Acta Scientific AGRICULTURE (ISSN: 2581-365X)

Volume 5 Issue 7 July 2021

Research Article

Integrated Treatment of Pig Production Wastewaters Using Pre-treatment with Biomass Ash and Bioremediation by Microalgae

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Received: June 08, 2021

Published: June 24, 2021

Abstract

Animal production leads to effluents with high loads of macro and micronutrients, and therefore with a huge potential of water bodies eutrophication. Conventional wastewater treatments are expensive, energy-consuming, release greenhouse gases (GHG), and produce a residual sludge. The use of microalgae for wastewater treatment allows recovery of nutrients (N, P, COD), minimize GHG emissions, and can significantly reduce costs relatively to conventional treatments. Microalgae have been used in the bioremediation of various effluents, such as sewage, manure, brewery, dairy, urban, among others.

In this work, piggery effluents were remediated by combining a physico-chemical pre-treatment with biomass ash and bioremediation with microalgae (*Chlorella vulgaris*, *Chlorella protothecoides* and *Tetradesmus obliquus*). The mixture of piggery effluent with biomass ash was stirred and fractionated by decantation to yield a liquid fraction and a solid precipitate. The fortification of the liquid fraction with olive-oil mill wastewater was also evaluated. Microalgae grown in the pre-treated effluent, in semi-continuous mode reached productivities of 258 and 237 mg $L^{-1}day^{-1}$ for *C. vulgaris* and *T. obliquus*, respectively. Both microalgae reached nutrient removal efficiencies of 100, 100, 90, and 100% for N, P, COD, and BOD_5, respectively. The microalgae composition was evaluated in terms of protein, sugar, lipid, fatty acids and ash contents.

The produced microalgae biomass was tested as biostimulants for the germination of wheat and watercress seeds with positive results, namely the fortification with *C. vulgaris* biomass produced an increase of 86% in the germination index of watercress seeds. The solid precipitate was tested as fertilizer for the germination of the same seeds, but the results were not as good as applying the algal biomass.

Keywords: Bioremediation; Wastewater Treatment; Piggery Effluents; Biomass Ash; Olive-oil Mill Wastewater; Fertilization

Introduction

Urban, agro-industrial and animal production wastewaters have been treated conventionally, by complex and costly technologies to avoid pollution of water streams, air and soils. Water contamination with pathogens and excess nutrients, is an environmental problem since it can lead to the eutrophication of fresh and marine ecosystems [1].

In 2018 the meat production sector in the European Community (EU28) amounted to 172,076.32 million EUR [2]. In that year,

EU produced 23.85 million tonnes of pork, and Portugal around 361,510 tonnes [3]. The pig sector has annual emissions of 668 million tonnes CO_2-eq., 27% of which corresponds to emissions from manure management. The total manure and feed emissions in industrial pig production systems was 5.2 kg CO_2-eq/kg carcass weight [4]. In Portugal it is estimated that around 12 million cubic meters of manure are produced annually [3]. The pig farm effluents are mostly composed by the natural production of faeces and urine and the wastewater from facility cleaning. Normally these effluents are sent to stabilization ponds and a fraction may be valorised by composting or anaerobic digestion. The stabilization ponds requires the existence of several ponds for the treatment process to take place over time, so the effluent could be transferred to the next pond as it matures [5]. Anaerobic digestion implies tight control of the operating parameters and a high dilution ratio of the effluents, so that microorganisms are not inhibited, being a limited solution for processing large volumes of piggery effluent [6].

The use of microalgae reduces to half the energy consumption of conventional treatment, allowing the recovery up to 90% of the nutrients present into wastewater, with the advantage of production a valuable biomass for animal feeding and agriculture uses, and/or feedstock for biofuel production, thus enhancing the productivity and sustainability of the whole process [7,8].

Conducting a physico-chemical pre-treatment in the agro-industrial effluents leads to an almost sterilization of the effluent, as well as the precipitation of several particles in suspension allowing a better light penetration and its use as culture medium, namely for microalgae.

Chemical precipitation is a widely applied pre-treatment for wastewater treatment that aims to remove ammonia-nitrogen, heavy metals and other non-biodegradable organic compounds [9,10]. This process involves the combination of metal cations and some soluble anions to form insoluble species that precipitate and are subsequently removed by sedimentation or filtration. Several chemical precipitating agents can be used in this process, such as lime (CaO), hydrated lime ($Ca(OH)_2$) and combinations of magnesium oxide (MgO) and phosphates (PO_4^{3-}) [11,12].

The advantages of chemical precipitation when compared to other methods are its simplicity and low implementation costs. But the constant consumption of the chemical agent and the need to eliminate the generated sludge may increase operating costs, impairing economic viability [13]. As such, minimizing costs for this process entails finding low-cost precipitating agents, with suitable chemical characteristics and that are available in significant amounts. Biomass ash is an inorganic by-product of solid biofuels' combustion that is generated in large amounts in industrial boilers or thermal power plants. Usually, this by-product contains high concentrations of silicon, aluminium, iron, calcium, and magnesium oxides, and is known for its precipitating capacity [14]. There is an increasing number of thermal power plants operating on forestry biomass or biomass wastes worldwide, generating around 480 million tons of ash [15]. This ash may be used for soil amendment or incorporated in construction materials. However, new valorisation processes should be proposed in order to manage such large quantities following environmentally friendly and sustainable criteria [14,16]. Chemical precipitation using fly or bottom ash is not very well documented in the literature and the works are mostly focused on the removal of metallic species from industrial wastewater [17]. Although chemical precipitation allows removal of several organic and inorganic contaminants, the process also involves extensive dissolution of ash components in the aqueous medium, namely calcium or magnesium cations, hydroxide ions or phosphates. Those soluble components yield high COD values and high pH values to the treated effluent. Consequently, the effluent must be acidified to return to neutrality and subjected to reverse osmosis or ion exchange processes to achieve regulated COD values [18].

