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Evaluation of microalgae as bioremediation agent for poultry effluent and biostimulant for germination

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ABSTRACT

This work addresses how a pre-treatment involving biomass ash influences the poultry effluent's bioremediation using three microalga strains, such as *Chlorella vulgaris*, *Chlorella protothecoides* and *Tetradesmus obliquus*. The undiluted effluent served as the culture medium for the growth, both in batch and semi continuous modes, and the remediation efficiency and biomass production yield were quantified. The combination strategy in batch mode, allowed removal efficiency of 100% for total nitrogen, more than 80% for total phosphorus and over 70% for chemical oxygen demand. Average biomass productivities for 10 days of 94.9, 76.2 and 72.0 mg L^{-1} day^{-1} were obtained for *T. obliquus, C. vulgaris* and *C. protothecoides*, respectively. Regarding semi-continuous strategy (28 days), the biomass productivities achieved were 245 and 194 mg L^{-1} day^{-1} for *T. obliquus* and *C. vulgaris*, respectively. Remediation rates of 100% for total nitrogen and phosphorus, and over 92% for COD were attained. The microalga composition was assessed for protein, sugar, lipid, and ash contents. The produced biomasses were tested as biostimulant and showed a 147% increase in wheat germination index, for the *C. vulgaris* microalga. The use of the precipitate from the biomass ash pre-treatment as fertilizer in germination tests was also assessed and results in an increase of 26%, for 10% of precipitate incorporation.

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1. Introduction

The poultry sector is currently the second largest contributor to the global meat production, being responsible for 39% of the total of 339 million ton carcass weight produced in 2019 — chicken meat represents 89% of the poultry tonnage (FAO, 2020). Poultry effluents are produced, in large volumes, in agro-industrial farms and slaughterhouses worldwide. They contain a significant organic load, including phosphorus and nitrogen compounds, emulsified fats, and particulate matter.

Usually, the organic residues of poultry slaughterhouse effluents are homogenized, thermally treated, and sent to a solid–liquid separator. While the decanted solid waste is used to produce pet diets, the liquid phase, with a high fat content, is subjected to a sequence of operations, such as flocculation, intended to reduce the organic load. The aqueous part goes for anaerobic digestion or to a water treatment plant, whereas the solid one, with high fat content, goes

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to landfill. The whole process entails high costs and a negative environmental impact, and is associated with a high potential of eutrophication (Agmar Ferreira et al., 2018; Martinelli et al., 2020; Oryschak and Beltranena, 2020). Some authors assessed the possible assembly of effluents stemming from multiple sources in a joint treatment using anaerobic co-digestion, and their findings were quite promising (Asses et al., 2019; Latifi et al., 2019).

Conventional effluent treatments require large amounts of chemicals and energy and are responsible for high greenhouse gas emissions and a sludge with no potential use/benefit — the use of microalgae reduces these drawbacks (Hjort et al., 2021). Microalgae provide the O_2 used by heterotrophic and autotrophic microorganisms to oxidize and/or assimilate organic carbon, as well as nitrogen and phosphorus (Moreno-Garcia et al., 2017; Patel et al., 2017). On the other hand, those same microorganisms provide the CO_2 that ensures an efficient microalga growth.

Bioremediation of wastewater using microalgae has been investigated in several effluents, including municipal and agro-industrial ones (Ferreira et al., 2019; Gramegna et al., 2020; Posadas et al., 2017). *Microalgae-based bioremediation* has the advantage of producing algal biomass that can be incorporated in different stages of the agro-industrial process, as well as used as feed in animal production (e.g., (Dineshbabu et al., 2019; Saeid et al., 2016)), as biofertilizer or biostimulant (e.g., (Navarro-López et al., 2020)).

There are several methods to conduct the pre-treatment in poultry slaughterhouse wastewater, namely electrochemical advanced oxidation, acid precipitation, cavitation-based processes and even nanotechnology assisted process (Hilares et al., 2021). These authors studied the use of acid precipitation in these effluents with H_2SO_4, achieving a removal of 80% of the COD, followed by the remediation with the microalga *Chlorella vulgaris*, which removed 83% of the COD that had remained after the pre-treatment. The pre-treatment with biomass ash was previously used for effluents from piggery (Viegas and Gonçalves, 2021), cattle (Viegas et al., 2021a) and landfill leachate (Viegas et al., 2021c). The microalgae-based remediation of poultry slaughterhouse effluents is still not much explored (Ferreira et al., 2018), except after using anaerobic digestion. The biomass ash pre-treatment of this effluent with subsequent use of microalgae has never been done before.

The poultry slaughterhouse wastewater after pre-treatment can be used as a culture medium for microalga growth, thus contributing to their remediation through the consumption of organic and inorganic nutrients, with the ensuing biomass production. Markou et al. (2016) studied the use of raw poultry litter leachate, however with the need of 10×, 15×, 20× and 25× dilution using the cyanobacteria *Arthrospira platensis* and the microalga *Chlorella vulgaris*. They found that the *C. vulgaris* has the best performance, being able to grow at 10x dilution, producing between 1.76 and 1.87 g L^{-1} at day 11, and exhibiting a superior consumption of nutrients (i.e., a higher bioremediation). Singh et al. (2011) reported a remediation of diluted (6% v/v) poultry litter from anaerobic digester, for the total N and P, of about (i) 65% and 85%, with the *Chlorella minutissima*, respectively and (ii) 70% and 88%, with the *Scenedesmus bijuga*, respectively. In a 29-day study involving *Scenedesmus obliquus* grown in poultry slaughterhouse effluent, productivities of 100 mg L^{-1} day^{-1} and remediations of 100% for total N and P were achieved (Ferreira et al., 2018).

The main objective of this work was (i) to assess the beneficial influence of a biomass ash pre-treatment in the microalga-based bioremediation of poultry slaughterhouse effluents and (ii) the valorization of the produced algal biomasses as biostimulant and precipitate (from pre-treatment) as fertilizer. The innovation of this approach is the use of biomass ash as a pre-treatment of poultry slaughterhouse effluents, which leads to the precipitation of dissolved and suspended compounds due to the rise in pH to 12–12.5.

2. Material and methods

2.1. Microorganisms and culture conditions

Three microalga species were selected for the treatment of the aviary effluents: *Chlorella vulgaris* (INETI 58, LNEG_UBB, Portugal) (Cv), *Chlorella protothecoides* (UTEX # 25 - USA) (Cp) and *Tetradesmus obliquus* (ACOI 204/07) (formerly known as *Scenedesmus obliquus*) (Coimbra University Culture Collection, Portugal) (To). All three microalga species were kept in the appropriate synthetic culture media: Cv and Cp in the Chlorella medium (UTEX, 2020), and To in the Bristol medium (UTEX, 2018). To evaluate the biomass productivity of the three microalgae grown in poultry effluent, a control synthetic growth medium (Chlorella or Bristol) was also used.

