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Biostimulant and biopesticide potential of microalgae growing in piggery wastewater

Alice Ferreira[a], Lusine Melkonyan[b,c], Sofia Carapinha[a], Belina Ribeiro[a], Daniel Figueiredo[d], Gayane Avetisova[b,c], Luisa Gouveia[a,d,*]

[a] LNEG, National Laboratory of Energy and Geology I.P., Bioenergy and Biorefineries Unit, Estrada do Paço do Lumiar 22, 1649-038 Lisbon, Portugal
[b] SPC "Armbiotechnology" NAS RA, 14 Gyurjyan Str., 0056 Yerevan, Armenia
[c] Yerevan State University, 1 Alex Manoogian, 0025 Yerevan, Armenia
[d] Green CoLab - Green Ocean Technologies and Products Collaborative Laboratory, Centro de Ciências do Mar do Algarve, Universidade do Algarve, Campus Gambelas, Edifício 7, 8005-139, Faro, Portugal

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ABSTRACT

Pig farming generates highly polluting wastewaters which entail serious environmental issues when not adequately managed. Microalgae systems can be promising for cost, energy and environment-efficient treatment of piggery wastewater (PWW). Aside from clean water, the produced biomass can be used as biostimulants and biopesticides contributing to a more sustainable agriculture.

Three microalgae (*Tetradesmus obliquus, Chlorella protothecoides, Chlorella vulgaris*) and one cyanobacterium (*Synechocystis* sp.) were selected after a preliminary screening in diluted wastewater (1:20) to treat PWW. The nutrient removals were 62-79% for COD (chemical oxygen demand), 84-92% for TKN (total Kjeldahl nitrogen), 79-92% for NH_4^+ and over 96% for PO_4^{3-}. *T. obliquus* and *C. protothecoides* were the most efficient ones.

After treating PWW, the produced biomass, at 0.5 g L^{-1}, was assessed as a biostimulant for seed germination, root/shoot growth, and pigment content for tomato, watercress, cucumber, soybean, wheat, and barley seeds. We observed an overall increase on germination index (GI) of microalgae-treated seeds, owing to the development of longer roots, especially in *T. obliquus* and *C. vulgaris* treatments. The microalgae treatments were especially effective in cucumber seeds (75-138% GI increase).

The biopesticide activity against *Fusarium oxysporum* was also evaluated at 1, 2.5 and 5 g L^{-1} of microalgae culture. Except for *Synechocystis* sp., all the microalgae tested inhibited the fungus growth, with *T. obliquus* and *C. vulgaris* achieving inhibitions above 40% for all concentrations.

1. Introduction

The ever-growing population has put an extreme pressure on agriculture to produce more food (Searchinger et al., 2019). Livestock farming practices have largely shifted to intensive animal farming to assure high yields of animal-derived products, but have led to negative impacts on the environment and public health (Anomaly, 2015).

In the European Union (EU), the majority of the protein consumed comes from animal sources (European Environment Agency, 2017). EU is currently one of the largest pig producers, with an average of 148 million pig heads over the last 10 years, according to Eurostat (2020). Consequently, this industry is estimated to generate 215 – 430 m^3/year (4-8 L/day/pig) of piggery wastewater (PWW) (García et al., 2017). PWW is generated from pig excreta and water used clean the hog housing sheds, containing a high organic load, ammoniacal nitrogen, and phosphorus.

While these pollutants are a problem for pig farms to handle, they can be valuable as low-cost and readily available nutrient and water sources for microalgae growth. The use of wastewater allows the reduction of microalgae biomass production below 5 €/kg at large scale (Acién et al. 2016). Microalgal-bacterial systems have already been used to treat PWW (e.g. Ferreira et al. 2018; García et al. 2017). They are commonly described as cost-efficient for nutrient recovery, providing a free process oxygenation, with reduced energy requirements and environmental impacts (Cuellar-Bermudez et al., 2017; Ferreira et al., 2018). In a perspective of circular bioeconomy, microalgae can recover the nutrients from piggery wastewaters, which can generate further income for the pig production facilities, as a source of biofuels (Batista et al., 2015; Ferreira et al., 2018, 2017), animal feed, fertilizers, stimulants and/or

* Corresponding author.
E-mail address: luisa.gouveia@lneg.pt (L. Gouveia).

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pesticides (Ferreira et al., 2019; García et al., 2017, 2018; Navarro-López et al., 2020; Posadas et al., 2017).

There is a growing trend on sustainable agriculture to promote low pesticide-input and the application of natural products, in detriment of minerals and chemicals, which are not only limited but can bring severe environmental problems (e.g., eutrophication, soil infertility, and biodiversity loss) (Bulgari et al., 2015; Calvo et al., 2014; Sharma et al., 2014). Catching this new wave, the use of biofertilizers, biostimulants, and biopesticides derived from microorganisms can promote seed germination, plant growth, flower set and fruit production (Bulgari et al., 2015; Colla and Rouphael, 2020; du Jardin, 2015; Singh et al., 2016), and expand the tolerance to abiotic (e.g. high salinity, drought, and frost) and biotic stresses (e.g. pathogens, pests, and insects) (Carvajal-Muñoz and Carmona-Garcia, 2012; Costa et al., 2019). All these aspects could be fulfilled by microalgae. They contain valuable compounds, such as amino acids, carbohydrates, minerals, trace elements, and phytohormones, among others (Colla and Rouphael, 2020; Górka et al., 2015; Khan et al., 2009). They can enhance plant growth by acting as an organic slow-release fertilizer to supply nutrients assimilated from wastewater and avoid the contamination of soils and water bodies with extreme nutrient loads (Coppens et al., 2016). Microalgae-based biostimulants can also improve nutrient uptake by plants and the soil structure and aeration, which may stimulate root growth (Bumandalai and Tserennadmid, 2019). Microalgae and cyanobacteria have also been shown to have antibacterial and antifungal activity (Costa et al., 2019; Renuka et al., 2018; Singh et al., 2016). However, deeper investigation is required on this agricultural biotechnological field (Costa et al., 2019).