Olive-oil mill wastewater (OMW) is a wastewater produced in large quantities in olive oil producing countries like Portugal. This residue is originated by the olive-oil extraction process and generates 1 to 1.6 m^3 of wastewater per tonne of olive fruit processed [19]. It constitutes a significant environmental problem mainly due to its high chemical oxygen demand (COD) and difficult biodegradability related to its antibacterial activity induced by the high quantity of polyphenols and tannins. In 2018 olive oil production in Portugal was 135,000 tonnes, resulting in a production of 725,000 to 1,161,000m^3 of OMW [20]. The olive-oil wastewaters has been previously tested for the growth of the microalga *Arthrospira platensis*, however, OMWs were used after a sodium hypochlorite treatment (NaClO) which significantly decreases the concentration of phenols and turbidity of the medium but also raises environmental problems [19].

Remediation studies with *Scenedesmus obliquus* were conducted in piggery effluents reaching remedies of 98% for nitrogen and 60% for COD, starting from effluents with an initial load of 14.2 gO_2 L^{-1} and 3.2 gN L^{-1} previously diluted in tap water to 5% (v/v).

Another study with *Chlorella vulgaris* and *Scenedesmus obliquus* reached biomass concentrations of 0.53 and 0.49 mg L^{-1}, respectively, after 20 days of cultivation. The achieved remediation rates were 58 and 50% for nitrogen and 28 and 27% for COD, with *C. vulgaris* and *S. obliquus*, respectively. In this case, the effluent had a relatively low COD (276 mgO_2 L^{-1}) and 56 mgN L^{-1} [21].

In this work, the treatment and valorisation of piggery wastewater was evaluated using a precipitation step in the presence of forestry biomass ash, followed by bioremediation with microalgae. Incorporating the ash into the piggery effluent is a way to enrich the culture medium in mineral components. The addition of OMW was also tested as an approach to the co-treatment of these two effluents. Following the effluent treatment, the microalgae and the precipitate obtained in the pre-treatment were analysed for their composition, and both were tested as biostimulants in germination tests of watercress and wheat seeds. The advantage of using biomass ash in the precipitation step is to value a residue from biomass combustion and avoid using specific chemicals to achieve the same purpose. The semi-continuous mode proposed for the bioremediation tests, aims to ensure a high production of algal biomass with a low concentration of nutrients in the last reactor, in order to improve the treatment efficiency.

Materials and Mthods

Culture medium

The piggery effluent consisting essentially of pig urine from suckling sows came from Raporal, from a farm near Setúbal, Portugal and was stored at 4°C. This effluent was pre-treated with biomass ash in a proportion of 120g per litre under agitation for 48h. The bottom ash sample used was supplied by Prélis Cerâmicas Lda. and is a by-product of the combustion of forestry biomass with a small percentage of polymeric residues of ceramic furnaces. The olive-oil mill wastewater (OMW) came from Beira Baixa - Portugal and was provided by Herdade da Tapada da Tojeira, an organic olive oil producer.

The trials were performed testing two conditions: piggery effluents pre-treated with ash (P+A) and piggery effluents pre-treated with ash plus 2% of olive-oil mill wastewater (P+A+O). The microalgae were also grown in a synthetic culture medium - BG-11 [22] that served as control (C).

Microorganisms and culture conditions

The tested microalgae were *Chlorella vulgaris* (INETI 58, LNEG_UBB, Portugal) (Cv), *Chlorella protothecoides* (UTEX 25, USA) (Cp) and *Tetradesmus* (formerly known as *Scenedesmus*) *obliquus* (ACOI 204/07, Portugal) (To). The trials were conducted in 1L Erlenmeyer flasks, agitated by an air flow of 15.2 L L^{-1}culture h^{-1}. The microalgae grew at room temperature (22°C ± 2°C), under artificial lighting (± 200 µE m^{-2} s^{-1}) with cycles of 12h light/12h dark. The inoculations were performed using approximately 16 mL of inoculum (2% of the final volume), in order to have an initial optical density (at 540 nm) between 0.2 and 0.4 [1].

Trials

Four sets of trials were performed: batch mode tests with the three microalgae and control (C - synthetic medium), piggery effluents pre-treated with ash (P+A), piggery effluents pre-treated with ash plus 2% of olive-oil mill wastewater (P+A+O) (1st set of trials), batch mode tests with *C. vulgaris* and *T. obliquus* with piggery effluent + ash (P+A) (2nd set of trials), semi-continuous mode tests with *C. vulgaris* and *T. obliquus* with periodic transfer of 50mL of (P+A) (3rd set of trials) and with periodic transfer of 100 mL of (P+A) (4th set of trials). The batch mode trials lasted for 12 days and semi-continuous for 20 days. The 2nd, 3rd and 4th trials were performed with *C. vulgaris* and *T. obliquus*, using a series of three reactors with 1L for each microalga (Cv-1, Cv-2, Cv-3 and To-1, To-2, To-3). In the 2nd trial the 3 reactors of each microalga served as replicates. In 3rd and 4th trials each reactor received every other day *x* mL of effluent from the previous reactor, and the first reactor received *x* mL of piggery effluent, according to Figure 1. In the 3rd trial the *x* was 50 mL and in the 4th trial the *x* was 100 mL.