The experiments were conducted in 1000 mL Erlenmeyer flasks agitated by an air flow of 15.2 L $L_{culture}^{-1}$ h^{-1} (air pump Stellar 380D, 5 W, Zhejiang, China) that keeps the culture (sealed with hydrophobic cotton) stirred. The microalgae grew at room temperature (23 °C ± 2 °C), under artificial lighting with LED fluorescent lamps (90 W), at ± 200 μE m^{-2} s^{-1} (digital luxmeter ROLINE, model RO 1332 A, Hong Kong, China) with alternate 12 h cycles of light and dark.

2.2. Culture medium

The effluent used in the tests was obtained from poultry slaughterhouse. This wastewater was achieved after the cooking of the poultry's remains that were not intended for human consumption, but will be used into feed for domestic animals containing a high amount of fat. The effluent belongs to the company Avibom, S.A., in Ramalhal (38°15'553.6" N, -9°26'28.9" W) located in the Torres Vedras region, Portugal, and was collected in May 2018. The effluent was collected in 20 L plastic containers and stored at 4 °C, to minimize chemical and biological changes. The tests were carried out for (i)

effluent directly obtained from the slaughterhouse (PE — raw wastewater from the poultry slaughterhouse) and (ii) same effluent pre-treated with ash (PE+A — raw wastewater from the poultry slaughterhouse plus ash). The pre-treatment consisted of adding 80 g L^{-1} of biomass ash (< 500 μm) to the effluent, followed by agitation with air flow for 1 h. The biomass ash (bottom ash) was supplied by the ceramics company Prélis Cerâmicas Lda, since it is a by-product of the combustion process taking place in the ceramics production industry (the furnaces employed by this industry use forestry biomass with a small percentage of polymeric residues as fuel). The biomass ash mineral components and chemical composition were determined through X-ray fluorescence spectrometry (Philips X Unique II spectrometer, Eindhoven, Netherlands). At the end of this process the pH was neutralized, and it was possible to obtain (i) a solid residue (from ash and suspended solids) and (ii) a poultry effluent with dissolved ash, intended for the microalga growth.

The effluents PE and PE+A were analysed for total nitrogen, nitrates, nitrites, total phosphorous (using Hanna instruments test Kits, Hanna Termo reactor HI839800-02 and Hanna Photometer HI83314-02, Cluj-Napoca, Romania), chemical oxygen demand (COD — through high range dichromate method from Hanna instruments test Kits, Cluj-Napoca, Romania), biochemical oxygen demand (BOD_5 — using WTW OxiTop IS 12, Weilheim, Germany) and total solids according to the methodology described in Standard Methods for the Examination of Water and Wastewater (Rice et al., 2017).

Regardless of the microalga species under consideration, the inoculations were performed using 20 mL of stock solution (inoculum) calculated to have an initial optical density (at 540 nm) of around 0.1 in the poultry effluent plus ash.

2.3. Experiments

Four sets of experiments were performed: (i) batch mode involving the three microalgae with the raw wastewater from the poultry slaughterhouse (PE), the raw wastewater from the poultry slaughterhouse plus ash (PE+A) and the control synthetic medium, and (ii) three sets of semi-continuous mode tests involving the two microalgae that performed best in the first set of tests with PE+A, namely the *C. vulgaris* and *T. obliquus* — second, third and fourth sets of tests, each requiring sequences of three 1 L reactors, one for each microalga (Cv-1, Cv-2, Cv-3 and To-1, To-2, To-3), that periodically received (every other day) a given amount of effluent from the previous reactor. The poultry effluents used in the semi-continuous mode and batch mode tests originated from the same farm but were collected at different times. Finally, the batch and semi-continuous modes tests lasted for 10 and 28 days, respectively.

In the second set of tests, the first reactor received 100 mL of poultry effluent with (PE+A) or without (PE) ash and the second and third reactors also received 100 mL of effluent from the previous reactor. Because, as expected, it was concluded that the PE+A performs much better than the PE, the third and fourth sets of tests were only carried out with PE+A. They differed from the second test set only in the fact that the reactors received 200 mL and 300 mL of effluent, respectively (instead of 100 mL). The hydraulic residence times (HRT) were 10, 5 and 3.3 days, respectively in the second, third and fourth test sets. Figure S1 (supplementary materials) provides a schematic representation of the performance of the semi-continuous tests.

It should be noted that, in the second test set, reactors 2 and 3 were supplemented weekly with aqueous $NaNO_3$ and KH_2PO_4, to achieve final concentrations in the culture medium of 20 mg N L^{-1} and 10 mg P L^{-1} (Ansari et al., 2017; Markou et al., 2016).

2.4. Microalga growth

During the experiments, samples were collected from the bioreactors every other day, to analyse the pH medium (pH Tester PH-108, China), N, P, and COD (using Hanna instruments test Kits, Cluj-Napoca, Romania) and microalgae growth by measuring the optical density at 540 nm (OD_{540}), using a spectrometer (Biochrom S4 Libra, Cambridge, England). Samples were also taken every week, to examine the microalgae dry weights by filtering the samples through a Whatman GF/C 47 mm filter.

In the batch mode tests, the culture was harvested by centrifugation at 7000 rpm for 5 min (Sigma 4K15, Osterode am Harz, Germany), whenever a microalga growth first led to a decrease in N, P and COD below the discharge limits. The liquid phase was also analysed for total nitrogen, nitrates, nitrites, total phosphorous, COD, BOD_5 and total solids, as in the beginning, and the biomass was dried at 45 °C for 48 h (Memmert U30 oven, Schwabach, Germany). In the semi-continuous mode tests, which lasted 28 days, part of the culture was used for analysis purposes and the remainder was used in the subsequent tests.

2.5. Microalga biomass characterization

Prior to the biomass characterization, the microalgae were grounded for 4 min at a speed of 25 s^{-1}, using a Retsch ball mill (model MM400, Haan, Germany). The total nitrogen present in the biomass was quantified by the modified Kjeldahl method (AOAC (Association of Official Analytical Chemists), 2006). The total protein was determined by multiplying the total nitrogen by the conventional conversion factor 6.25 (Jones, 1931). The sugar content was determined by quantitative acid hydrolysis, using the method proposed by Miranda et al. (2012), optimized for the extraction of sugar from the microalga biomass, followed by the method of the phenol-sulphuric reagent (Dubois et al., 1956), intended for the determination of the total sugar content.