Europe is currently the biggest market for biostimulants, with around 8.5 million hectares of area treated in 2016 (Liebig et al., 2020). This has amplified the need for a harmonized European Regulation for placing biostimulants on the market. Thus, on 2019, a new Fertilizing Products Regulation (FPR) (EU) 2019/1009 was published including biostimulants for the first time as CE-marked fertilizing products on 2022 (Regulation (EU) 2019/1009, 2019). The Global Biostimulant Market was estimated to be valued at USD 2.6 billion in 2019 and is expected to grow 11.24% through 2025 (MarketsandMarkets, 2020).

Considering all the aspects presented, the research on microalgae for agriculture is a very relevant and promising topic. Our work aimed to combine microalgae cultivation with piggery wastewater treatment to generate clean water and bioproducts (bio-fertilizers, stimulants, and pesticides) to respond to an eco-friendlier approach for sustainable agriculture. For this, we did a screening of several microalgae (*Tetradesmus obliquus, Chlorella protothecoides, Chlorella vulgaris*, and *Neochloris oleoabundans*) and cyanobacteria (*Synechocystis* sp. and *Nostoc* sp.) to treat piggery wastewater to select the most successful one(s) in nutrient removal efficiency, and with the best biomass quality for agricultural products. The obtained microalgal biomass was evaluated for germination, root and shoot growth, and pigment content in different seeds, such as watercress, tomato, cucumber, barley, wheat, and soybean. Their biopesticide effect was also investigated against the fungus *Fusarium oxysporum*. The production of biostimulants and biopesticides from microalgae cultivated in wastewaters is yet an unexplored approach, and to the best of our knowledge, few studies address this. Thus, we believe our work can offer an important contribution to better understand the potential of microalgae for agricultural purposes.

2. Materials and methods

2.1. Effluent and microalgae

The piggery wastewater was collected from a stabilization pond in a local pig farm from Valorgado in Herdade do Pessegueiro (39°00′09.0"N 8°38′45.5"W) (Glória do Ribatejo, Portugal) during the month of May. This PWW corresponds to the liquid fraction of pig slurry after separation from solid manure.

The microalgae tested were *Synechocystis* sp. PCC 6803 (Amsterdam University, Netherlands), *Tetradesmus obliquus* (formerly known as *Scenedesmus obliquus*) (ACOI 204/07, ACOI Culture Collection, Coimbra University, Portugal), *Chlorella protothecoides* (also known as *Auxenochlorella protothecoides*) (strain 25,UTEX Culture Collection, Austin University, USA), *Chlorella vulgaris* (INETI 58, 90 LNEG_UB, Portugal), *Neochloris oleoabundans* (UTEX #1185, UTEX Culture Collection, Austin University, USA), and *Nostoc* sp. PCC 9202 (Instituto de Bioquímica Vegetal y Fotosíntesis, Seville, Spain).

2.2. Microalgae/Cyanobacteria screening

A screening was carried out to select the microalgae or cyanobacteria which were able to grow in PWW. The different species tested were inoculated in small flasks using different dilutions (1:20, 1:10, 1:5, 1:2, 1:1) of PWW with tap water as the cultivation medium and were kept at room temperature (23-25°C), under continuous artificial light conditions (3 fluorescent lamps of 18W, Philips TL-D) at light intensity of 41 μE m^{-2}. s^{-1}, and orbital agitation at 150 rpm (G-25 incubator shaker (New Brunswick Scientific Co, USA).

2.3. Wastewater treatment experiments

The microalgae and cyanobacteria capable of growing in 1:20 PWW - *Synechocystis* sp., *T. obliquus, C. protothecoides*, and *C. vulgaris* – were used for further treatment experiments to evaluate their performance on nutrient removal. Because most species are microalgae, from this point forward, *Synechocystis* sp. (cyanobacterium) will be referred to as a microalga as well when mentioning all the species tested, just to simplify the writing.

The microalgae cultures were cultivated in 5 L bubble columns photobioreactors (PBRs) using the same 1:20 PWW as medium, at a working volume of 4 L. The cultures were maintained at room temperature (23-25°C) under continuous illumination (3 fluorescent lamps of 36 W and 6 of 18 W, Philips TL-D) at an average light intensity of 53 μE m^{-2} s^{-1}. The aeration was supplied at 0.15 vvm (air volume (L) per volume of culture medium (L) per minute (m)) through aquarium pumps. After 19 days of cultivation, the microalgae cultures were left to settle for 24 h at room temperature to concentrate the biomass. The supernatant was collected for further analysis. The microalgal biomass was further concentrated by settling for more 24 h at 4°C for germination, plant growth and pesticide trials.

2.3.1. Microalgae growth

The assessment of microalgae growth was monitored by measuring the optical density of the culture samples, at 540 nm (Rocha et al., 2003), against distilled water, using a Hitachi U-2000 spectrophotometer. In addition, the biomass dry weight and the ash free dry weight (AFDW) were determined through gravimetry by drying the samples at 105°C overnight and incinerating at 550°C for 1 h, respectively. The biomass productivity was calculated from the final biomass concentration, given by the AFDW at the end of the cultivation period of 19 days.

