Wastewater nutrient recovery using twin-layer microalgae technology for biofertilizer production

Inmaculada González, Natalia Herrero, José Ángel Siles, Arturo F. Chica, M. Ángeles Martín, Carlos García Izquierdo and José María Gómez

ABSTRACT

This study evaluates the feasibility of advanced biofilm microalgae cultivation in a twin layer (TL) system for nutrient removal (N and P) as the tertiary treatment in small wastewater treatment plants (WWTPs) located in sensitive areas. Furthermore, the potential valorisation of microalgae biomass as a component of bio-based fertilizers is assessed. *Scenedesmus* sp. was chosen among 33 microalgae strains for inoculation of TL due to its high growth rate and its nutrient uptake capacity. The tests carried out in the prototype were markedly efficient for total soluble and ammoniacal nitrogen removal (up to 66 and 94%, respectively). In terms of potential valorisation of microalgae, the nutrient content was 5.5% N (over 40% protein), 8.8% P_2O_5 and 1.5% K_2O, high enzymatic activity, very low levels of heavy metals and no detectable pathogen presence. However, in the formulation of solid-state bio-based fertilizers, the microalgae proportions in blends of over 2% of microalgae led to negative effects on ryegrass (*Lolium perenne L.* ssp.) and barley (*Hordeum vulgare* ssp.). The obtained results demonstrate that TL represents a promising technology, which allows efficient tertiary treatment of urban wastewater and the production of high-quality bio-based fertilizer.

Key words | bio-based fertilizers, microalgae, tertiary treatment, twin layer system, wastewater treatment plant

Inmaculada González
Natalia Herrero
José María Gómez
BIOMASA PENINSULAR S.A.,
Calle Constancia 38, Bajo, 28002 Madrid,
Spain

José Ángel Siles (corresponding author)
Arturo F. Chica
M. Ángeles Martín
University of Cordoba (Spain) – Department of Inorganic Chemistry and Chemical Engineering,
Campus Universitario de Rabanales,
Ctra. N-IV, km 396, Edificio Marie Curie, 14071 Córdoba,
Spain
E-mail: *a92siloj@uco.es*

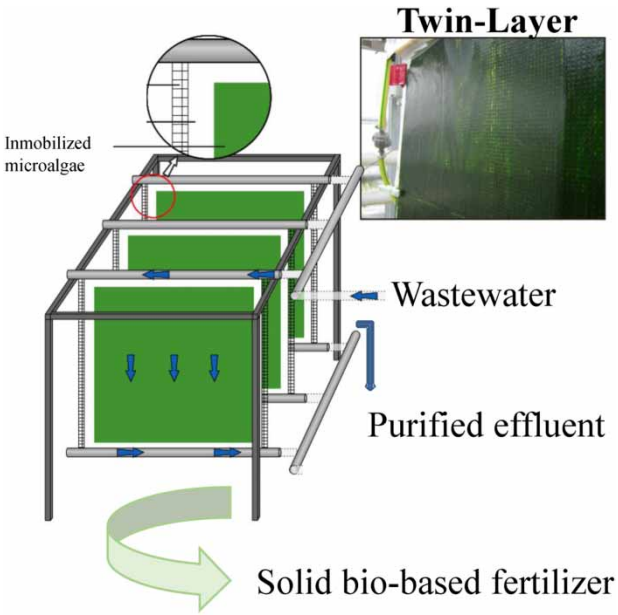
Carlos García Izquierdo
Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC),
Campus Universitario de Espinardo,
P.O. Box 164, CP 30100 Murcia,
Spain

HIGHLIGHTS

- Urban wastewater was subjected to tertiary treatment by twin-layer microalgae.
- Ammoniacal nitrogen was succesfully removed from wastewater by *Scenedesmus* sp.
- Microalgal biomass met the requirements to be used as a solid bio-based fertilizer.

doi: 10.2166/wst.2020.372

GRAPHICAL ABSTRACT



INTRODUCTION

Currently, there are extremely rural areas with low population density and without wastewater treatment plants (WWTP) where nutrient removal (tertiary treatment) is non-viable through traditional technologies (2016 Spanish Environmental Profile 2017).

In the European Union, small urban agglomerations are usually those with a population of less than 2000 inhabitants – coinciding with the limit established by Directive 91/271/EEC – below which wastewater only requires 'adequate treatment'. However, small urban areas can exhibit problems common to medium and large populations, either because of their industrial activity or because of the variation in the number of inhabitants in different seasons. In this regard, the treatment of wastewater derived from populations smaller than 500 equivalent inhabitants (EI) usually face inconveniences derived from the fact that the engineered design hardly accomplishes the effluent quality requirements on nutrients established by the legislation. Furthermore, as the size of the population decreases, the cost of wastewater treatment per inhabitant increases.

The secondary treatment of wastewater mainly removes soluble organic matter, and the adaptation of existing facilities for the reduction of nitrogen and phosphorus levels is complicated due to their low operational flexibility. Although the decrease in the concentration of phosphorus might be achieved by chemical precipitation with iron and aluminum salts, this process involves the supply of chemical reagents and the production of large amounts of chemical sludge to be separated in settling clarifiers (Mehta *et al.* 2015). However, the reduction of nitrogen is more complex because this is carried out through the biological treatments of nitrification and denitrification (Thakur & Medhi (2019). But several treatment units, internal and external recirculation flows, consequent prolonged aeration conditions, high energy demand and/or low organic loads are required for an efficient nitrogen removal rate. Hence, these technologies might be technically and economically unsustainable for small populations.

In this context, there are low-cost technologies that are commonly employed at small and medium WWTPs (<500 inhabitants), such as rotating biological contactors, which allow the removal of soluble organic matter. Nevertheless, sometimes, it is necessary to upgrade these facilities to fulfil the regulatory discharge limits regarding N and P concentration in the effluents. The adoption of nutrient removal processes in these WWTPs is not easy due to their markedly low operational flexibility (Cortez

et al. 2008). A wide range of microalgae-based technologies have been developed to overcome the technical constraints of the conventional nutrient removal processes. High rate algal ponds (HRAPs) with suspended microalgae are the most widely employed nowadays due to their low maintenance requirements. This type of configuration involves low maintenance costs and low algae biomass productivity but high space requirements. In addition, the separation of algae biomass from the treated water is considered to be a major challenge in this system (Stephens et al. 2010; Sturm & Lamer 2011).