The determination of the lipid content was made through a soxhlet apparatus for 6 h, with n-hexane solvent and about 1 g of algal biomass. The composition of the lipidic fraction, in terms of fatty acids, was determined by means of a GC–MS analyser - *Gas Chromatography coupled with Mass Spectrometry* (Focus GC, Polaris Q – Thermo, Austin, USA), equipped with a DB-5 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: (i) initial value of 40 °C, held for 1 min, (ii) increase to 150 °C, at a rate of 10 °C/min, value held for 15 min, and (iii) increase to 250 °C, at 5 °C/min, immediately followed by an increase to 280 °C, at 10 °C/min, value held for 10 min. The transfer line and ion source temperatures were 250 °C and 230 °C, respectively. The fatty acids present in the n-hexane solvent were identified by comparing their mass spectra with those existing in the NIST and WILEY databases and with the retention time and mass spectra of the corresponding standards. The fatty acid methyl esters were prepared by adding equal parts of sample and methanolic KOH (2N) (Nagappan et al., 2019). Finally, the moisture and ash content of the algal biomass were determined gravimetrically, weighing 1 g of biomass in triplicate and drying it at 105 °C for 24 h in an oven (Memmert U30 oven, Schwabach, Germany) for moisture and then submitting it to 550 °C in a muffle furnace (Nabertherm L3/1106, Lilienthal, Germany) for 2 h for ash content, according to the method described in APHA (American Public Health Association) (2012).

2.6. Precipitate analysis

Concerning the precipitate obtained from the mixture of poultry effluent and biomass ash, its proximate and ultimate compositions were determined. The moisture (M), ash content (Ash) and volatile matter (VM) were determined gravimetrically, following the methods prescribed in ASTM 949-88, 830-87, and 897-88, respectively. The fixed carbon (FC) content was obtained as the difference between the full dry mixture and the ash plus volatile matter contents. The ultimate composition analysis was performed by means of a Thermo Finnigan elemental analyser (CE Instruments Model Flash EA 112 CHNS series, Austin, USA). The oxygen content was obtained as the difference between the full dry ashless mixture and the C plus H plus N plus S contents. The mineral composition was determined evaluated through ICP-AES (Inductively Coupled Plasma — Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima, Edison, USA). The conductivity and pH were determined using a Mettler Toledo MC226 conductivity meter (Schwerzenbach, Switzerland) and a Crison MicropH 2001 pH meter (Alella, Spain), respectively.

2.7. Seed germination tests

The germination tests of *Triticum aestivum* (wheat) and *Nasturtium officinale* (watercress) were done using the microalgae biomasses obtained from the fourth test, after the poultry wastewater remediation, as advocated by Zucconi et al. (1981). The *C. vulgaris* and *T. obliquus* cultures were used in concentrations of 0.2 and 0.5 g L^{-1}.

Seed germination tests using precipitate extracts were also done, following the guidelines of Monteiro et al. (2011). The compost sample was (i) dried (80 °C), (ii) sieved (2 mm) (on a vibratory sieve shaker, Retsch, Haan, Germany) (iii) mixed with distilled water at 60 °C, in the proportions of 0 (control), 5, 10, 20, and 40%, and subsequently (iv) stirred with a magnetic stirrer for 3 h. This procedure was also applied to the ash used in the pre-treatment, in proportions of 5 and 10%. The aqueous extracts were then filtered through filter paper (Whatman 2).

In the germination tests, 3 mL of (i) microalga cultures, (ii) precipitate extracts or (iii) ash extracts were pipetted into 90 mm diameter Petri dishes lined with sterile absorbent paper. 50 watercress and 50 wheat seeds were put in each box (with 3 replications per treatment), which was then sealed with parafilm and placed in an incubator at 28 °C and for 5 days without light. The germination index, recorded after the fifth day, is determined by means of:

$$\textit{Germination index}\,(\%) = \frac{G \times W}{G_C \times W_C} \times 100 \tag{1}$$

where G is the number of germinated seeds, W is the seedling weight — Gc and Wc are the values of these same parameters in the control case (distilled water).

2.8. Statistical analysis

Duplicate tests were performed for the microalgae growth and triplicate ones for the germination tests — for all the analyses, data were reported as mean ± standard deviation (SD). Parameters such as the productivity, biomass composition or germination index were compared using the ANOVA analysis of variance with one-way, carried out using the IBM SPSS statistical 23 software. The mean values obtained were compared by means of the Tukey HSD test and the correlation observed was deemed statistically significant when $p < 0.05$.

Table 1
Compositions of the poultry effluents without (PE) and with (PE+A) ash in the batch mode and semi-continuous mode tests (mean ±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 ash content (g L^{-1})	Initial optical density
Batch mode tests	PE	**205** ±19	61 ± 8	**6.3** ±0.9	1.4 ± 0.1	3.5 ± 0.5	1.0 ± 0.1	0.750 ± 0.04
	PE + A	105 ± 11	5 ± 0	1.3 ± 0.1	0.0 ± 0.0	**15.6** ±1.6	13.1 ± 0.7	0.029 ± 0.00
Semi-continuous mode tests	PE	**271** ±24	59 ± 7	**5.6** ±0.3	2.4 ± 0.1	5.7 ± 0.2	2.6 ± 0.2	0.948 ± 0.03
	PE + A	186 ± 14	11 ± 1	1.5 ± 0.1	0.4 ± 0.0	**17.2** ±0.8	15.7 ± 0.2	0.020 ± 0.00

Table 2
Main chemical composition of the biomass ash used in the pre-treatment (wt. %) (mean ±SD, n = 3).

Parameter	Value	Parameter	Value
pH	13.02 ± 0.03	SO_3	0.92 ± 0.01
CaO	65.9 ± 0.2	P_2O_5	0.76 ± 0.01
Cl	11.5 ± 0.04	Na_2O	0.56 ± 0.01
SiO_2	6.59 ± 0.06	MnO	0.17 ± 0.01
Al_2O_3	3.97 ± 0.04	BaO	0.163 ± 0.002
MgO	3.16 ± 0.02	ZnO	0.088 ± 0.001
TiO_2	2.52 ± 0.06	SrO	0.086 ± 0.001
Fe_2O_3	2.28 ± 0.03	Cr_2O_3	0.068 ± 0.006
K_2O	1.18 ± 0.03	CuO	0.058 ± 0.003

3. Results and discussion

3.1. Microalga growth and remediation

The compositions of the poultry effluents, without (PE) and with (PE+A) ash, used in the different tests are given in Tables 1 and 2 provides the chemical composition of the biomass ash used in the pre-treatment.