In the 3rd and 4th trials (semi-continuous mode) reactors 2 and 3 were also supplemented with aqueous $NaNO_3$ to a final concentration of 20 mgN L^{-1} in the culture medium, every week [23] and with aqueous KH_2PO_4 to a final concentration of 10 mgP L^{-1}.

Figure 1: Scheme of the transfer process for the 3rd and 4th trials. In the 3rd trial *x* is 50 mL and in the 4th trial *x* is 100 mL.

Microalgae growth evaluation

During the trials, samples were collected every other day to analyse total N, P, COD, BOD_5 and total solids content and total ash content in the medium according to the Standard Methods for Water and Wastewater [24]. Total phenolics were measured by the Folin-Ciocalteu method adapted by Singleton., *et al*. [25]. Samples were also taken every week to evaluate biomass dry weight by filtering the samples through a Whatman GF/C 47mm. In the 1st and 4th trials after the decrease of N, P, and COD below the discharge limits (15, 10 and 150 mg L^{-1}, respectively) [26], the culture was harvested by centrifugation at 7000 rpm for 5 minutes. The supernatant was evaluated for all the same parameters as in the beginning, and the biomass was dried at 45°C for 48h. For the 2nd and 3rd trials at the end of the experiment part of the culture was used for analysis and the rest was used for the following trial.

Microalgae biomass characterization

The algal biomass was grounded using a Retsch ball mill - model MM400 for 4 min at a speed of $25s^{-1}$ and characterized for total protein, total carbohydrate, and total lipid contents. Total nitrogen was quantified by the modified Kjeldahl method [27]. Total protein was calculated by multiplying total nitrogen content by the conventional conversion factor of 6.25 [28]. Carbohydrate content was determined by quantitative acid hydrolysis according to Miranda., *et al*. [29] followed by the phenol-sulfuric method [30]. Lipid content was determined after Soxhlet extraction with n-hexane for 6 hours. 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), 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 250ºC, at 5 ºC/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) [31]. Finally, the moisture and ash content of the algal biomass were determined according with the method described in APHA [32].

Precipitate analysis

The proximate and ultimate compositions of the precipitate was evaluated using standard methods. Moisture (M), ash content (Ash) and volatile matter (VM) were determined gravimetrically according to the ASTM methods 949-88, 830-87, and 897-88, respectively. Fixed carbon (FC) was established by difference. Ultimate analysis was performed using an elemental analyser (Thermo Finnigan - CE Instruments Model Flash EA 112 CHNS series). Oxygen content was achieved by difference. The mineral composition was evaluated through ICP-AES (Inductively Coupled Plasma - Atomic Emission Spectrometer, Ultima). Conductivity and pH were determined using a conductivity meter (Mettler Toledo) and a pH meter (Crison MicropH), respectively.

Seed germination tests

The germination tests of *Triticum aestivum* (wheat) and *Nasturtium officinale* (watercress) were done by the method proposed by Zucconi., *et al*. [33], using *C. vulgaris* and *T. obliquus* biomass produced in piggery wastewater, at fortification levels of 0.2 and 0.5 g L^{-1}. Seed germination tests using precipitate extracts were also done following a method adapted from Monteiro and colleagues [34]. The dried precipitate sample was sieved (< 2 mm) and mixed with distilled water at 60°C in the proportions of 0 (control), 5, 10, 20, and 40% and subsequently stirred with a magnetic stirrer for 3 hours. This procedure was also applied to the ash used in the pre-treatment, at 5 and 10%. The aqueous extracts were then filtered through filter paper (Whatman 2) and used in the germination tests at volume of 3 mL/Petri dish. The seeds to be tested (50 watercress and 50 wheat seeds) were distributed over the Petri dishes lined with sterile absorbent paper, with 3 replicates per treatment. The Petri dishes were sealed with parafilm and placed in an incubator at 28ºC, without lighting for 5 days. The germination index was determined by the Equation (1):

$$Germination\ index\ (\%) = \frac{G \times W}{G_C \times W_C} \times 100 \text{-------- (1)}$$

Where G is the number of germinated seeds and W is the seedling weight. Gc and Wc are the same parameters, but in the control (distilled water).

Statistical analyses

Trials were performed in duplicate for microalgae growth and in triplicate for germination tests and for all the analysis, data were reported as mean ± standard deviation (SD). The parameters such as productivities, biomass composition 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 $p < 0.05$.

Results and Discussion

Pre-treatment evaluation and precipitate characterization

The chemical composition of the biomass ash used in the pre-treatment of the piggery effluent is reported in table 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.

Parameter	Value	Parameter	Value
pH	13.0 (± 0.03)	SO_3	0.92 (± 0.010)
CaO	65.9 (± 0.20)	P_2O_5	0.77 (± 0.010)
Cl	11.5 (± 0.04)	Na_2O	0.56 (± 0.010)
SiO_2	6.6 (± 0.06)	MnO	0.17 (± 0.008)
Al_2O_3	4.0 (± 0.04)	BaO	0.16 (± 0.002)
MgO	3.2 (± 0.02)	ZnO	0.088 (± 0.001)
TiO_2	2.5 (± 0.06)	SrO	0.086 (± 0.001)
Fe_2O_3	2.3 (± 0.03)	Cr_2O_3	0.068 (± 0.006)
K_2O	1.2 (± 0.03)	CuO	0.058 (± 0.003)

Table 1: Main chemical composition of biomass ash (wt. %).