In recent years, other advanced technologies that use immobilised microalgae have gained attention since they facilitate the recovery of the nutrient-rich microalgae and their valorisation as biofuel, animal feed or bio-based fertilizers (Acién et al. 2016). Twin layer (TL) is an advanced biofilm microalgae cultivation technology that offers important advantages in comparison with the traditional suspended algae growth for nutrient removal processes and facilitates the recovery of the nutrients, closing the global biogeochemical cycle of N and P. TL has limited space requirements (36–250 L/kg dry microalgae biomass) in comparison with raceway ponds (2000–2,850 L/kg dry microalgae biomass), and the microalgae productivity is higher than 150–300 kg dry weight/m^3 in the advanced porous substrate bioreactor system (PSBRs) and 0.35–0.50 kg dry weight/m^3 in raceway ponds (Podola et al. 2017). Furthermore, light is absorbed much more efficiently by microalgae, and the residence times are lower (Schultze et al. 2015). Moreover, this technology could complement low-cost traditional technologies. However, this alternative treatment requires a custom configuration, design and adaptation in new and already implemented WWTPs. Consequently, further research is required to evaluate its treatment efficacy at full scale especially in the effluents derived from small populations, depending on the type of wastewater, secondary treatment configuration and type of microalgae inoculated. The main limitation of the TL technology is the need to remove particles from the secondary treatment so as not to collapse the drippers through which wastewater is provided to the system. Even being an extensive biological system, verticality reduces the space requirement, although this is still significant.The objective of this research study is twofold: (a) to demonstrate the technical and environmental feasibility of a prototype-scale TL system as an advanced nutrient removal technology (tertiary treatment of urban wastewater) and (b) to close the N and P cycles through the recovery of nutrients in the form of fertilizers based on microalgae.

METHODS

Microalgae selection and cultivation: scaling-up

Studies at the laboratory scale

The productivity and efficiency of the TL system for the treatment of the effluent of the secondary treatment (SWW) is highly dependent on the selection of algal strains to inoculate the system. In the first screening test, a wide range of algal strains (33 types) were grown and isolated in a laboratory-scale TL system using wastewater from a WWTP in the province of Córdoba (Spain) as the nutrient source. Their average growth (expressed in dry weight) over 12 days was determined. This TL system was constructed with a glass fiber sheet (40 × 10 cm, only on one side and five laboratory-scale systems) hanging in a plexiglass tube (Figure 1).

Wastewater was supplied via PVC tubes connected to a peristaltic pump and dripped on the glass fiber at the top of the sheet. Water was transported over the whole length of the glass fiber by gravity and, at the end, dripped back into

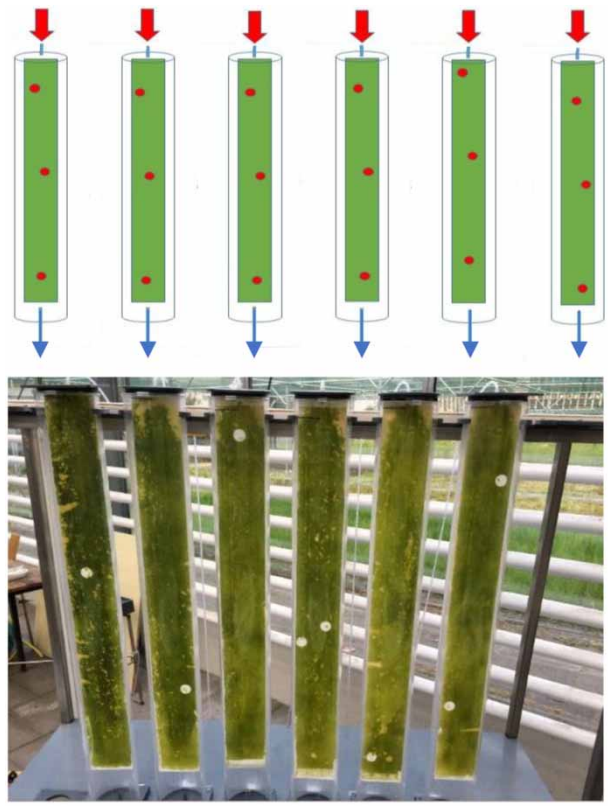


Figure 1 | Laboratory-scale TL system. The wastewater flow is shown by red and blue arrows, while red circles simulate regular biomass samples. The full colour version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wst.2020.372.

a reservoir bottle. The system was supplied with 1 L, which was exchanged twice a week to avoid nutrient limitation for the microalgae. The algae were inoculated on round polycarbonate filters of 25 mm diameter by filtering the liquid culture. The inoculated filters were simply placed on the wet glass fiber, and they stuck by self-adhesion. About 30 filters fit on one glass fiber sheet.

After their inoculation in the laboratory-scale TL system, a preliminary species identification of the strains was carried out based on microscopical observations. The strains that were selected after the previous screening were further identified by sequencing their 18S rRNA coding DNA sequence and comparing it to the database sequences (Melkonian Laboratory-Microalgal research at the University of Cologne, https://www.uni-due.de/biology/ccac/). The established cultures were maintained by the Culture Collection of Algae Cologne (CCAC) and thus are available for further studies. Once a single cell or a colony of each strain was isolated from the established cultures (enrichment cultures in defined culture media, isolation with microcapillary under an inverted microscope, capillary spraying of cells on agar plates and colony selection), it was placed in a sterile growth medium (BG11 or Waris-H + Si) and was transferred regularly (every 3 weeks) to fresh medium. This assured the survival of the sterile cultures, where no wastewater was added, and produced enough biomass for the screening study.