Although the effluents used do not have high nitrogen and phosphorus load, the initial COD load is very high (6140 mg O_2 L^{-1} and 5630 mg O_2 L^{-1}).

This mineral waste was mainly composed of CaO (65.9%) and contained several other water-soluble components such as MgO or Fe_2O_3; the alkalinization potential of this ash is expressed by its pH of 13.0, that corresponds to the equilibrium pH in aqueous solution. This biomass ash had some heavy metals such as copper, chromium, manganese, and zinc, but the amounts present are almost vestigial, being far below the limits for incorporation in the soil (Portuguese Ministry of the Environment, 2009a).

The batch mode experiment ran for 10 days — at the 10^{th} day, (i) the nitrogen and phosphorus available fell below their discharge limits (15 and 10 mg L^{-1}, respectively) and (ii) the COD in the medium PE+A reached the discharge limit of 150 mg O_2 L^{-1} (Portuguese Ministry of the Environment, 1998).

Regarding biomass productivities in batch mode obtained with the three microalgae grown in the control medium and poultry effluents PE and PE+A, it was observed that the highest and lowest biomass productivities were obtained with the microalgae *T. obliquus* grown in the ashless effluent PE (94.9 ± 2.8 mg L^{-1} day^{-1}) and control medium (50.0 ± 5.3 mg L^{-1} day^{-1}), respectively. On the other hand, the microalgae *C. vulgaris* and *C. protothecoides* grew better in the effluent PE+A than in its PE counterpart (76.2 vs. 65.1 and 72.0 vs. 61.2 mg L^{-1} day^{-1}, respectively) - note also that the biomass productivity in the control medium is the intermediate one in both cases. Calixto et al. (2016) grew *Chlorella* sp. in a bio-compost of chicken excrements and in raw chicken manure, attaining biomass productivities of 6.8 and 4.3 mg L^{-1} day^{-1}, respectively, in 17 days — these values are considerably lower than those obtained in the present study. However, it should be noted that Markou et al. (2016) grew *Chlorella vulgaris* in (20x) diluted poultry litter leachate for 11 days, achieving productivities ranging from 160 to 169 mg L^{-1} day^{-1}. Moreover, Ferreira et al. (2018) grew *Scenedesmus obliquus* in poultry slaughterhouse effluent (COD of 3.7 g O_2 L^{-1} and 122.7 mg N L^{-1}) for 29 days and achieved a productivity of 100 mg L^{-1} day^{-1}.

In the batch mode tests, the remediation capacity for total nitrogen was 100% in all variants. Regarding phosphorus, the microalgae grown in PE+A completely remedied it, whereas in PE the remediation was 82.0 ± 2.5%. Similar studies with poultry effluents also found a remediation close to 100%, for total nitrogen and phosphorus (Ferreira et al., 2018). However, the remediation achieved with diluted poultry litter anaerobic digester, for total nitrogen and phosphorus, was of about 65% and 85%, for *Chlorella minutissima*, and 70% and 88%, for *Scenedesmus bijuga* (Singh et al., 2011). For the poultry effluent PE, the COD remediation was above 93% with the three microalgae, whereas for the PE+A this remediation varied between 70% (Cp) and 83% (To). While the BOD_5 was remediated to almost 100% by the three microalgae in PE, in the case of PE+A the Cp microalga exhibited significantly better performance: a remediation rate of 75%, compared with 39% (Cv) and 47% (To). Despite lower COD levels in the diluted poultry litter leachate, Markou et al. (2016) attained lower remediation rates (between 45 and 82%).

Table 3
Average biomass productivities and final bioremediation rates for the Cv (*Chlorella vulgaris*), Cp (*Chlorella protothecoides*) and To (*Tetradesmus obliquus*) microalgae in the pre-treatments and first to fourth tests (PE — poultry effluent; PE+A — poultry effluent with ash).

		Microalga	Biomass productivity (mg L^{-1} day^{-1})	Total nitrogen removal (%)	Total phosphorus removal (%)	COD removal (%)	BOD_5 removal (%)
Pre-treatment (batch)		–	–	49	92	79	97
Pre-treatment (semi-continuous)		–	–	50	81	73	85
1st Test (Batch mode)	PE	Cv	65.1	**100**	**86**	**96**	**100**
		Cp	61.2	**100**	82	94	99
		To	**94.9**	**100**	82	93	99
	PE + A	Cv	76.2	**100**	**100**	75	39
		Cp	72.0	**100**	**100**	70	**75**
		To	**79.7**	**100**	**100**	**83**	47
2nd Test (100 mL)	PE	Cv	193.6	50	38	94	**99**
		To	234.0	**68**	**41**	**96**	**99**
	PE + A	Cv	141.9	92	22	92	**100**
		To	**244.5**	**98**	**81**	**100**	95
3rd Test(200 mL)	PE + A	Cv	151.5	**95**	28	**94**	**99**
		To	**204.1**	**95**	**97**	**94**	**99**
4th Test (300 mL)	PE + A	Cv	140.6	71	61	55	**99**
		To	**181.8**	**82**	**89**	**56**	93

Table 3 shows the average algal biomass productivities during the different tests and the remediation rates for each microalga and experiment. The highest biomass productivity was obtained for *T. obliquus* (244.5 ± 5.1 mg L^{-1} day^{-1}) in the second test with PE+A, while the second highest was obtained for the same microalga with PE in the second test. In fact, *T. obliquus* always outperformed *C. vulgaris* concerning productivity and bioremediation. However, in the fourth test no microalga managed to reach the required limits for effluent discharge for the COD parameter (664.0 ± 6.4 and 676.2 ± 8.5 mg O_2 L^{-1}) and total nitrogen (34.2 ± 2.1 and 53.7 ± 2.8 mg N L^{-1}), the first and second values concern *T. obliquus* and *C. vulgaris*, respectively.

Fig. 1 displays the evolution of COD in the last reactor (Cv-3 and To-3) over the three semi-continuous mode tests. The figure shows that, in the second test, the discharge limit for COD is reached after 8 days and remains fairly constant. In the third test the discharge limit is reached after 12 days, while in the fourth test no discharge limit is ever reached for the COD — thus, it can be concluded that the microalgae considered do not have the capacity to remedy such a large addition volume (300 mL of poultry effluent) every other day. To ensure that the treatment is effective (i.e., the effluent can be released from the last/third reactor), the addition volume should not exceed 200 mL at a time, which means 600 mL weekly or 20% of the total volume. The algal productivity during the semi-continuous mode tests increased over time (28 days) in the first, second and third tests. It decreased in the fourth tests where it always decreased, further showing that cultures are not able to multiply at a sufficient rate.