The piggery effluent was treated with 120g L^{-1} of biomass ash. This treatment had a yield of 89.8% (wt.) of pre-treated liquid effluent and 10.2% (wt.) of precipitate.

The raw piggery effluent, the piggery effluent pre-treated with ash (P+A) and the piggery effluent pre-treated with ash plus olive-oil mill wastewater (P+A+O) were evaluated and had the characteristics presented in table 2.

Conducting a physico-chemical pre-treatment leads to an increment of the pH to 12.5, as well as the precipitation of several particles in suspension allowing a better light penetration and its use as culture medium, namely for microalgae. The pre-treatment with ash results in an effluent with a COD and optical density at least 50% lower and a BOD_5 decrease of about 23%, making this effluent much less organically charged. On the other hand, the pre-treatment considerably increases the total solids of the effluent, as well as its ash content.

The precipitate obtained after the pre-treatment of the piggery effluent with biomass ash is mainly composed of ash and some suspended solids that were found in the effluent. The composition of the precipitate is presented in table 3.

The precipitate had an extremely high alkaline pH and low moisture (27.5%). The organic matter was quite low (5.2%) however, most agricultural soils have levels of 1 to 2%. The presence of organic matter generates benefits to the soil as it conserves moisture and soil aggregation [35]. The presence of calcium, magnesium, and potassium cations are important as they are essential for the development of plants. The C/N ratio is used to check the stability of nitrogen in the compost.

Composts with low C/ N ratios (< 15) indicate that their decomposition can provide high amounts of nitrogen, high ratios indicate nitrogen is stable in the compost and it will be less accessible to be assimilated by plants [36]. The precipitate can be considered as a source of P, as it has a high content of this nutrient (5.2g kg^{-1}), and since the C/P ratio is not very high, phosphorus mineralization is not too complex. The precipitate has a low amount of micronutrients and heavy metals in its constitution, so its incorporation in the soil would never exceed the legal limits allowed for sludge applications [37].

Microalgal productivity and remediation process

The 1st and the 2nd tests ran for 12 days because after this time the discharge limits for total N, P, and COD were reached for poultry effluent plus ash (P+A). The 1st test demonstrate that the three microalgae were able to grow in the control and tested effluents, achieving average productivities of 18.9, 30.9, and 16.1 mg L^{-1} day^{-1} for Cv, Cp, and To, respectively in P+A+O, and 15.8, 11.4, and 19.2 mg L^{-1} day^{-1} for Cv, Cp, and To, respectively, in P+A (Figure 2).

The addition of a reduced quantity of OMW led to an increase in the productivity of microalga *C. protothecoides.* The remaining microalgae do not present significant differences between P+A and P+A+O. Although, microalgae growth was higher for P+A+O, than for P+A, except for *T. obliquus*.

For 2nd, 3rd, and 4th tests, the two algae with the best growth and remediation performance were selected, therefore *C. vulgaris* and *T. obliquus* were chosen.

The hydraulic retention time in the 3rd test was 20 days and in the 4th test was 10 days.

Parameter	Units	Batch mode tests			Ash remediation efficiency (%)	Semi-continuous mode tests		Ash remediation efficiency (%)
		RP	(P+A) (120 g L^{-1})	(P+A+O)		RP	(P+A) (120 g L^{-1})	
pH		6.9 ± 0.1	13.1 ± 0.2	12.8 ± 0.2	-89.9	6.8 ± 0.2	12.5 ± 0.2	-83.8
Total N	mg N L^{-1}	1138.7 ± 15.1	913.7 ± 6.9	913.1 ± 11.5	19.8	860.1 ± 4.3	690.01 ± 5.1	19.8
Kjeldahl N	mg NH_3 L^{-1}	1137.3	912.5	911.7	19.8	860.0	690.0	19.8
Nitrates	mg NO_3^- L^{-1}	7.78 ± 0.82	1.10 ± 0.1	0.71 ± 0.01	85.9	0.046± 0.010	0.007 ± 0.000	84.8
Nitrites	mg NO_2^- L^{-1}	0.00 ± 0.00	0.33 ± 0.14	0.01 ± 0.00	-	0.003 ± 0.000	0.052 ± 0.008	-1633.3
Total P	mg P L^{-1}	13.2 ± 1.0	6.2 ± 1.4	6.8 ± 0.3	53.0	26.2 ± 0.1	6.4 ± 0.0	75.6
O.D.	540nm	1.83 ± 0.04	0.77 ± 0.02	1.067 ± 0.022	58.1	1.593 ± 0.043	0.198 ± 0.009	87.6
COD	mg O_2 L^{-1}	2150.0 ± 31.3	1043.4 ± 58.3	2100.5 ± 63.2	51.5	2300.0 ± 91.4	1171.4 ± 40.4	49.1
BOD_5	mg O_2 L^{-1}	1050.0 ± 26.6	800.0 ± 11.3	970.0 ± 35.3	23.8	1250.0 ± 70.7	970.0 ± 14.1	22.4
Phenols	mg L^{-1}	25.8 ± 2.4	17.2 ± 0.3	87.2 ± 4.0	33.3	n.d.	n.d.	-
Total solids	g L^{-1}	10.1 ± 0.5	27.7 ± 0.3	29.9 ± 0.1	-174.3	6.3 ± 0.0	14.3 ± 0.1	-127.0
Ash content	g L^{-1}	6.2 ± 0.2	24.0 ± 0.4	24.0 ± 0.2	-287.1	4.0 ± 0.1	10.9 ± 0.1	-172.5

Table 2: Characterization of the raw piggery effluent (RP), piggery effluent pre-treated with ash (P+A) and piggery effluent pre-treated with ash and olive-oil mill wastewater (P+A+O).
n.d. – not determined.