The most productive strain was provided by the microalgae biotechnology team of the Institute of Botany of the University of Cologne and was subsequently inoculated at a higher scale of photo-bioreactors to produce the microalgae in batches. In this case, microalgae was cultivated in suspension at laboratory scale in two stages: at first, in conical flasks (1 L) and at a second stage in plastic tubular photo-bioreactors (plastic containers) (8 L bags). This experimental setup allowed the withdrawal of samples and analytical monitoring of the cultivation process. For strain maintenance, conical flasks (1 L) were filled with approximately 600 mL of growth medium and some of the provided algal suspension. For medium preparation, water was autoclaved in the flasks and a fertilizer concentrate was added (Sportsmaster WSF Spring & Summer) at a concentration of 330 mg/L as well as magnesium sulphate heptahydrate at 20 mg/L. The algal biomass was added after the solution had fully cooled down (5 mL of concentrated microalgae).

After cultivation, the harvested suspensions were concentrated using the natural settling capacity of the algal strain. The biomass was subsequently concentrated by short centrifugations (1,000 rpm for 4 minutes). After centrifugation, the microalgae were kept in refrigeration at 4 °C before inoculation in the TL prototype.

Prototype scale TL

The TL consisted of a total of 18 modules – distributed in 3 lines of 6 modules – and all were connected to work in a single process (Figure 2). Each module (stainless steel box) occupied a footprint area of 1.44 m^2 and its dimensions were 1.2 m wide, 1.2 m long and 1.80 m high. The structure of the module was made of stainless steel. On this metal structure hung the sheets containing the immobilised microalgae. Each structure contained two cultivation sheets, which received the wastewater from the secondary treatment effluent (rotational contactors for organic matter removal) located in a WWTP in the Province of Córdoba (Spain). This WWTP serves 4,100 inhabitants, and its effluent is discharged into an environmentally sensitive area.

Each cultivation sheet or layer comprises two ultrathin, microporous outer layers, where microalgae are immobilised by self-adhesion, and one macroporous inner layer (source layer) through which wastewater is distributed. The dimensions of the source layer and substrate layer were around 1.0 m wide and 1.5 m high, so the total cultivation or growth area is 2.656 m^2 per sheet.

Water was pumped by a pressure group with automatic resetting from the secondary WWTP to the decanter located before the TL system. In addition, 4 pressure relief valves were installed in the upper parts of the main line for purging the possible air bubbles inside the pipes and to help maintain the pressure appropriated in the net.

The above-mentioned decanter was installed to reproduce the secondary decanter of the WWTP because only a fraction of the flow rate derived from the secondary treatment was treated in the TL system. Subsequently, an opaque 1,000 L tank was installed to prevent or minimise the growth of algae inside the tank and consequently reduce the amount of solids in the system. It was used as a reservoir inlet for the TL system, where water level sensors regulated a pump and assured constant supply. Flowing from this reservoir, the water was filtered through a sand filter and a second coil filter installed to remove particles larger than 5 μm prior to being actively pumped to the TL system. These measures were necessary in order to protect the drippers that irrigated the TL from clogging. Each layer had an irrigation tube with 14 drippers that emitted 0.5 L/h, produced 7 L/h per sheet and obtained a total of approximately 252 L/h for the entire prototype or WW (6 m^3/d).

