URL. accessed 8.19.20. <https://utex.org/pages/algal-culture-media-recipes>.
- Vonshak, A., Maske, H., 1982. Algae: growth techniques and biomass production. In: Hall, J.C. (Ed.), *Techniques in Bioproduction and Photosynthesis*. Pergamon Press, Oxford, pp. 66–77.
- Yu, K.L., Lau, B.F., Show, P.L., Ong, H.C., Ling, T.C., Chen, W.H., Ng, E.P., Chang, J.S., 2017a. Recent developments on algal biochar production and characterization. *Bioresour. Technol.* 246, 2–11. <https://doi.org/10.1016/j.biortech.2017.08.009>.
- Yu, K.L., Show, P.L., Ong, H.C., Ling, T.C., Chi-Wei Lan, J., Chen, W.H., Chang, J.S., 2017b. Microalgae from wastewater treatment to biochar – feedstock preparation and conversion technologies. *Energy Convers. Manag.* 150, 1–13. <https://doi.org/10.1016/j.enconman.2017.07.060>.
- Zhang, L., Pei, H., Yang, Z., Wang, X., Chen, S., Li, Y., 2019. Microalgae nourished by mariculture wastewater aids aquaculture self-reliance with desirable biochemical composition. *Bioresour. Technol.* 278, 205–213. <https://doi.org/10.1016/j.biortech.2019.01.066>.
- Zheng, N.Y., Lee, M., Lin, Y.L., 2020. Co-processing textile sludge and lignocellulose biowaste for biofuel production through microwave-assisted wet torrefaction. *J. Clean. Prod.* 268, 122200. <https://doi.org/10.1016/j.jclepro.2020.122200>.
- Zucconi, F., Forte, M., Monaco, A., De Bertoldi, M., 1981. Biological evaluation of compost maturity. *Biocycle* 22, 27–29.