Parameter	Units	Precipitate	Parameter	Units	Precipitate
Moisture	%	27.5 ± 0.8	C/N		69.8
Ash content	%	81.2 ± 0.3	C/P		44.5
Volatile Matter	%	17.0 ± 0.9	Nitrogen	mg.g^{-1}	1.5
Organic matter	%	5.24 ± 07	Phosphorus	mg.g^{-1}	5.2
pH		12.3	Calcium	mg.g^{-1}	233.2
Electrical conductivity	dS.m^{-1}	6.2	Magnesium	mg.g^{-1}	20.5
Bulk density	m/v	1.29	Potassium	mg.g^{-1}	2.3

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

In 3rd and 4th experiments a supplementation of $NaNO_3$ and KH_2PO_4 in reactors 2 and 3 of each microalga was used. Since the N:P molar ratio of the piggery effluent used in the semi-continuous mode was 108:1, which indicates that phosphorus concentration may be limiting for microalgal growth, it would be necessary to supplemented the medium with phosphorus [38].

In the semi-continuous mode, the 3rd and the 4th tests last for 20 days. The highest productivity was reached in the 3rd test with

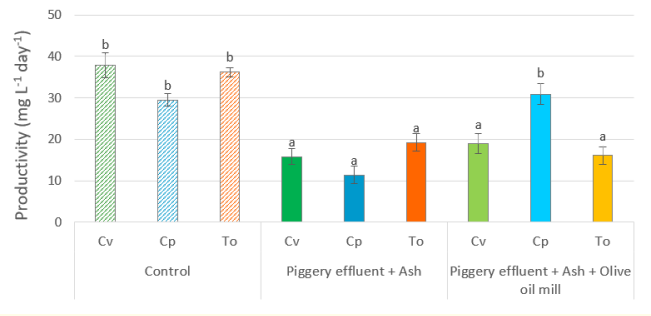

Figure 2: Average productivity for the 1st test in 12 days (mean ± SD, n = 3). (Control; Piggery effluent + ash; Piggery effluent + ash + olive oil mill wastewater; Cv - *Chlorella vulgaris*; Cp - *Chlorella protothecoides*; To - *Tetradesmus obliquus*). Values with different index letters show significant differences with $p < 0.05$.

258.2 ± 7.1 and 236.7 ± 8.9 mg L^{-1} day^{-1} for *C. vulgaris* and *T. obliquus* respectively (Figure 3).

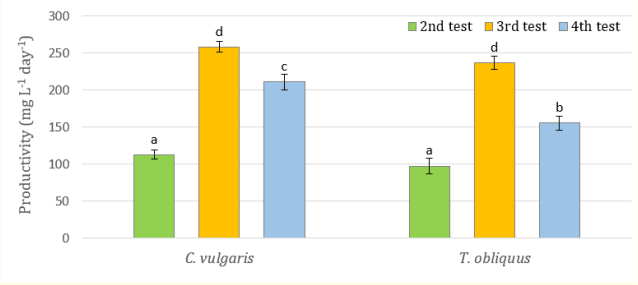

Figure 3: Average biomass productivity of the 2nd (12 days), 3rd (20 days) and 4th (20 days) tests for *Chlorella vulgaris* and *Tetradesmus obliquus* (mean ± SD, n = 3). Values with different index letters show significant differences with $p < 0.05$.

The growth of the microalgae led to an effective removal of nitrogen and phosphorus and other components contributing to COD (Table 4).

The removal efficiency was superior to 82% for N and more than 77% for P in the batch mode. The three tested microalgae have a high potential to remove the N, P, and total solids. Regarding COD

			Total Nitrogen (%)	Total Phosphorus (%)	COD (%)	BOD_5 (%)	Ash (%)	Phenols (%)	Optical density (%)
1st test (Batch)	Control	Cv	99 c	34 c	-	-	27 b	100 d	51 c
		Cp	99 c	43 d	-	-	26 b	100 d	34 b
		To	100 c	42 d	-	-	29 b	100 d	4 a
	P+A	Cv	91 b	77 e	88 c	99 a	61 c	65 c	95 e
		Cp	93 b	100 g	82 bc	100 a	59 c	59 c	98 e
		To	89 b	100 g	89 c	99 a	61 c	33 a	99 e
	P+A+O	Cv	98 c	91 fg	73 b	98 a	58 c	45 b	90 e
		Cp	99 c	100	76 b	99 a	56 c	40 b	90 e
		To	100 c	86 f	76 b	99 a	57 c	45 b	90 e
2nd test (Batch)		Cv	100 c	100 g	90 c	100 a	15 a	-	96 e
To			100 c	100 g	91 c	100 a	14 a	-	99 e	
3rd test (50 mL)		Cv	99 c	22 b	89 c	99 a	28 b	-	97 e
To			99 c	38 cd	91 c	99 a	23 b	-	99 e	
4th test (100 mL)		Cv	78 a	6 a	40 a	98 a	12 a	-	91 d
To			77 a	30 c	39 a	97 a	10 a	-	94 e	

Table 4: Remediation rates for the microalgae (n = 3) (Cv - *Chlorella vulgaris*, Cp - *Chlorella protothecoides*, To - *Tetradesmus obliquus*) in the four tests (Control and Piggery effluents).