2.3.2. Nutrient removal

The initial raw and diluted (1:20) PWW were characterized in terms of ammonia and total Kjeldahl nitrogen (TKN), chemical oxygen demand (COD), and phosphorus, according to the standard methods by APHA (2005), as previously described by Ferreira et al. (2017). Ammonium nitrogen was quantified using an ion selective electrode Crison code: 96 63 (Crison-HACH). TKN was determined by the standard method 4500-N_{org} B with adaptation. The COD determination was carried out by the Open Reflux method – Method 5220-B (APHA, 2005). A commercial kit was used for the measurement of phosphorus (Phosver 3-Powder Pillows, Cat. 2125-99, Hach) at 890 nm, using a HACH DR/2010 spectrophotometer.

To evaluate the efficiency of microalgal-based treatment, the same analyses were performed for the final effluent at the end of the cultivation runs, after settling and filtration.

2.3.3. Microalgae biomass characterization

The biochemical composition of the microalgal biomass was determined in terms of proteins, sugars, and fatty acids. Total sugars (carbohydrates) content was determined through the phenol-sulfuric method (DuBois et al., 1956), following quantitative acid hydrolysis extraction (Hoebler et al., 1989). Protein content was estimated through the Kjeldahl method and calculations were conducted applying the conversion factor 5.95 (López et al., 2010; Waghmare et al., 2016). A detailed description of the methods was already made by Ferreira et al. (2017).

The elemental composition of the microalgae/cyanobacteria biomass was analyzed by x-ray fluorescence (XRF) spectroscopy, using a NitonTM XL3t analyzer (Thermo Fischer Scientific, USA). The analysis was conducted exposing samples of freeze-dried biomass in proper cuvettes to XRF, under helium-rich atmosphere.

2.4. Seed germination study

The seed germination/plant growth experiments were carried out in Petri dishes with Whatmann filter paper, with 8 seeds of each plant, in duplicate. The plants tested were cucumber (*Cucumis sativus*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), soybean (*Glycine max*), watercress (*Nasturium officinale*), and tomato (*Licopersicon esculentum*). For each plant, five treatments were done: control (distilled water) and four microalgae cultures of 0.5 g L^{-1} each (*Synechocystis* sp., *T. obliquus, C. protothecoides*, and *C. vulgaris*). The microalgae cultures were adjusted to the desired concentration (0.5 g L^{-1} for germination and growth experiments, and 1, 2.5 and 5 g L^{-1} for biopesticide trials) by adding distilled water. All samples were incubated at room temperature (25 ºC) in the dark for 5 days followed by sunlight in the remaining 5 days. During the experiment, the samples were watered daily with the same amount of distilled water to keep the filter paper humid.

2.4.1. Root and shoot growth

At the end of 10 days, the seedlings were carefully separated and measured with a ruler and the results registered for comparison between the microalgae treatments and the control with the distilled water.

2.4.2. Germination index

The germination index (GI) of each sample was determined according to Zucconi et al. (1981) by the following equation:

$$GI(\%) = \frac{G \times L}{G_W \times L_W} \times 100 \quad (1)$$

Where G and L are the number of germinated seeds and the root length in the case of the microalgae extracts and G_w and L_w are the same parameters for the control (distilled water).

2.4.3. Chlorophyll and carotenoid contents

The chlorophyll *a, b* and carotenoid contents were evaluated spectrophotometrically according to Sumanta et al. (2014) using 80% acetone as the solvent. The grown sprout leaves, from each plant were collected and grinded in 5 mL of acetone; the samples were homogenized for 2 min in vortex followed by 20 min centrifugation 13000 × g in a 2-6E centrifuge (Sigma, Switzerland); the resulting supernatant was separated and a volume of 0.5 mL of supernatant was mixed with 4.5 mL of 80% acetone; this solution was then measured in a U-2000 spectrophotometer (Hitachi, Japan).

The chlorophyll *a* (C_a), chlorophyll *b* (C_b) and total carotenoids (C_{arot}) were calculated through the following equations (Sumanta et al., 2014):

$$C_a(\mu g/mL) = 12.25 \times A_{663} - 279 \times A_{646} \quad (2)$$

$$C_b(\mu g/mL) = 21.5 \times A_{646} - 5.1 \times A_{663} \quad (3)$$

$$C_{arot}(\mu g/mL) = \frac{1000 \times A_{470} - 1.82 \times C_a - 85.02 \times C_b}{198} \quad (4)$$

2.5. Biopesticide trials

The biopesticide bioassays were done against the fungus *Fusarium oxysporum* in sterile Petri dishes. Potato Dextrose Agar (PDA) was used as culture medium (4 mg L^{-1} potato starch, 20 mg L^{-1} dextrose, 15 mg L^{-1} agar). Due to the vast bacterial load coming from the effluent, tartaric acid (10% w/v) was used to decrease the medium's pH to 3.5 and, thus, inhibit the bacterial growth, according to manufacturer instructions. First, agar was poured into the Petri dishes until half, and 4 holes were done using Oxford towers. After the agar solidification, the PDA was added. The microalgae suspensions were poured into the holes and the fungus was placed in the middle. Sterile distilled water was used as control. The Petri dishes were then incubated in the dark at 25°C for 10 days.

The inhibition percentage is calculated using the Eq. 5, where PD and CD correspond, respectively, to the diameter of the fungi growth with microalgae suspension and in the control (distilled water), respectively.

$$Inhibition\ (\%) = 100 - \left(\frac{PD}{CD} \times 100\right) \quad (5)$$

3. Results and discussion

3.1. Microalgae screening

To choose the best microalga(e) for the PWW treatment, a preliminary screening was done with six microalgae - *Synechocystis* sp., *T. obliquus, C. protothecoides, C. vulgaris, N. oleoabundans*, and *Nostoc* sp. cultivated in diluted PWW (1:20). Several dilution factors were tested (1:20, 1:10, 1:5, 1:2, 1:1). However, 1:20 was the only one that provided the adequate nutrient content (especially for ammonium) for the microalgae growth as well as a suitable light penetration. Their growth was monitored through optical density for 23 days (Fig. 1). Only *N. oleoabundans* and *Nostoc* sp. were not able to grow in the diluted PWW, and consequently they were excluded for the following experiments. Except for *Synechocystis* sp., which started to grow right after being inoculated, the other microalgae took around 10 days to acclimatize to the effluent conditions (lag phase).