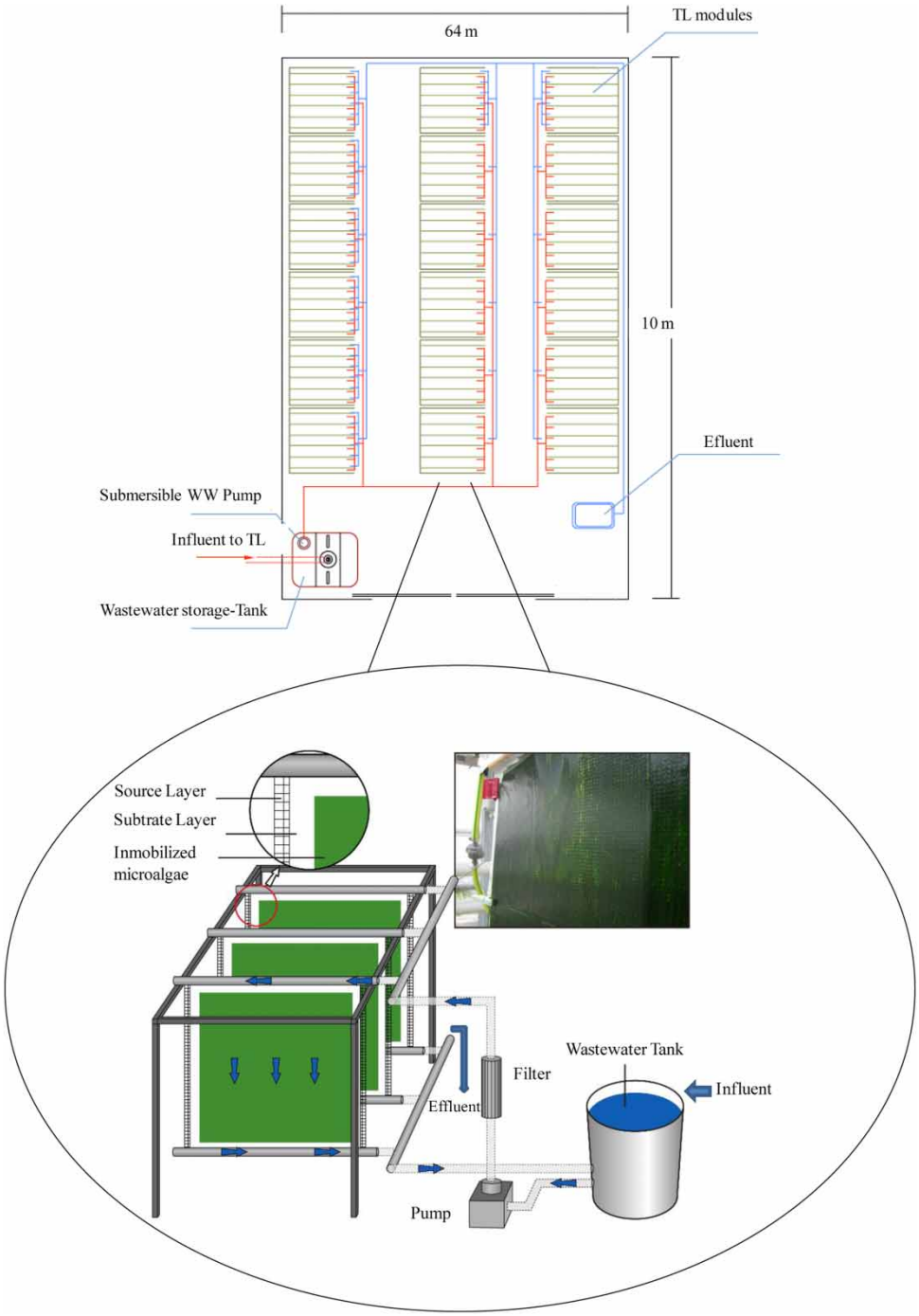


Figure 2 | Diagram of the TL prototype used as tertiary treatment.

The pressure of the water flow was adjusted to 1.5 10^5 Pa by an electronic pressure control (CPH-Tecnoplus 15).

Each drip line was separated from the others by using ball valves to operate them individually, and in the case of the sheets, this was done with every second sheet in order to facilitate the maintenance works without stopping the system.

After water passed through the layer, it was collected in the drainage tubes suspended at the lower part of the layer. Another transversal drainage collection pipe collected the outflow for each line and from here, the treated water was carried by gravity to a tank where a pump redirected the outflow back to the water inlet of the WWTP.

In order to control and maintain the humidity and temperature conditions, the prototype was installed inside a reinforced polycarbonate greenhouse with a metal support structure, a weather station and a Zenital window, which were both connected by a module control unit. Through the weather station, it was possible to measure parameters such as wind speed, external humidity, external temperature, internal temperature and internal humidity. It was also equipped with an auxiliary ventilation and irrigation system.

The construction details were as follows: width – 6.4 m; length – 10 m; height under gutters – 3 m; roof peak height – 4.3 m; entry – 2 frontal doors – 2.5 m × 3 m; zenithal window – 10 m in length and motorised control.

Lightweight glazing requires fewer metal supports; thus, the overall cost of a polycarbonate greenhouse project, glazing and systems included, can be up to 25% less than its equivalent in glass. Roofs designed for polycarbonate glazing offer a larger surface for light to penetrate and less shadow from the metal roof purlins and frames. Interestingly, a standard polycarbonate greenhouse structure has up to 7% more transparent surfaces than an equivalent-size glass house.

Each layer of the TL prototype was inoculated with 3 g TS/m^2 of microalgae under different storage conditions to evaluate the differences in nutrient uptake and microalgae growth (fresh microalgae were directly cultivated in the laboratory-scale bio-reactors and centrifuged for concentration; lyophilized microalgae (−50 °C and 15 Pa) were not dried previously and microalgae were conserved in refrigeration at 4 °C). The microalgae biomass was generally harvested after two weeks of harvesting by manual scraping of the cultivation layers without any need for mechanical dewatering (i.e. centrifugation).

Agronomic assessment of microalgae as bio-based fertilizer

The harvested microalgae were used after lyophilisation as a component of fertilizers in solid state formulations (individually and mixed with vegetable compost) to evaluate the effect of the direct application of microalgae to an agricultural soil at greenhouse scale. For these preliminary trials, different doses of microalgae and compost were evaluated depending on their nitrogen content (one dose was considered to have a normal level, another dose had a lower level and another had a higher level). Table 1 summarises the experiments carried out with both microalgae and compost in soil and controls. The mixtures of the substrates were homogenised and stored at 4 °C until the analysis was carried out in plastic pots containing 150 g of agricultural soil. The agronomic assessment was performed at greenhouse scale on barley and ryegrass in quadruplicate, and each pot was sown with 0.8 g of seeds.

Table 1 | Description of the different treatments used in the barley and ryegrass crop

Treatment	Acronyms	Composition
1	$SM_{2\%}$	Soil + microalgae 2%
2	$SM_{4\%}$	Soil + microalgae 4%
3	$SM_{6\%}$	Soil + microalgae 6%
4	$SM_{9\%}$	Soil + microalgae 9%
5	SC_{110}	Soil + compost 110 kg N/ha
6	SC_{170}	Soil + compost 170 kg N/ha
7	SC_{220}	Soil + compost 220 kg N/ha
8	$SM_{2\%}C_{170}$	Soil + compost 170 kg N/ha + microalgae 2%
9	$SM_{4\%}C_{170}$	Soil + compost 170 kg N/ha + microalgae 4%
10	$SM_{6\%}C_{170}$	Soil + compost 170 kg N/ha + microalgae 6%
11	$(NH_4)_2SO_4$	Control ammonium sulphate (170 kg N/ha)
12	C	Control (soil)

Chemical analysis

Wastewater characterisation

The wastewater influent and the effluents of the TL were analysed by the following parameters: pH, total chemical oxygen demand (COD, g O_2/L), soluble total organic carbon (TOC, mg/L), soluble total carbon (TC, mg/L), soluble inorganic carbon (IC, mg/L), soluble total nitrogen (Ns, mg/L), ammoniacal nitrogen (N-NH_4^+, mg/L), phosphorous (P-PO_4^{3-}, mg/L) and biochemical oxygen demand (BOD_5, mg/L), which were analysed in accordance with APHA Standard Methods (APHA 1989).

Monitoring microalgae cultivation

Suspended solids, which were measured through optical density at 680 nm, total solids (TS, g/kg), total fixed solids (FS, g/kg) and total volatile solids (VS, g/kg) were also analysed in accordance with APHA Standard Methods (APHA 1989).