Note: Values with different index letters show significant differences with $p < 0.05$.

removal, microalgae in P+A was shown to be effective in reducing COD. In the case of P+A+O the microalgae were able to significantly reduce N and P, but the COD was not reduced to levels that would allow its discharge. The raw effluent P+A+O had a higher COD (2100.5 mgO_2 L^{-1}) and the values attained were 511.3 and 574.9 mgO_2 L^{-1}, for To and Cv, respectively.

In the 3rd test the remediation to allow the effluent release was reached after 8 days of transfers. Although remediation rates are low for phosphorus (22 and 38%), the discharge value had already been reached before remediation (6.4 mgP L^{-1}). In the 4th test the COD remediation never achieved the required levels for discharge (150 mg O_2 L^{-1}). When the rate is increased to 10% (4th test), its capacity for total N, P and BOD_5 remediation remains, but the COD levels do not fall in the same proportion, as can be seen in Figure 4. Consequently, the discharge limit values of 150 mg O_2 L^{-1} were not reached.

Other study with piggery effluents reached biomass yields of 0.53 g L^{-1} and 0.49 g L^{-1} for *T. obliquus* and *C. vulgaris*, respectively, higher than those achieved in the present study. However the starting effluent had only 1/20 of the total nitrogen of the current study, and the removal rate for nitrogen and phosphorus was only 49% and 18% for *C. vulgaris* and 58% and 24% for *T. obliquus,* respectively [21].

Figure 4: Progress of COD in the reactors of the 2nd test and in the last reactor of 3rd and 4th tests, Cv - *Chlorella vulgaris*, To - *Tetradesmus obliquus* (mean ± SD, n = 3).

Biomass composition

The characterization of the algal biomass from piggery effluents was made in terms of protein, sugar, lipid, and ash contents (Figure 5).

Protein is the most abundant content, independently the microalgae specie or culture conditions. There was a tendency of an ash increase from the 1st to 4th test. The microalga with the highest protein content was *T. obliquus* (51.3%) in the (P+A), the one that had the highest sugar content was *C. protothecoides* (51.8%) in (P+A+O) and the highest lipid content was again To (16.0%) in (P+A+O).

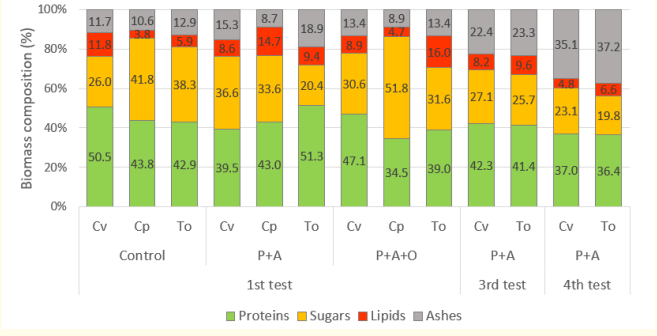

Figure 5: Characterisation of the biomass (% DW) for the 1st, 3rd and 4th tests (mean, n = 3). (Control - synthetic medium; (P+A) - piggery effluent + ash; (P+A+O) - piggery effluent + ash + olive oil mill wastewater; Cv - *C. vulgaris*; Cp - *C. protothecoides*; To - *T. obliquus*).

The lipidic fraction composition is presented in figure 6 and as can be seen, there is a clear predominance of unsaturated fatty acids, mainly oleic acid (C18:1), conjugated linoleic acid (CLA) and linoleic acid (C18:2). The most representative saturated fatty acid is palmitic (C16:0). In semi-continuous tests, both microalgae showed a tendency to have a higher amount of palmitic acid, mainly due to the reduction of CLA, that in the case of 4th test, it does not exist at all.

Application of microalgal biomass

The use of microalgae in human and animal nutrition is not new. However, for its use in animal feed it is necessary that the production costs of microalgae be reduced. One of the approaches to achieve this reduction is to combine the production of microalgae to the effluent's treatment produced by the respective animals, in a perspective of circular economy.

Piggery feed

Swine compete directly with the human food chain because they eat mainly cereals and soybeans, so there is a strong inter-

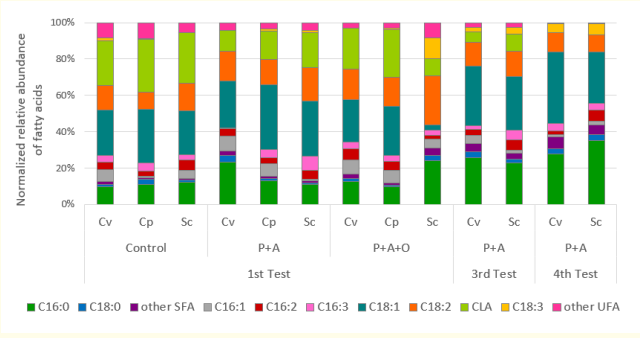

Figure 6: Characterisation of microalgae fatty acids for the four tests (mean, n = 3). (Control - synthetic medium; P+A - piggery effluent + ash; P+A+O - piggery effluent + ash + olive oil mill wastewater; Cv - *C. vulgaris*; Cp - *C. protothecoides*; To - *T. obliquus*).

est in introducing microalgae in pig feed [39]. There are several studies showing the benefit of introducing microalgae in the pigs' diet. Namely with regard to daily weight gain, to average daily feed intake and feed conversion ratio. These studies with durations varying between 2 and 8 weeks, introduced in the pigs' diet (piglets, female pigs, weaned piglets and weaned castrated male swine) between 0.1 (*Chlorella vulgaris*) to 5.51% (*Schizochytrium* sp.) of microalgae [40]. The microalgae *Arthrospira maxima* and *Arthrospira platensis* were also tested with positive results regarding the pigs' daily weight gain. It was reported that *Arthrospira platensis* may increase up to 15-26% of average daily gain, with no effect on backfat thickness [41]. In addition, a beneficial effect on the intestinal development of these mammals was also detected, particularly for *Chlorella*, enhancing the control of mild digestive disorders, without compromising the digestibility of nutrients [40]. Other studies with additions of 10 and 15% of algal biomass in pig feed for 28 and 42 days could enhance growth and decreased plasma uric acid concentrations [42].