3.2. Treatment performance

Table 1 presents the initial composition of raw and diluted (1:20) PWW. This wastewater has very high levels of COD (7232 mg O_2 L^{-1}) and ammonia (3150 mg NH_4^+ L^{-1}), which are inhibitory for microalgae growth (Collos and Harrison, 2014). The values are much higher than ones usually reported in studies using PWW for microalgae cultivation, mainly because PWW underwent anaerobic digestion before algae cultivation (Ayre et al., 2017; Uggetti et al., 2014; Wang et al., 2013). Furthermore, the PWW has a very dark brown color which can hinder the light penetration and thus the photosynthetic growth. A dilution of 1:20 was then required to significantly decrease ammonium and color until levels that are adequate for microalgae growth.

Synechocystis sp., *T. obliquus, C. protothecoides*, and *C. vulgaris* allowed nutrient removal efficiencies that are depicted in Table 2.

All microalgae were able to efficiently treat the wastewater since they all achieved high removal efficiencies, with ammonium removals above 79% and near complete removal of phosphate. Regarding COD removal, *C. vulgaris* and *T. obliquus* gave the best results (79 and 73%, respectively). Furthermore, *T. obliquus* and *C. protothecoides* achieved productivities higher than 30 mg L^{-1} d^{-1}, while *Synechocystis* sp. and

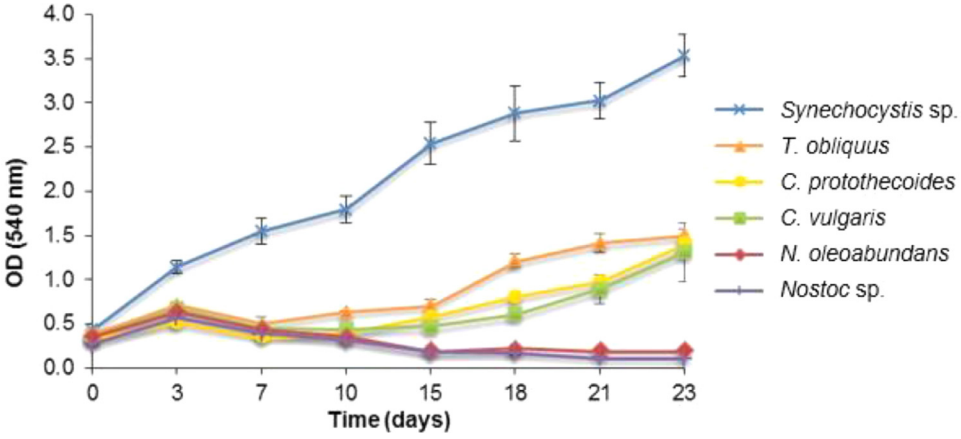

Fig. 1. Growth curves of *Synechocystis* sp. (×), *Tetradesmus obliquus* (▲), *Chlorella protothecoides* (●), *Chlorella vulgaris* (■), *Neochloris oleoabundans* (◆), and *Nostoc* sp. (+) cultivated in diluted piggery wastewater (1:20) for 23 days (mean ± standard deviation, n=2).

Table 1
Piggery wastewater (PWW) composition in terms of pH, chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), ammonium nitrogen (NH_4^+) and phosphate (PO_4^{3-}) (mean ± standard deviation, n=2). Legislation values are depicted in Portuguese law (Decree-Law No 236/98, 1998).

PWW	pH	COD (mg O_2 L^{-1})	TKN (mg L^{-1})	NH_4^+ (mg L^{-1})	PO_4^{3-} (mg L^{-1})
Raw	7.70	7232±89	3500±420	3150±56	117.2±2.3
Diluted (1:20)	7.90	335±0	175±21	158±3	5.86±0.11
Legislation	6-10	150	15	10	10

Table 2
Productivity (in ash free dry weight) and nutrient removal efficiency (mean ± standard deviation, n=2) after 19 days of wastewater treatment with the microalgae *Synechocystis* sp., *Tetradesmus obliquus, Chlorella protothecoides* and *Chlorella vulgaris* in 5L PBRs.

Microalgae	Productivity (mg L^{-1} d^{-1})	Nutrient Removal efficiency (%) COD	TKN	NH_4^+	PO_4^{3-}
Synechocystis sp.	23.7±2.6	61.6±5.5	88.0±5.7	92.4±0.1	90.1±0.0
T. obliquus	31.6±0.0	73.1±3.3	89.6±4.7	87.5±0.4	98.1±0.0
C. protothecoides	36.8±7.9	68.4±2.2	92.0±1.6	92.0±0.0	98.5±0.0
C. vulgaris	22.4±3.9	79.2±3.5	84.0±2.3	79.4±0.1	98.6±0.3

C. vulgaris were around 22-23 mg L^{-1} d^{-1}. The final pollutants composition of the treated water after microalgae cultivation was still slightly above the permitted discharged limits (Table 1) for nitrogen pollutants TKN (14-28 mg L^{-1}) and NH_4^+ (12-32 mg L^{-1}), while COD levels are under the limits (70-128 mg O_2 L^{-1}). Thus, it would be necessary a longer treatment period to fully treat the wastewater to comply with the Portuguese legislation (Decree-Law No 236/98, 1998).