Bio-based fertilizer characterisation

Complete physico-chemical characterisation was performed to control the quality of the harvested microalgae and compost following the methodology proposed by the US

Department of Agriculture and the US Composting Council (Thompson *et al.* 2002). Heavy metal contents (mg/kg) of the samples (Cu, Cd, Cr, Ni, Pb, and Zn) were evaluated throughout the process and analysed by atomic absorption spectrophotometry (spectrophotometer model AAnalyst 100/300; Perkin Elmer). Micro and macronutrients (mg/kg) were analysed by inductively coupled plasma-optical emission spectrometer (ICP-OES) quantitative determination, which is an elemental analysis technique. *Salmonella* sp. and *Escherichia coli* were also determined by the Agriculture and Fisheries Management Agency of Andalusia (Regional Ministry of Agriculture, Fisheries and the Environment) according to the methodology proposed by UNE-EN ISO 6579:2003 and ISO 7251:2005, respectively. Total organic carbon (TOC) and total nitrogen (N) were determined in a Carlo Erba Elemental Analyser. Phospholipids were extracted using the Bligh and Dyer method (1959) and then fractioned and quantified using the procedure described by Frostegard *et al.* (1993). Basal respiration was determined with an infrared gas analyser (PBI-Dansensor, CheckMate II) (Hernández & García 2003). Soil phosphatase activity was determined using the method by Tabatabai & Bremmer (1969), while β-glucosidase activity was determined with the method by Tabatabai (1982) and Eivazi & Tabatabai (1988). Urease activity was determined using the method by Kandeler *et al.* (1999). The results of the enzymatic activities were the average analyses performed in triplicate and were expressed as $\mu g^{-1}\,h^{-1}$ based on dry composite weight. The germination index (GI) was calculated according to the procedure suggested by Zucconi & De Bertoldi (1987). The growth chambers were equipped with control over temperature (28 °C), humidity (75% relative humidity), and light conditions (darkness) for germination tests in petri dishes with filter paper and 8 seeds of barley (*Hordeum vulgaris*). A volume of 2 mL of different microalgae extracts alone or as an additive of compost (diluting five, ten and twenty times three preliminary concentrations: 0.05, 0.075 and 0.10% of microalgae corresponded to the nitrogen dosage of 110 (low dosage), 170 (normal dosage) and 220 kg N/ha (high dosage) and a mixture of compost and microalgae (0.2% compost +0.028% microalgae) were added. The petri dishes were placed in a germination chamber for 5 days. After germination, the number of germinated seeds was recorded, and the lengths of the seedling roots and shoots was measured. All the treatments were carried out by quadruplicate with distilled water as control.

RESULTS AND DISCUSSION

Microalgae selection

After taking liquid samples from the WWTP under study, 33 strains were isolated, and a list of all the isolates is shown in Table 2. They included diatoms, cyanobacteria and chlorophyceaen algae. Unicellular as well as filamentous strains were isolated. In some cases, a definite assignment was not possible and there only a description is given. All the cultures were free of fungi but, in some cases, may have contained bacteria. This does not influence the performance of the tertiary treatment because wastewater naturally contains a high quantity of bacteria and natural algae.

Figure 3 shows the values of production yields obtained by cultures in the laboratory-scale TL technology once the growth rate remained constant after 12 days of cultivation. As shown, the productivities ranged from 3.6 ± 0.2 g/m^2·d to 12.2 ± 2.5 g/m^2·d. Based on the results, the five most productive strains were selected for further evaluation. A major criterion was the growth rate when grown on wastewater, with *Scenedesmus* sp. (1) showing again the highest productivity under the operational conditions evaluated. This strain was consequently selected for further assays. From microscopical observations and differences in growth in the stock cultures as well as in the first screening, it is clear though that these strains are clearly different and no re-isolates from the same strain.

In a subsequent preliminary experiment, *Scenedesmus* sp. was used to evaluate the nutrient uptake performance (nitrogen and phosphorus) from wastewater under the same experimental conditions (Figure 4). Water was recirculated for sufficient time to achieve a nutrient reduction that allowed the discharge of wastewater into the water bodies as established in the Directive 91/271/EEC (Royal Decree 509/1996; Royal Decree-Law 11/1995). As can be observed, the concentration of nitrogen and phosphorus decreased over time. By fitting the experimental data to a decreasing model, the time necessary to reach the desired nitrogen and phosphorus concentration for discharge can be calculated. A nitrogen concentration of 9 mg/L, corresponding to 80% reduction, was reached after 2.6 d. With regard to the removal of phosphorus, the same reduction percentage was achieved after 2.9 d. The main results showed that if an exact scale-up is assumed (similar wastewater quality, light cycle and weather), one square meter of algal growth surface could treat 140 L of the effluent of the secondary

Table 2 | List of strains isolated from WWTP

Genus name/description	Strain code	Growth medium for maintenance	Microscopical aspect	Scale
Coccoid green alga	7	BG11		– 8 μm
Coccoid green alga	2	BG11		– 8 μm
Coccoid green alga	3	BG11		– 8 μm
Coccoid green alga	6	BG11		– 8 μm
Coccoid green alga	5	BG11		– 8 μm
Coccoid green alga	4	BG11		– 8 μm
Pediastrum sp.	1	BG11		– 40 μm
Scenedesmus sp.	2	BG11		– 8 μm
Uronema sp.	2	BG11		– 40 μm
Oocystis sp.	2	BG11		– 8 μm
Uronema sp.	1	BG11		– 40 μm
Oocystis sp.	3	BG11		– 8 μm
Chlorella sp.	2	BG11		– 8 μm
Chlorella sp.	5	BG11		– 8 μm
Scenedesmus sp.	4	BG11		– 8 μm
Scenedesmus sp.	3	BG11		– 8 μm
Navicula sp.	4	Waris-H + Si		– 20 μm
Phormidium sp.	1	Waris-H + Si		– 8 μm
Uronema sp.	3	Waris-H + Si		– 8 μm
Navicula sp.	3	Waris-H + Si		– 20 μm
Filamentous cyanobacterium	1	Waris-H + Si		– 10 μm
Scenedesmus sp.	5	Waris-H + Si		– 10 μm