There is scant information about the effect of adding microalgae to the diet of sows and gilts and how it affects the reproductive cycle. However, microalgae are a source of DHA and protein, two major components of the sperm membrane. As a result, these two compounds are added to boar diets in order to improve sperm quality [39]. A study with the addition of 150g *Schizochytrium* sp./Kg points to an improvement in the mobility of sperm [43]. On the other hand, the existing information shows the added value of DHA ingestion during gestation and lactation due to the immunomodulatory properties of DHA to improve the immunological status of piglets before and after birth [39].

Biostimulant activity

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% [44]. Other examples were done by Deepika and Mubarak Ali [45] using *Chorococcum* sp. to promote growth in *Cucumis sativus*, *Solanum lycopersicum*, *Capsicum annuum*, and *Vigna radiata*, with good results. Grzesik., *et al.* [46] 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*. [47] had positive results when using *C. vulgaris* for *Hibiscus esculentus* development and its role in enhancing soil fertility. Likewise, Marks., *et al*. [48] applied a liquid slurry to soil of live cells of *Chlorella* sp. from wastewater treatment concluding there is an enhancement of soil fertility.

Plant growth is influenced by phytohormones and aminoacids, among others that can be found in different sources, namely microalgae. This phytohormones includes gibberellins, auxins, cytokinins, ethylene and abscisic acid [49]. To establish the biostimulating capacity of microalgae in seed germination, a complete chemical analysis including the content of amino acids and phytohormone profile should be performed. However, it is simpler to directly test the effect of microalgae biomass on seed development through germination tests. The potential biostimulant activity was evaluated determining the germination index (GI) of the control, with distilled water (100%), and the GI obtained with microalgae biomass of Cv or Cp and To obtained in the last test of each of the tested agro-industrial effluents. If the GI is higher than 100% it is considered that there is a biostimulating activity. Figure 7 shows the results of the tested microalgae cultures, at two concentrations (0.2 and 0.5 gL^{-1}) on the germination of the two species of seeds (wheat and watercress).

Both microalgae had a positive effect on seed germination, between 16% and 86%. The biostimulant effect was more evident in watercress seeds, especially for Cv - 0.2 g/L with an increment of 86%. Cv - 0.5 g/L had similar effect on watercress and wheat with an increment around 62%. Other authors have also obtained promising results of a 40% increase in the germination of watercress

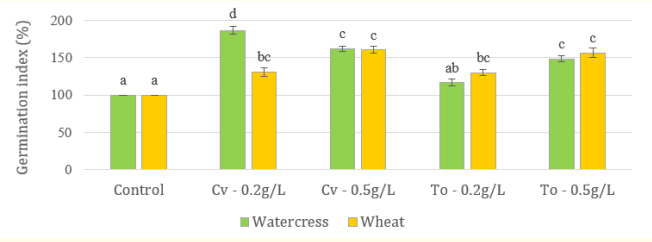

Figure 7: Germination index of wheat and watercress seeds, using the two microalgae cultures (Cv-*Chlorella vulgaris* and To- *Tetradesmus obliquus*), at two concentrations (0.2 and 0.5 g/L).

seeds, using algae biomass of *S. obliquus*, obtained in the treatment of brewery effluents [50]. The results obtained in the present study showed that the use of algal biomass grown in piggery effluent has the capability to be used as a biostimulant in seed germination, to a more sustainable agriculture practice. Both microalgae biomass had important macro and micronutrients for plant nutrition (Table 5), namely calcium, potassium, magnesium, and sodium which could be, besides phytohormones and amino acids, the responsible for the good results.

The results achieved in this investigation provide clear evidence about the benefits of using microalgal biomass produced in agro-industrial effluent as biostimulant for seed germination. A more efficient and sustainable use of resources can be achieved with the replacement of synthetic fertilizers by microalgae-based biostimulants/fertilizers.

	Al	B	Ba	Ca	Cu	Fe	K	Mg	Mn	Na	P	Si	Sr	Zn
Piggery - Cv	0.36	0.01	1.03	8.34	0.04	0.09	**6.28**	2.60	0.03	2.63	0.00	0.23	0.29	0.09
Piggery - To	1.27	0.01	3.82	3.42	0.14	0.50	3.29	0.26	0.09	3.51	0.00	0.66	0.39	0.23

Table 5: Chemical characteristics of microalgae biomass used in biostimulant germination tests, presented in mg/g (Cv - *Chlorella vulgaris*, To - *Tetradesmus obliquus*).

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.

Precipitate as fertilizer

The germination index of watercress and wheat seeds for precipitate extracts, for biomass ash extracts and for control is shown in figure 8. The results obtained demonstrate that the precipitate presented some toxicity for the watercress seeds, since the germination index showed a decline close to 40% compared to the control. However, in the case of wheat, there was an increase in the germination rate of 16.2% for incorporations of 5% of precipitate. Basically, both precipitate or ash, have a negative influence on watercress and wheat seed germination. Nevertheless, its incorporation on the soil could have a different behaviour. The precipitate had a higher concentration of minerals than the ash, namely aluminium, boron, barium, copper, iron, manganese, sodium, titanium, and zinc.