Nonetheless, it is important not to forget that the effluent was previously diluted with a significant amount of water to adjust its composition to microalgae growth. However, this is not a viable strategy for large scale application, from the economic and environmental point of view. However, this strategy is adequate for the purpose of the present work, which was to select the microalgae that could simultaneously grow by treating PWW, and have effect on plant germination, growth, and protection.

To upgrade the present work, we are looking for alternative strategies to avoid the use of fresh water, which is a scarce resource. A stronger inoculum to start the microalga culture as well as the injection of CO_2 could be used to control the pH range (6-7) and shift the chemical equilibrium from NH_3 to NH_4^+, which is less toxic for microalgae (Ayre et al., 2017). Moreover, pre-treatment processes could be applied aiming to reduce the ammonia toxicity and decolorize the effluent, to avoid the need of using water for dilution (Depraetere et al., 2013; Kim et al., 2014).

3.3. Microalgae biomass composition

The biochemical and mineral composition of the microalgae biomass is available in Table 3. All microalgae grown in PWW are rich in proteins owing to the higher nitrogen content of the wastewater, which is used by microalgae for protein synthesis. Moreover, it indicates that they are growing in adequate conditions. *Synechocystis* sp. presents the highest protein content as expected (47.3%), but also the other strains presented significant contents (above 35%). These protein rich wastewater-grown microalgae could then be a key source of amino acids, such as tryptophan and arginine which are metabolic precursors of phytohormones (Chiaiese et al., 2018). Hence, the microalgae are expected to have a stimulating effect on the growth and yield of plants. On the other hand, *C. protothecoides* has the highest carbohydrate content (32.7%), while the others had very similar contents (25-27%). Some studies have already evidenced that microalgae polysaccharides promote plant growth, nutrient uptake, and extend plant tolerance to stress (El-Naggar et al., 2020; EL Arroussi et al., 2018, 2016; Farid et al., 2019).

All the listed macro- and microelements are essential minerals for plant physiology and development, being part of several cellular mechanisms, such as ion fluxes, osmosis, salt tolerance and even as co-factors for enzymes. Macronutrients are normally found in plants within a range of 1000 to 15000 ppm (dry weight) and micronutrients concentrations 100 to 10000 times lower (Delhaize et al., 2015). Considering these val-

Table 3
Biochemical (protein and carbohydrates) and mineral composition (mean ± standard deviation, n=2) of *Synechocystis* sp., *Tetradesmus obliquus, Chlorella protothecoides,* and *Chlorella vulgaris* grown in diluted (1:20) piggery wastewater.

Composition	Synechocystis sp.	T. obliquus	C. protothecoides	C. vulgaris
Protein (%)	47.3±2.5	34.5±2.1	34.4±0.8	38.3±0.9
Carbohydrates (%)	25.1±0.2	25.5±0.1	32.7±0.6	26.8±2.7
	Mineral content (ppm)			
N	77000±1400	57960±280	57750±910	64330±1050
P	3193±89	3634±91	4104±90	3571±100
K	26249±252	17957±199	16394±185	16007±285
Ca	9331±227	42303±405	30901±338	24268±461
S	12415±144	13392±148	13991±146	11451±193
Mg	<LOD	<LOD	<LOD	<LOD
Fe	222±27	74.8±25	273±28	350±28
Cl	1211±32	5173±57	2391±50	781±27
Mn	<LOD	<LOD	<LOD	<LOD
Zn	434±12	188±8	447±14	561±13
Cu	124±13	78.5±13	120±13	135±13
Mo	6.4±1.0	7.3±1.0	6.6±1.0	7.7±1.0
Ni	<LOD	<LOD	<LOD	<LOD

LOD: limit of detection

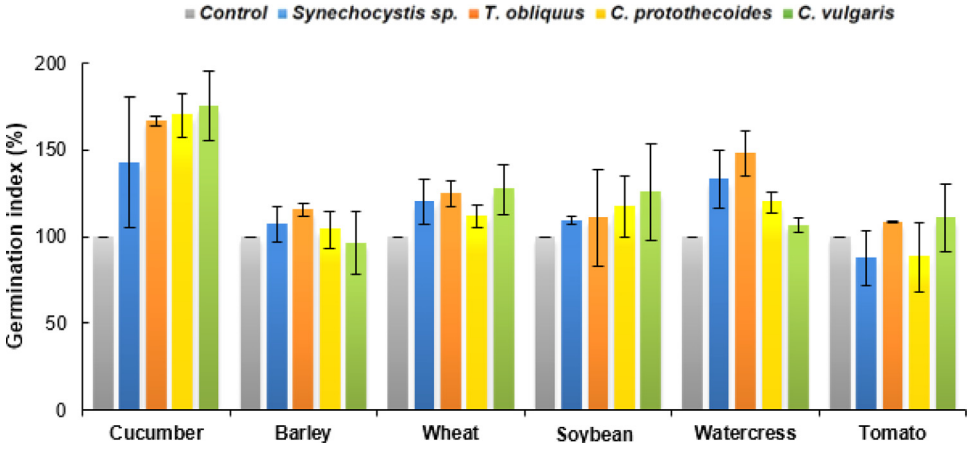

Fig. 2. Germination index (%), considering distilled water as the control (100%) for the 6 different seeds treated with different microalgae suspensions (*Synechocystis* sp., *Tetradesmus obliquus, Chlorella protothecoides,* and *Chlorella vulgaris)* grown in diluted piggery wastewater at a concentration of 0.5 g L^{-1}. Error bars indicate standard deviation (n=2).

ues, the mineral contents of the cultivated microalgae are within the specified range and can, therefore, meet the plant necessities, which is an indication of the potential of these microalgae to act as biofertilizers.