(*continued*)

Table 2 | continued

Genus name/description	Strain code	Growth medium for maintenance	Microscopical aspect	Scale
Chlorella sp.	1	BG11		– 8 μm
Coccoid green algae	1	BG11		– 8 μm
Sickle shaped algae (Unidentified)	1	BG11		– 8 μm
Stigeoclonium sp.	1	BG11		– 80 μm
Oocystis sp.	1	BG11		– 8 μm
Chlorella sp.	4	BG11		– 8 μm
Chlorella sp.	3	BG11		– 8 μm
Navicula sp.	2	Waris-H + Si		– 8 μm
Navicula sp.	1	Waris-H + Si		– 8 μm
Scenedesmus sp.	6	Waris-H + Si		– 8 μm
Scenedesmus sp.	1	Waris-H + Si		– 8 μm

Growth media BG11: Naumann *et al.* (2013). Waris-H + Si: MCFadden & Melkonian (1986).

treatment in the same amount of time (2.9 days, as the concentration of phosphorous is more restrictive).

Microalgae cultivation in suspension at laboratory scale

The assays carried out in suspension mode in laboratory-scale photobioreactors allowed the growth of *Scenedesmus* sp. to be monitored, as well as the variation in the characterisation of the culture medium with the experimental time (60 d). Table 3 shows the main analytical results obtained at the beginning and end of the experiments carried out in the photo-bioreactors. As can be observed, there were some differences among the most relevant variables in terms of pH, alkalinity and conductivity, which is probably due to the lower value of the latter at the end of the experiment (related to the concentration of dissolved salts) and which enabled CO_2 to be dissolved in the culture medium. Alkalinity quantifies inorganic carbon, ammonia nitrogen, phosphate and other species. With regard to the concentration of total organic carbon (TOC), this variable decreased slightly with time, which is probably due to the activity of heterotrophic bacteria in the culture medium. On the other hand, a marked reduction in the concentration of different forms of nitrogen was observed, while the concentration of phosphorus did not decrease significantly. A similar trend was reported by De Alva *et al.* (2013), who reported higher organic nitrogen removal from municipal wastewater, as nitrogen is generally required at higher quantities for microbial metabolism.

With regard to the quantification of the fresh microalgae growth, this was carried out in a more intensive manner through the optical density at the wave length with the maximal absorbance (680 nm). This variable was proportional to the concentration of solids contained in the mixing liquor of the bioreactors. Figure 5 shows the variation in absorbance with the experimental time. As can be observed, the growth of microalgae in the culture medium led to an increase of absorbance in such a manner that its final concentration was found to be $1{,}960 \pm 35$ mg VS/L. It is worth noting that microalgae did not stop growing throughout the

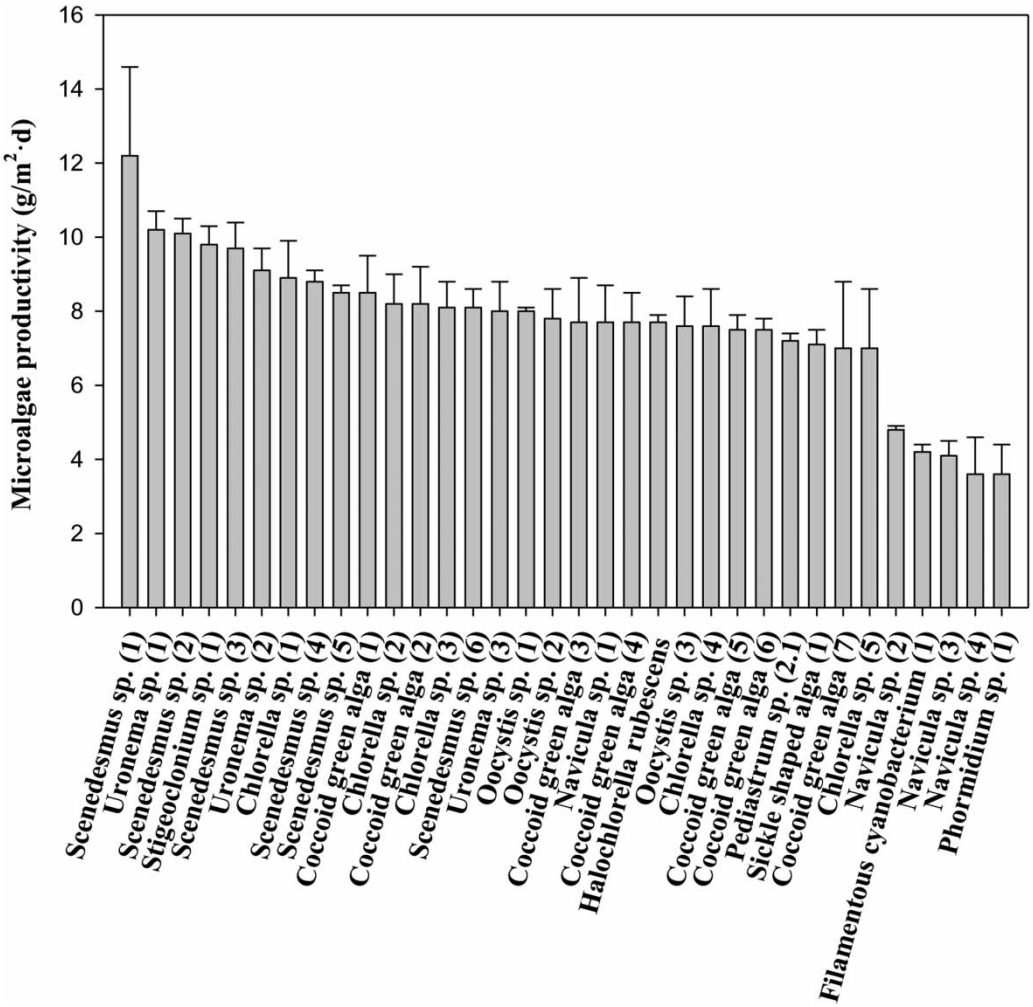


Figure 3 | Microalgae productivity calculated from the final biomass weight after 12 d of cultivation (error bars represent standard deviation, $n = 3$).