Supplementation of nitrogen and phosphorus was found to be necessary in the second tests, which involve only the addition of 100 mL of effluent every other day — in these tests it was necessary to add $NaNO_3$ twice and KH_2PO_4 once during the 28 days. Supplementation was not necessary in the third and fourth tests, probably because the addition of a larger effluent volume met the microalga culture needs.

3.2. Biomass composition

The compositions of the algal biomasses produced were obtained by quantifying the protein, carbohydrate, lipid, and ash contents at the end of each test (batch or semi-continuous mode). Fig. 2 provides the biomass percentage composition, in dry weight, for first tests (batch mode) involving the three microalgae cultivated in the control medium, poultry effluent (PE) and poultry effluent + ash (PE+A).

The microalgae with the highest protein contents were those grown in PE and also the Cv and Cp grown in their control media. The remaining cases showed significantly lower amounts of protein, namely all those involving microalgae grown in PE+A. Concerning the sugar contents, the To grown in the Bristol medium had 57.4%, while the microalgae grown in PE had an average of 47.8 ± 4.7% and those grown in PE+A a much lower value (29.8 ± 5.4%). Regarding the lipid contents, To was the microalga with the highest lipid contents: 24.9% in PE and 27.2% in the control medium. The remaining cases had lower values with relatively small differences between them: their average is 12.6 ± 2.5%. The algae ash contents are significantly higher in the PE+A cases, since this medium contains much more dissolved salts — To has the highest value, followed by Cv and Cp. In the remaining cases, the ash content varies considerably, between 29.5 ± 2.1% (Cp in the control medium) and 5.0 ± 0.3% (To in the control medium). Comparatively speaking, the *T. obliquus* grown in the same kind of effluent (slaughterhouse poultry) shows lower values of sugars (36.2%) and lipids (19.8%), protein and ash content was not determined (Ferreira et al., 2018).

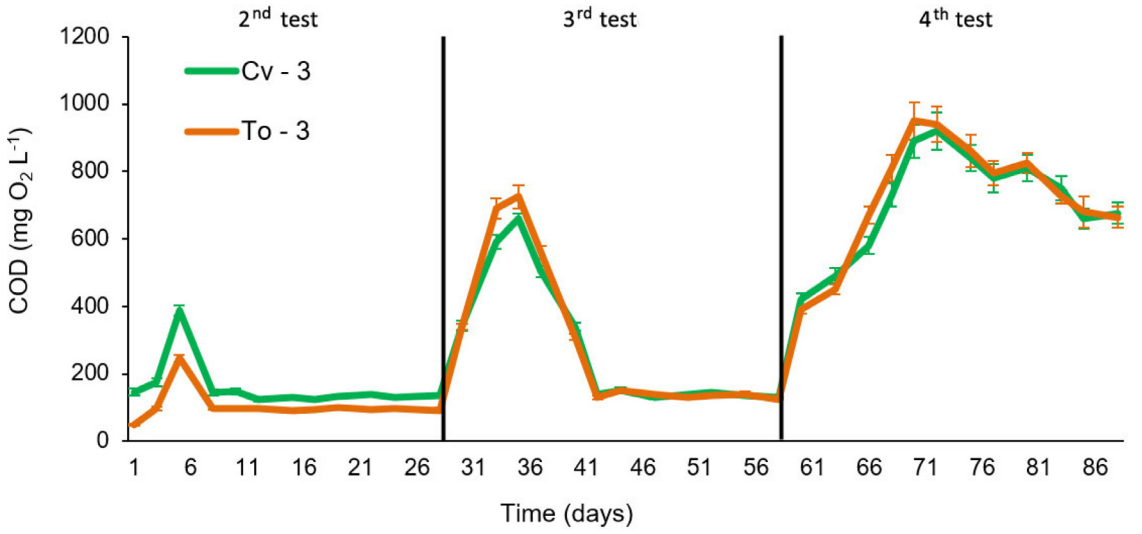

Fig. 1. COD evolution in the last reactor of PE+A (poultry effluent plus ash) in the second to fourth tests, for Cv-3 (*Chlorella vulgaris* in the 3rd reactor) and To-3 (*Tetradesmus obliquus* in the 3rd reactor) (mean ± SD, n = 3).

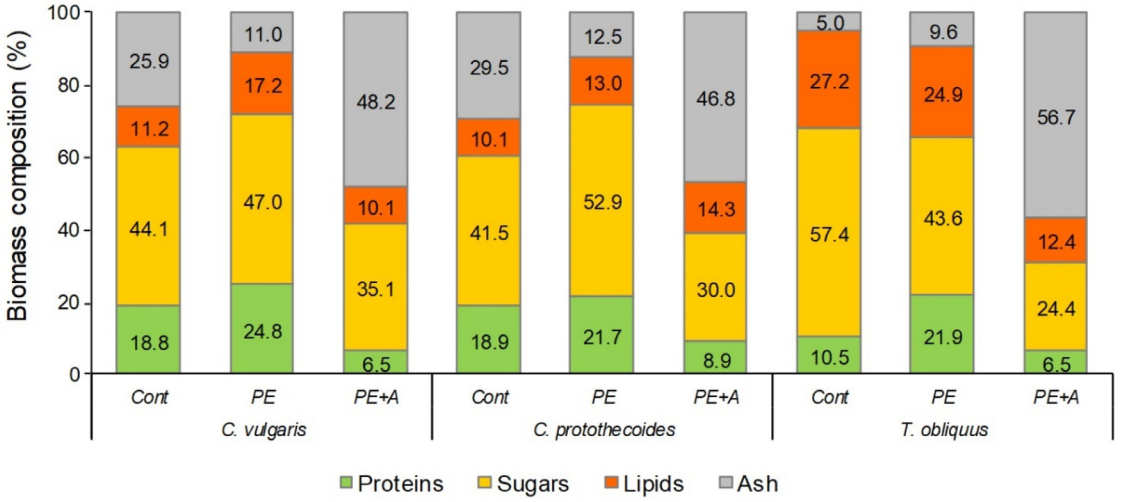

Fig. 2. – Biomass percentage composition, in dry weight, for the first tests (batch mode) involving the microalgae *Chlorella vulgaris* (Cv), *Chlorella protothecoides* (Cp) and *Tetradesmus obliquus* (To) in the control medium (Cont.) and poultry effluent without (PE) and with (PE+A) ash (mean, n = 3).

Fig. 3 provides similar results for the three semi-continuous mode tests but involving only the microalgae Cv and To in poultry effluent without (PE) and with (PE+A) ash.