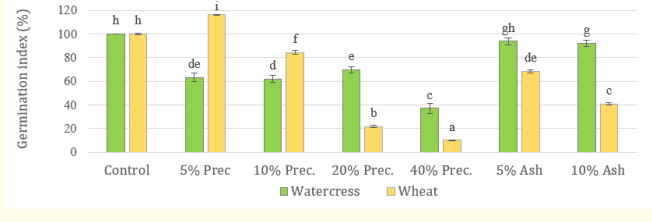

Figure 8: Germination index in watercress and wheat seeds (mean ± SD, n = 3) for control and different aqueous extract of precipitate and biomass ash (Prec. - Precipitate aqueous extract; Ash - Ash aqueous extract).

Process integration and production potential

In order to make feasible and appealing the microalgae production for animal feed, it is necessary that the economic evaluation of the production process is profitable compared to feed alternatives.

Currently, about 20,000 tonnes of microalgae are produced per year worldwide, mainly in raceway ponds [51]. These open systems have as main disadvantages the loss of large amounts of water by evaporation and the contamination with biomass from different sources, which strongly limits the use of the biomass, on the other hand, it presents lower operating costs. Closed systems (photobioreactors) reduce the loss of water, allow to operate with higher cell concentrations and dramatically reduce the risk of contamination [52]. The choice of the production system will therefore depend on the purpose of the biomass that is intended to be produced.

One of the most significant aspects of this assessment is to consider an integrated system. When the objective lies only in the production of biomass, the cost of this biomass becomes higher and is only justified for high-value products. On the other hand, if there are several resources and products involved, in a concept of biorefinery and circular economy it is more likely that the system will become profitable.

If considering the economic and environmental costs of treating agro-industrial effluents, the use of microalgae can be rewarding because in addition to obtaining the treated effluent, we have the added value of obtaining algal biomass.

It is important to consider that during the production of microalgae and respective effluents' treatment, significant amounts of atmospheric CO_2 are absorbed to form algal biomass.

Regarding the porcine sector, assuming an agro-industrial farm with around 1000 animals, which together produce daily 5.98 m^3 of manure (according to the data in point 1), this would represent a requirement of treating 12 m^3 of piggery effluent at every 2 days (Figure 9).

In this situation, to carry out the pre-treatment, 1436 kg of biomass ash would be necessary, resulting in 10.7 m^3 of effluent to be bioremediated by microalgae and 1721 kg of precipitate that could be integrated into the soil as a fertilizer. In this system, 5.55 kg of algal biomass (Cv) would be obtained every 2 days and 10.7 m^3 of treated effluent, which could be discharged in municipal collectors or used for washing and irrigation of feed cultures for pigs. This algal biomass could be integrated into the pigs' diet produced on the farm, however, since it is a reduced amount of biomass, it could only be used as a weekly supplement in the order of 20 g per animal. Alternatively, if the treated effluent were destined for agricultural irrigation, there would be no need to separate the algae from the medium and both products could be used for irrigation.

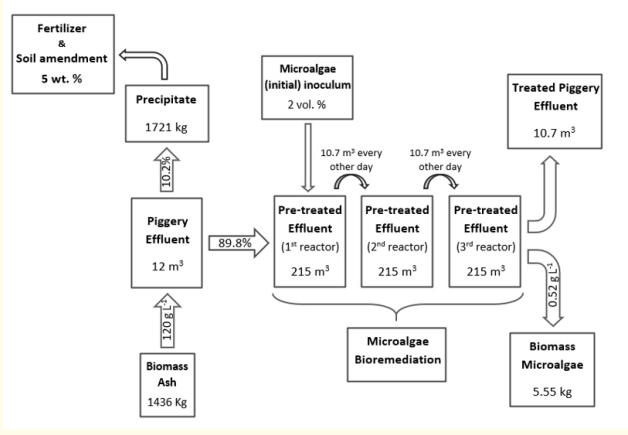

Figure 9: Graphical representation of the piggery effluent treatment process with quantity flows for every two days inputs.

Conclusion

Piggery effluent pre-treated with forestry ash is suitable to the growth of the tested microalgae (*Chlorella vulgaris*, *Chlorella protothecoides* and *Tetradesmus obliquus*) and the incorporation of 2% olive-oil wastewaters did not have a negative effect on microalgae growth. These microalgae achieved excellent rates of bioremediation for total nitrogen, total phosphorus, and COD. The produced microalgae showed high protein contents what suggests its possible use as a supplement for pig feed, in a circular economy strategy. However, further testing would be necessary to validate the incorporation of microalgae in the pig diet in order to select appropriate doses and ensure animal safety conditions. Also, the biomass carbohydrate components could be used to produce hydrogen (by dark fermentation) or ethanol (by alcoholic fermentation). The precipitate obtained after the pre-treatment may serve as fertilizer, after appropriate formulation. The treated effluent can be used as a water resource to irrigate crops or washing operations of animal production facilities. The microalgae biomass could be used as a biostimulant for seed germination with good results in the case of wheat and watercress, thus promoting the production of animal feed.

Acknowledgments

This work has been supported by FCT - Fundação para a Ciência e Tecnologia within the PhD Grant SFRH/BD/131178/2017 and R&D Units Project Scope: UIDP/04077/2020.

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