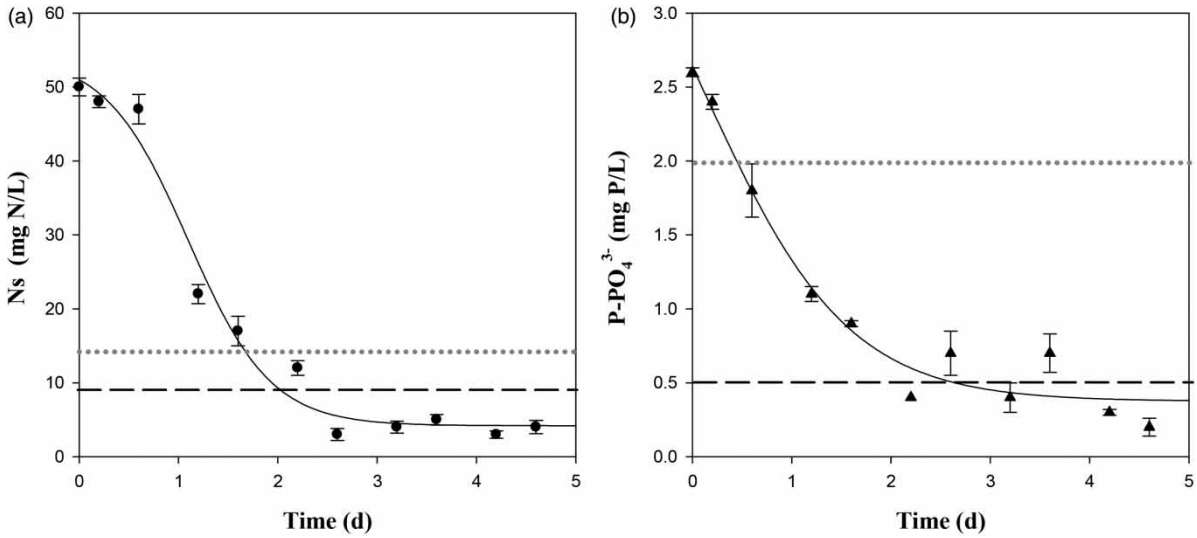


Figure 4 | Removal of soluble total nitrogen (a) and phosphorus (b) from SWW by *Scenedesmus sp.* in the TL. One phase decay curve fitting (gray line); error bars represent standard deviation ($n = 3$); dotted lines correspond to maximal discharge concentrations of EU directive; dashed lines represents reduction of 80% of the initial concentration.

Table 3 | Analytical monitoring of *Scenedesmus* sp. (1) growth in photobioreactors at laboratory scale (fresh biomass; duration: 60 d)

Parameters	Start	End
pH	7.32 ± 0.1	8.35 ± 0.01
Conductivity (μS/cm)	371 ± 5	270 ± 0.02
Alk (mg $CaCO_3$/L)	181 ± 1	259 ± 0.5
TS (mg/L)	400 ± 25	2,200 ± 55
MS (mg/L)	75 ± 15	240 ± 40
VS (mg/L)	325 ± 20	1,960 ± 35
TOC (mg/L)	57 ± 1	33 ± 1
Ns (mg/L)	117 ± 1	9 ± 1
N-NH_4^+ (mg/L)	1.69 ± 0.10	<DL (0.10)
N-NO_2^- (mg/L)	<DL (0.02)	<DL (0.02)
N-NO_3^- (mg/L)	7.77 ± 0.10	1.15 ± 0.01
P-PO_4^{3-} (mg/L)	3.17 ± 0.01	3.08 ± 0.01

DL, Detection limit.

experimentation. Further studies allowed the quantification of the growth yield of microalgae under the study conditions, which reached a mean value of 2.92 ± 0.70 g TS/m^3·d. Other laboratory-scale studies that used suspension cultures reported a biomass production of 0.24 g/L·d (dry weight basis) and complete removal of N from the wastewater (Doria *et al.* 2012).

Considering the effect that a pretreatment for storage might have on the activity of the harvested microalgae, the lyophilisation and storage at 4 °C were evaluated. In Figure 5, the growth of *Scenedesmus* sp., which was subjected to different pretreatments measured through absorbance at 680 nm, is plotted as a function of time under different conditions. As expected, the best conditions to grow microalgae under the study conditions were found to be the use of fresh algae harvested from photobioreactors. The worst situation took place when microalgae was directly lyophilized. In a more specific way, an initial lag phase was observed for lyophilized microalgae, while its subsequent growth rate became stable and slow. In addition, a brown coloration was observed initially that does not appear in the culture of fresh algae, which has a clear green coloration. Consequently, when microalgae were directly lyophilized, their growth was inhibited or had slowed down. Initial discoloration was observed at the beginning of re-seedling, and even after adding new nutrients, the growth did not reach the same rate as when fresh microalgae was used. Nevertheless, lyophilisation might be an interesting pretreatment of microalgae for other uses where transport or further approaches are to be implemented.