The comparison between Figs. 2 and 3 makes it possible to conclude that the compositions of the microalgae grown in the semi-continuous mode tests are very similar to those observed in the batch mode tests. It is clear that a pre-treatment with ash leads to microalgae with (i) higher sugar and lipid contents, and (ii) less proteins.

Fig. 4 shows the variation of the fatty acids present in the microalgae grown in PE and PE+A poultry effluents (and also in the control medium, in the batch mode tests). Regarding the fatty acid composition of the microalgae biomasses, it consisted of a mixture of (i) unsaturated fatty acids, including C16:1, C16:2 and oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and conjugated linoleic acid (CLA), and (ii) saturated fatty acids, including palmitic (C16:0), stearic (C18:0), and lignoceric (C24:0), regardless of the algae species and growth medium.

A high content of oleic acid was observed in all the microalgae, particularly for *T. obliquus*: 60.3% and 59.6%, respectively for PE+A and PE. *C. vulgaris* also has a fairly high content when grown in PE+A (48.2%). Note that oleic acid is the predominant methyl ester in olive oil and is beneficial in preventing heart problems in humans, as it lowers blood cholesterol levels and regulates insulin and blood pressure (Jones et al., 2015). In chicken production, diets rich in oleic acid

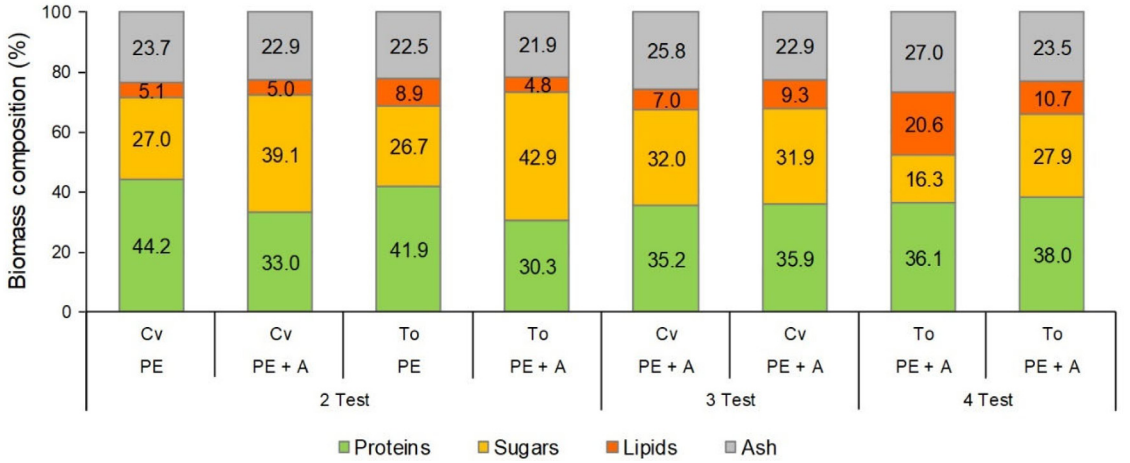

Fig. 3. Biomass percentage composition, in dry weight, for the three semi-continuous mode tests involving the microalgae *Chlorella vulgaris* (Cv) and *Tetradesmus obliquus* (To) (mean, n = 3).

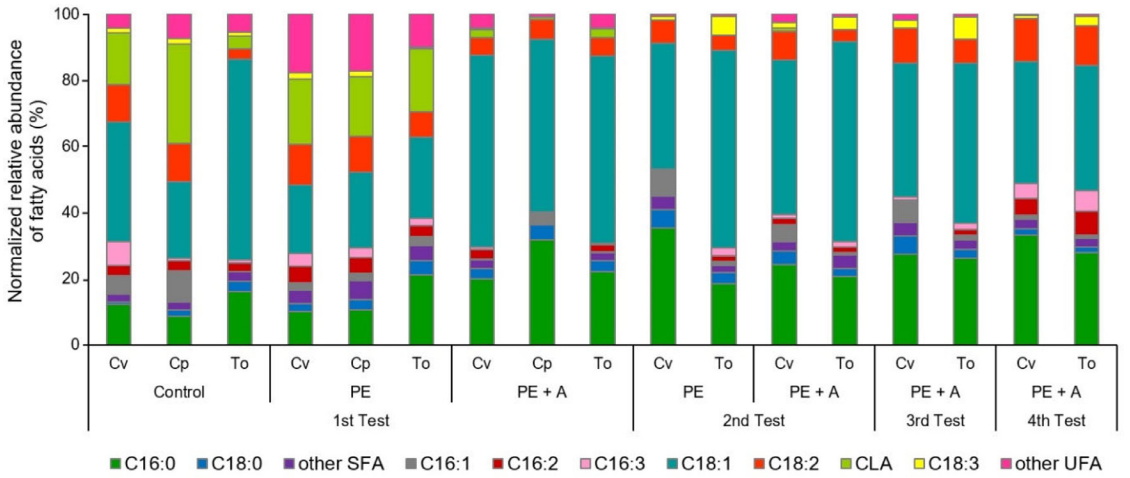

Fig. 4. Fatty acids percentage composition in the microalgae (Cv - *Chlorella vulgaris*, Cp - *Chlorella protothecoides* and To - *Tetradesmus obliquus*) grown in the control medium (first tests) and in the poultry effluent without (PE) and with (PE+A) ash (all tests).

were found to improve the meat and egg quality (Toomer et al., 2020, 2019). Linoleic acid, mostly, and conjugated linoleic acid are two methyl esters exhibiting also reasonable contents in the analysed algae, mainly in the batch mode tests in the control media and PE. It is worth noting that linoleic acid consistently appears in all tests for the three microalgae considered, although its percentages do not differ considerably, it is fair to say that the highest values are obtained for *Chlorella vulgaris*. On the negative side, it must be recognized that, as also reported by Calixto et al. (2016), palmitic acid (another methyl ester) also appears in all tests for the three microalgae, with the highest percentages occurring in the semi-continuous mode tests involving *C. vulgaris*. Several studies mention the benefits of CLA with respect to animal and human health, as it stimulates the immune function with protective effects against cancer, obesity, diabetes and atherosclerosis in both animal studies and different human cell lines (Yang et al., 2015). With respect to chicken feed, it was also found that even a small CLA increase in the basal diet had a noticeable influence in reducing abdominal fat and cholesterol in the liver and eggs of laying hens (Wang et al., 2019).