3.4. Effect of microalgae as biostimulants for seed germination, plant growth, and pigment content

3.4.1. Germination index

It is considered that a germination index (GI) of 100% corresponds to control samples, where seeds were treated with distilled water. Therefore, only microalgae suspensions leading to GI higher than 100%, are considered to have biostimulant activity. The results are shown in Fig. 2a.

In general, it is possible to observe that all microalgae studied have shown a positive effect on the germination index for the tested seeds. This effect seems to be especially clear in cucumber seeds, where GI values were at least 99% higher compared to control (distilled water). On the other hand, the positive effect was lower in the case of tomato seeds. It is also important to notice that *T. obliquus* generated GI values higher than 100% in all the tested seeds, and the highest GI average values. *C. vulgaris* gave the best results for cucumber, wheat, and tomato seeds, while *T. obliquus* had the greatest impact on barley and watercress seeds. This is in accordance with results obtained previously by Navarro-López et al. (2020), where *T. obliquus* grown in brewery wastewater was also shown to have biostimulant potential in watercress seeds. Likewise, the same was shown in barley seeds (Ferreira et al., 2019). For soybean, *Synechocystis* sp. increased 65% the GI compared to the control, standing out from all the other microalgae. Because soybean seeds have a high protein contents, the demand for N is extremely high during seed formation. To fulfill this, they can fixate N_2 in a symbiotic process with soil borne rhizobia bacteria (Mcgrath et al., 2013). *Synechocystis* sp. can also fixate N_2, and thus, can explain why it had a major effect on soybean seeds.

Microalgae and cyanobacteria can synthesize a remarkable diversity of biologically active molecules, such as fatty acids, phytohormones (e.g. auxins, cytokinins, gibberellins, etc.), polysaccharides and phenolics (Cuellar-Bermudez et al., 2017; Renuka et al., 2018; Ronga et al., 2019). However, the action mode of these compounds in plant development is still not well explored. Thus, it is not completely clear why *T. obliquus* and *C. vulgaris* promoted best results than *Synechocystis* sp. in the present work, when the latter has a higher N content. Nevertheless, we can hypothesize that the first two may have optimal contents in some components that might provide a higher effect of seed germination and plant growth. For example, Plaza et al. (2018) saw that *Scenedesmus* spp. showed a higher concentration of phytohormones compared to *Arthrospira* spp., promoting a higher increase in root dry weight of *Petunia x hybrid* plants.

3.4.2. Shoot and root growths

The results for shoot and root development of each type of seed tested are displayed in Fig. 3. The microalgae studied had an overall positive effect on plant roots, with lengths higher than the control. However, the effect of microalgae on plant shoots was not significant.