3.3. Application of microalgal biomass as biostimulant

The influence of various substances on the germination and development capacity of plants can be analysed in several ways. The direct application of algal biomass in seed germination was evaluated through the germination index of wheat and watercress seeds. The potential biostimulant activity was calculated comparing the germination index (GI) of the control medium with distilled water (100%) with those achieved when using the *C. vulgaris* and *T. obliquus* obtained in

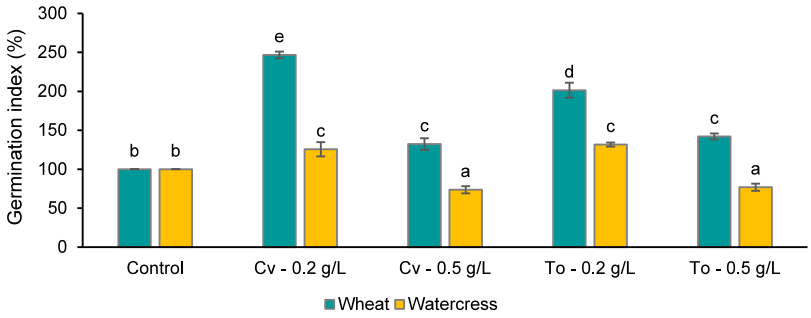

Fig. 5. Germination index for the control medium and the two microalgae cultures (Cv – *Chlorella vulgaris* and To – *Tetradesmus obliquus*) in two concentrations (0.2 and 0.5 g L^{-1}), as an indicator of the potential biostimulant activity (mean ± SD, n = 3).

Table 4
Analytical characterization of the precipitate (mean ±SD, n = 3).

Parameter	Units	Value	Parameter	Units	Value
Moisture	%	11.0 ± 0.2	C/N		122.4
Ash content (Ad%)	%	69.8 ± 0.2	C/P		44.9
Volatile Matter	%	28.2 ± 0.4	Nitrogen	g Kg^{-1}	0.8
Organic matter	%	6.0 ± 0.2	Phosphorus	g Kg^{-1}	4.8
pH		10.5	Calcium	g Kg^{-1}	208.0
Electrical conductivity	dS m^{-1}	5.8	Magnesium	g Kg^{-1}	20.2
Bulk density	m/v	1.48	Potassium	g Kg^{-1}	0.6

the fourth experiment at 0.2 and 0.5 g L^{-1} concentrations (Fig. 5) - note that a GI higher than 100% indicates the occurrence of biostimulant activity.

Both microalgae have a positive influence on the seed germination — between 26% and 147%. The biostimulant activity is more pronounced for the wheat seeds, mostly for Cv - 0.2 g/L (147% increase) and To – 0.2 g/L (101% increase). The addition of ash to the effluent may have also contributed to the positive effect on seed germination, as it contributes with essential minerals to plant development. From the obtained results and confirmed by previous works, the best biostimulant effect is attained from lower concentrations (e.g., Navarro-López et al. (2020)), probably due to an inhibition factor related to higher concentrations. Similar studies involving microalgae grown in cattle and aquaculture effluents reported increases of (i) 77% (To – 0.2 g/L) for watercress seeds and 70% (Cp – 0.2 g/L) for wheat seeds in cattle effluent (Viegas et al., 2021a), and (ii) 238% (Cv – 0.5 g/L) and 80% (To – 0.2 g/L) for watercress and 98% (To – 0.2 g/L) and 84% (Cv – 0.5 g/L) for wheat in aquaculture effluent (Viegas et al., 2021b).

The results achieved in this investigation provide clear evidence about the benefits of using microalgal biomass produced in poultry effluent as biostimulant for seed germination. Besides the phytohormones and aminoacids, both biomasses (Cv and To) contain several important macro and micronutrients for plant nutrition (Ca, K, Mg, Fe, P, Na, and Zn), which are most likely responsible for the beneficial effects. Some heavy metals at trace levels were detected in microalgae biomass (Table S1 — supplementary materials), namely copper and zinc, as these metals are often present in poultry feed, consequently in the effluent and later on absorbed by the microalgae (Muhammad et al., 2020). The replacement of synthetic fertilizers by biostimulant/fertilizer-based microalgae may lead to a more efficient use of resources and a more sustainable agriculture practice.

3.4. Precipitate characterization and germination tests

The precipitate obtained after pre-treating the poultry effluent with biomass ash mostly contains ash and suspended solids previously present in the effluent. Table 4 provides the characterization of the precipitate.

The precipitate had an alkaline pH and an extremely low moisture (11%). At this stage, it is worth noting that the ideal situation corresponds to the range between 30% and 60% (Wang et al., 2020) - however, it should also be pointed out that a higher moisture leads to higher transportation costs, since it affects the soil bulk density. Organic matter consists of carbon-based amount present in the compost and, ideally, its percentage should be higher than 50%. The precipitate considered contains a substantially lower value (6.0%) which, depending on the precipitate proportion incorporated into the soil, may compromise the soil aggregation and/or moisture retention (USDA, 2010). The presence of bases, such as Ca, Mg and K, enriches the soils in such elements, which are essential to plant nutrition — this is an important aspect in Portugal, where most soils exhibit low levels of bases (particularly calcium). The C/N ratio determines the stability of the nitrogen present in each compost — when this ratio is high, the nitrogen is bonded and already stable, which means that it is less accessible to be assimilated by plants and, therefore, the incorporation of nitrogen fertilizers is required. A compost with a C/N ratio below 20 can supply significant amounts of nitrogen, since they are more susceptible to

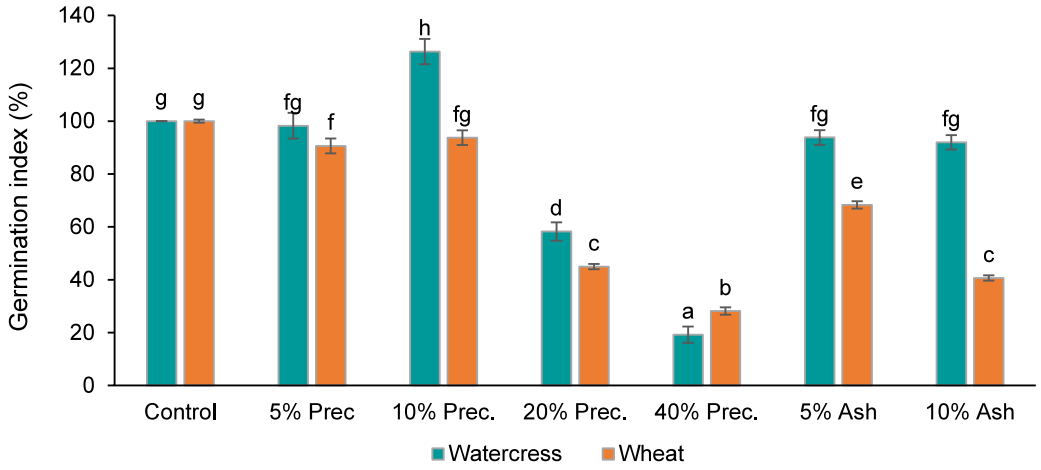

Fig. 6. Germination index of watercress and wheat seeds (mean ± SD, n = 3) for the control medium and different aqueous extracts of precipitate and biomass ash (Prec. — Precipitate aqueous extract; Ash — Ash aqueous extract).

decomposition (Deng et al., 2020). In view of this knowledge, the value 122.4 indicates that the nitrogen in the compost is practically inaccessible to the plants. Because the precipitate has a high phosphorus content (4.8%), it constitutes a significant source of this nutrient — however, it was found that the organic P mineralization becomes more difficult as C/P increases. Regarding micronutrients and heavy metals, the application of the precipitate incorporates in the soil amounts much lower than both the maximum values allowed and those prescribed by the Portuguese authorities for the agricultural use of sludge originating from the processing of organic products (DL276/2009 - (Portuguese Ministry of the Environment, 2009b)).

Because watercress is a plant very sensitive to excess mineral elements and organic phytotoxic substances, it is commonly used as a test plant (Luo et al., 2018). These phytotoxic substances are formed during composting and their presence indicates that the compost has not yet reached the required maturity (Muscolo et al., 2018). Since wheat is a cereal that integrates human food and many animal diets, it was also chosen for the seed germination test. Fig. 6 provides the germination index of watercress and wheat seeds for the control medium, precipitated aqueous extracts and ash aqueous extracts.

The results show that wheat is more susceptible to the presence of the precipitate or ash than watercress. These germination tests show that a positive effect, with respect to the control medium, is obtained in only one case: incorporation of 10% of precipitate in the germination of watercress seeds — the germination index increases by 26%. Although all the remaining cases exhibit negative effects, such effects are, with one exception, more pronounced in the wheat seeds — the exception is the incorporation of 40% of precipitate, when the germination index decreases are very large for both seeds. At this stage, it is worth noting that Zucconi et al. (1981), who developed the procedure adopted in this work, are of the opinion that germination index drops below 10%, with respect to the control medium, are not significant and suggest that the precipitate has an adequate degree of maturation. According to this criterion, meaningful toxicity is only present in watercress seeds when 20% or 40% of the precipitate is incorporated. On the other hand, such toxicity is only absent in wheat seeds when 5% or 10% of the precipitate is incorporated — in particular, the incorporation of biomass ash is always toxic, regardless of the amount. Finally, it should still be pointed out that the incorporation of the precipitate in the soil may alter their expected behaviour, due to the interaction with the soil nutrients.

3.5. Operational costs of laboratory-scale microalgal treatment in this study

The operating costs of semi-continuous effluent treatment carried out by microalgae during the 28 days were limited to the lamps' consumption and the agitation of cultures by the air pump. During this period the lamps consumed 30.2 kWh and the pump 3.4 kWh to obtain 4.8 L of treated effluent. It is worthy to mention that at the laboratory level, consumption was not optimized, and the lamps were underused. Implementing this strategy on a large scale, it should be outdoors and with natural lighting. There are large facilities demonstrating this technology (with the capacity to treat 3000 m^3 day^{-1}) such as AQUALIA, a company in Spain, which result in very low consumption for the treatment of effluents (0.2 kWh m^{-3}) with the production of significant amounts of biogas (12,775 kg ha^{-1} per year) and 90 t ha^{-1} per year of biomass for biofertilizer applications, reflecting what can be achieved in the treatment of effluents with microalgae (Acién et al., 2017). In the present study, microalgae could be used for animal feed, after overcoming the legal issues, and/or to improve the productivity of agricultural crops used on animal feed, in a real circular bio-economy.

3.6. Process integration and production potential

According to the results obtained in this study, and assuming a farm of broiler chicken production with 10,000 birds, where 450 chickens are slaughtered per day (22 days per month) after 34 days of growth. According to the existing data (González-García et al., 2014), a farm with these dimensions would originate about 3.35 m^3 of slaughterhouse cooking water daily. To proceed with the pre-treatment of this effluent, approximately 605 kg of biomass ash would be needed every 2 days (Figure S2 — supplementary materials). Which would result in 802 kg of precipitate, that could be integrated into the soil as a fertilizer, and 7.05 m^3 of effluent ready to be remedied by microalgae. At the end of the remediation process, 7.05 m^3 of treated effluent and 3.45 kg of *T. obliquus* algal biomass would be obtained (or 2.73 kg if *C. vulgaris* were chosen). In this case the treated effluent could be used for irrigation and the algal biomass obtained every two days at the end of the process is reduced so it could only be used in the chicken feed as a weekly food supplement of 2% or integrated as a biostimulant in the production of chicken feed crops.

3.7. Practical applications and future research prospects

The study developed aims to shed light on the use of simple and inexpensive processes in the treatment of poultry slaughterhouse effluents. Therefore, it can be applied by agricultural farms and/or companies without the need for large investments. The obtaining algal biomass can be included in feed of the produced animals or used in agricultural crops by the farmers as an important asset of the process, increasing the sustainability and the circular bio-economy. In the future, it would be important to develop the transfer process on a large scale and test its real applicability, namely in terms of the application of the precipitate to the soil and its interactions with it. There are still some technological, regulatory, and market-related barriers but the results of this study and similar ones, emphasizes a huge potential for sustainable development as long as the economic, social and environmental challenges are addressed.

4. Conclusion

Poultry slaughterhouse effluents are suitable for the growth of microalgae and enable an efficient remediation after pre-treated with 80 g L^{-1} of biomass ash. It is possible to operate an efficient semi-continuous treatment system with successive transfers between reactors if transferred effluent does not exceed 20% of each reactor volume, with an HRT of 5.

Microalgae biomass showed good composition to be used in poultry diets or as supplement, allowing for the supply of a balanced amount of protein and a healthy range of unsaturated fatty acids.

This integrated approach enables to (i) reducing ash deposition in landfills, (ii) recovering nutrients from the effluent, (iii) use ash from the precipitate as fertilizer, (iv) re-use water in slaughterhouses, (v) use water for irrigation purposes, (vi) discharge water in water bodies, (vii) use microalga biomass in wheat seeds to increase productivity and lower costs of poultry feed, in a circular bioeconomy platform.

CRediT authorship contribution 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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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2021.102048.

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