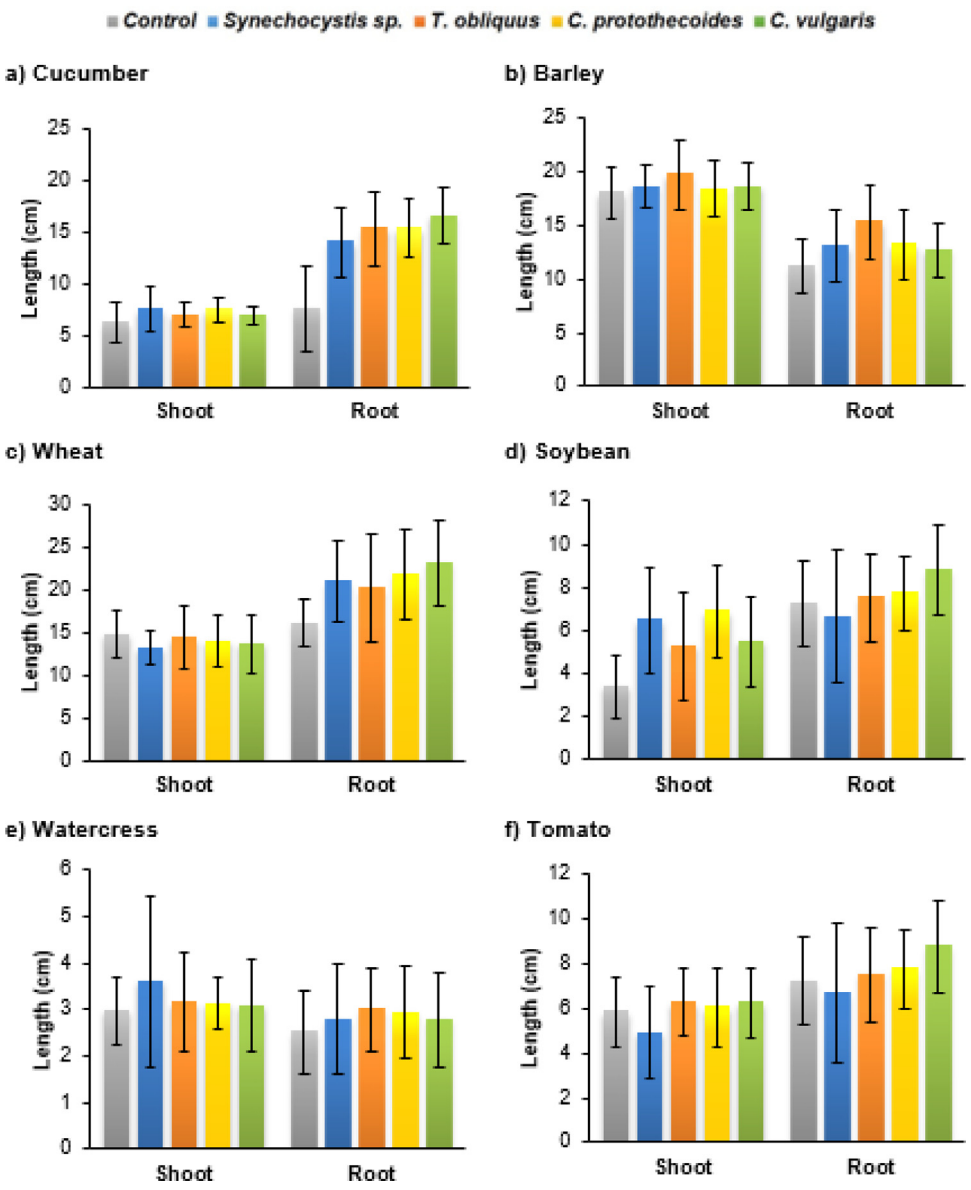

Fig. 3. Average shoot and root length for (a) cucumber, (b) barley, (c) wheat, (d) soybean, (e) watercress and (f) tomato seeds with distilled water (control) and treated with microalgae biomass suspensions of *Synechocystis* sp., *Tetradesmus obliquus, Chlorella protothecoides*, and *Chlorella vulgaris*, after a 10-day cultivation period. Error bars indicate standard deviation (n=2).

The positive effect on roots were especially evident in cucumber and wheat (100% and 33.5% average length increase, respectively). Furthermore, most seeds treated with *C. vulgaris* and *T. obliquus* originated plants with longer roots. Similar trends were obtained by Bumandalai and Tserennadmid (2019) using *C. vulgaris* suspensions, at different concentrations, to treat cucumber seeds. They highlighted that 0.25 g L^{-1} of algal suspension is the best treatment for root and shoot lengths, being more effective in the root, like in the present study. Nevertheless, the same authors show up an increased germination for tomato seeds, being the best results obtained at 0.17 g L^{-1} with *C. vulgaris* biomass. For higher concentration, they observed an inhibitory effect on the plant growth. This last result might suggest that the microalga concentration used (0.5 g L^{-1}) might be excessive for tomato plants, negatively affecting their growth. This is especially clear in the case of *Synechocystis* sp. treatment, which has a higher protein concentration (47.3%) and, consequently, of amino acids and/or polyamines, which could inhibit seed growth at concentrations exceeding the optimum (Navarro-López et al., 2020; Tarakhovskaya et al., 2007). Nonetheless, in the case of cucumber seeds, it can be said that the concentration applied (0.5 g L^{-1}) was beneficial for the plant roots as shown by Navarro-López et al. (2020) with *T. obliquus* treating cucumber seeds.

Regarding shoot lengths, only in soybean plants the increase in treated seeds was more perceptible, especially in the case of *Synechocystis* sp. and *C. protothecoides* (above 90% increase). In the case of barley, seeds treated with *T. obliquus* stood out from other microalgae (almost 9% increase in shoot length). This is accordance with the previous study done by Ferreira et al. (2019), where *T. obliquus* grown in brewery wastewater also showed a promising effect on barley seeds. In wheat shoots, the microalgae had a negative effect which could also be explained by the reasons presented before regarding the high concentration of microalgae in the present study, just like showed by Kumar and Sahoo (2011) for *Triticum aestivum* var. Pusa Gold (wheat) seeds treated with seaweed extract at concentrations above the optimum. Moreover, Rachidi et al. (2020) obtained significant differences in shoot length of tomato seeds treated with microalgae (*Arthrospira platensis, Dunaliella salina*, and *Phorphorydium* sp.), unlike the present results, but not for the root lengths, similar to the present study.

These results could be expected due to the application method, where the seed is soaked in the microalgae suspensions, reaching the roots first and slowing spreading to the other parts of the plant. In addition, the cultivation period of 10 days could be short for some of the plants tested. Moreover, foliar application

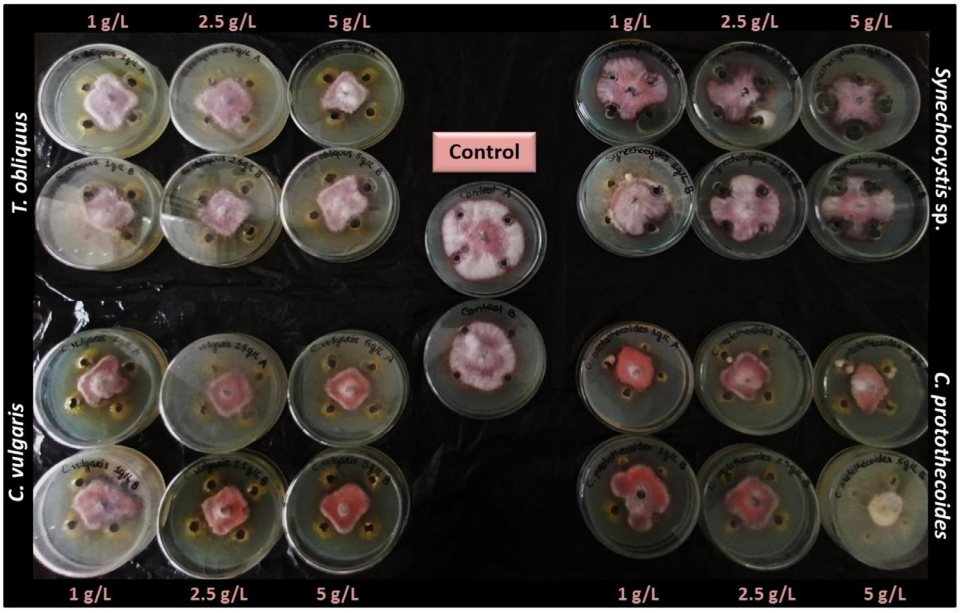

Fig. 4. Biopesticide activity of the microalgae suspensions (*Synechocystis* sp., *Tetradesmus obliquus, Chlorella prototheocoides*, and *Chlorella vulgaris*), at different concentrations (1, 2.5 and 5 g L^{-1}), against pathogen *Fusarium oxysporum*.

of microalgae could promote a better development in plant shoots (Plaza et al., 2018).

3.4.3. Chlorophyll and carotenoid contents

The application of microalgae to enhance pigment content was evaluated in all plants tested and the results are shown in Fig. S1 (see Supplementary Material).

Considering the overall results, the chlorophyll *b* and total carotenoid concentrations in the six plants studied were not affected by the microalgae suspensions in comparison with distilled water samples, unlike the results shown by Mutale-joan et al.(2020) (for both) and Rachidi et al. (2020) (for carotenoids) in tomato plants. However, for chlorophyll *a* there was a slight increment in plants treated with microalgae, similar to the results obtained by Jimenez et al. (2020) and Rachidi et al. (2020) using microalga *Monoraphidium* sp. and *Arthrospira platensis*, respectively, to treat tomato seeds. This was more noticeable for cucumber, where there was an enhancement of chlorophyll *a* in the plants treated with microalgae. For soybean and wheat, the seeds treated with *Synechocystis* sp. revealed higher chlorophyll *a* content than the control, and in tomato, *T. obliquus* stood out from the others. Thus, the results suggest that the microalgae tested will probably have a more pronounced effect on chlorophyll rather than in carotenoids. However, more detailed analysis is needed to further conclude the potential effect of microalgae biomass on the photosynthetic pigments of the plants.

3.5. Effect of microalgae as biopesticide

Having been found several cyanobacteria strains, macroalgae and some microalgae with pesticide activity (Costa et al., 2019; Renuka et al., 2018), trials were conducted to understand if any of the four microalgae used in this work – *Synechocystis* sp., *T. obliquus, C. prototheocoides* and *C. vulgaris* – could have an impact on the growth of the fungus *Fusarium oxysporum*. Different concentrations of microalgae suspension were studied (1, 2.5 and 5 g L^{-1}). The results are shown in Fig. 4.

In Fig. 4, it is possible to observe the inhibition of fungi growth in most samples containing microalgae suspensions, when compared to control. The only microalga that seemed to have an insignificant effect on the fungi growth is *Synechocystis* sp.

These observations can be confirmed by calculating the inhibition halo, which is presented in Table 4. As expected, the inhibition is related to the microalgal concentration. Even at the lowest concentration (1 g L^{-1}), *C. vulgaris* and *T. obliquus* allow an inhibition percentage higher than 40%, which were maintained with the increased biomass concentration (except for *C. prototheocoides* which increased from 35 to 53% when the concentration increased from 1 to 5 g L^{-1}). The inhibition effect of *C. vulgaris*, grown in different media, against *F. oxysporum* was already reported by Vehapi et al. (2018). In another study by Vehapi et al. (2020), *C. vulgaris* followed by *C. prototheocoides* showed the strongest antifungal effect against various apple-infecting fungi, such as *Aspergillus niger, Alternaria alternata*, and *Penicillium expansum*. Moreover, the authors attributed the major antifungal activity to compounds like terpenes, alkaloids and polypeptides found in *C. vulgaris* (Vehapi et al., 2020).

Table 4
Inhibition percentage of *Fusarium oxysporum* by *Synechocystis* sp., *Tetradesmus obliquus, Chlorella prototheocoides* and *Chlorella vulgaris* at different concentrations (1, 2.5, and 5 g L^{-1}).

Microalgae	Concentration (g L^{-1}) 1	2.5	5
Synechocystis sp.	10.5±6.5	3.2±0.4	0.0±0.0
T. obliquus	43.5±0.0	43.5±1.6	46.8±1.6
C. prototheocoides	35.1±10.9	36.7±0.4	53.2±4.0
C. vulgaris	45.6±3.6	48.8±0.4	49.6±0.4

Given these results, the assessment of their biopesticide potential should be extended to other pathogenic microorganisms (fungi and bacteria) that could have a greater impact on crop productivity. Moreover, lower concentrations should be studied to determine if microalgae suspensions can be used at the same concentrations for both biostimulant and biopesticide activities.

4. Conclusions (233)

Microalgae can recover nutrients and water from livestock wastewater to promote a more sustainable use of these resources on agriculture, with full respect for public health and the environment. The introduction of microalgae as biofertilizers, biostimulants and biopesticides is a promising approach to reduce or even replace the use of non-renewable chemicals, without compromising plant productivity.

On this perspective, our study offers some insights on how microalgae can connect wastewater treatment to agriculture, especially when both are within the same context of livestock farming. Our work showed that microalgae like *Tetradesmus obliquus* and *Chlorella vulgaris* not only have the capability cleaning piggery wastewater by collecting nutrients from it, but can also simultaneously promote seed germination, root growth, and plant protection.

The present work helped us identify the difficulties associated with microalgae cultivation in PWW, which was extremely useful to discover the need of a pre-treatment step to reduce ammonia and color levels to improve the microalgae-based treatment in undiluted PWW. Finally, we were able to select the most robust microalgae for both wastewater treatment and agriculture use, which will be further studied in more broad and in-depth studies.

Regardless, we believe that the development of innovative products (e.g., biofertilizers, biostimulants, and biopesticides) to promote crop yield and quality, while minimizing the agricultural carbon footprint, could be one of the main application of microalgae used for wastewater treatment, within a circular bioeconomy approach.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2021.100062.

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