Microalgae cultivation at prototype scale

Once the layers of the TL system were inoculated with fresh *Scenedesmus* sp, one layer was monitored throughout its length. Samples were taken at different distances from the inlet (15, 30, 45, 60, 75 and 90 cm). This procedure allowed

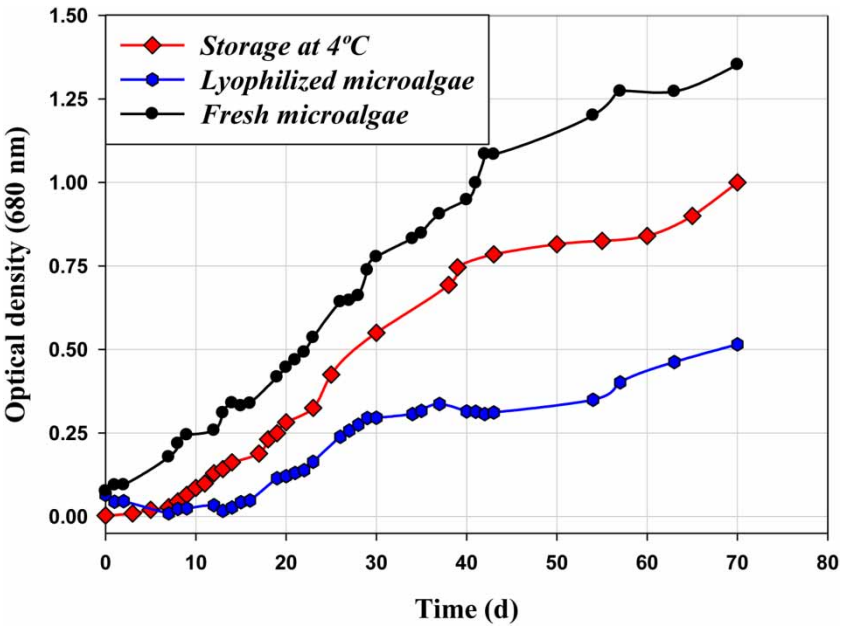


Figure 5 | *Scenedesmus* sp. growth quantified through density at the maximal absorbance wave lengths. ($n = 3$; standard deviation < 0.01 in all cases).

to quantify the percentage of nitrogen and phosphorus contained in the harvested biomass as a consequence of its removal from wastewater, which was also characterised. Figure 6 shows microalgae are richer in N and P in the highest part of the layer because they are in contact with a higher concentration of nutrients, while their concentration remained stable at the highest lengths. The average values of nutrient content in microalgal biomass were found to be $5.53 \pm 1.17\%$ for N and $8.8 \pm 2.54\%$ for P as P_2O_5, where both are expressed in dry weight.

In parallel, with increase in length, microalgae growth rate decreased from 5.66 to 1.17 $g/m^2 \cdot d$ within the range of 0–90 cm. It is worth noting that the liquid effluent of the same layer fulfilled the requirements established by the EU directive. Consequently, the dimensions chosen for building the TL prototype might be adequate to treat the SWW under study.

With regard to the operation of the TL prototype at pilot scale, monitoring of the different variables in the influent and effluent of the TL at different sampling points also allowed quantification of its efficacy in terms of N and P removal, among other informative variables. It should be noted that different layers were inoculated with fresh *Scenedesmus* sp., lyophilized microalgae or with microalgae stored at 4 °C as a pretreatment before inoculating the surface of the layers. The total duration of the experiment was 90 days. Table 4 shows the most representative results (mean values and standard deviation) of the physico-chemical characterisation of the influent and the effluent obtained under the different conditions previously explained.

As can be observed, the removal of ammoniacal nitrogen and total soluble nitrogen was not significantly different among different layers. A similar trend was observed in terms of phosphorus removal. Microalgae removed soluble nitrogen, mainly in the form of ammoniacal nitrogen. Specifically, the efficacy in the removal of

Table 4 | Physico-chemical characterisation of influent wastewater and effluents of the TL prototype

	Influent	Fresh and centrifuged microalgae	Lyophilized microalgae	Microalgae stored at 4 °C
Ns (mg/L)	57 ± 1	21 ± 1	23 ± 1	23 ± 1
$N\text{-}NH_4^+$ (mg/L)	24.7 ± 0.1	1.4 ± 0.2	1.7 ± 0.1	1.0 ± 0.01
$P\text{-}PO_4^{3-}$ (mg/L)	5.4 ± 0.1	3.4 ± 0.02	2.9 ± 0.1	2.9 ± 0.2
TC (mg/L)	174 ± 1	107 ± 1	114 ± 1	102 ± 1
IC (mg/L)	124 ± 1	67 ± 1	66 ± 1	67 ± 1
TOC (mg/L)	50 ± 1	40 ± 1	48 ± 1	35 ± 1

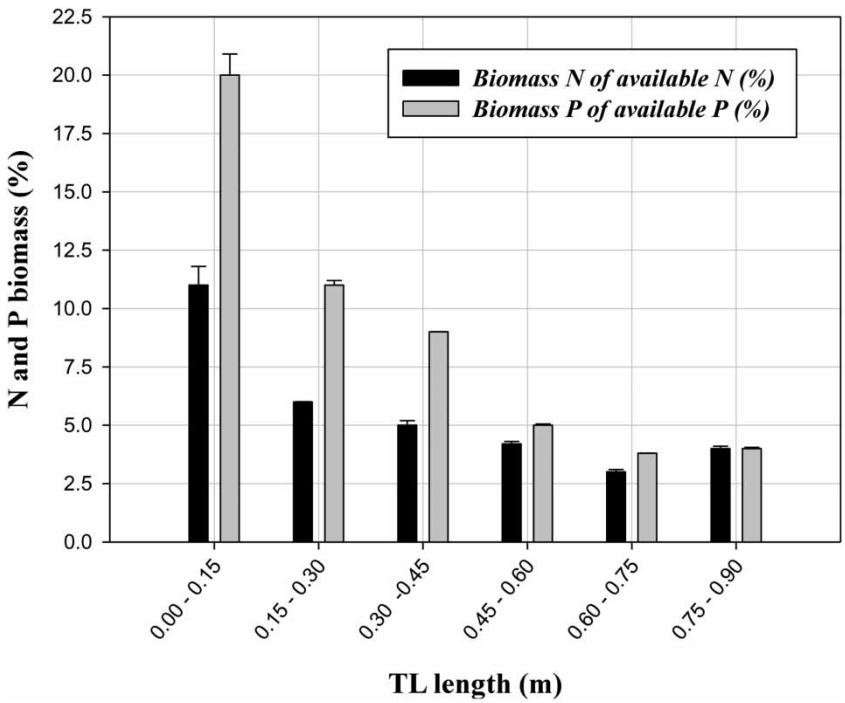


Figure 6 | Percentage of nitrogen and phosphorus (as P_2O_5) contained in biomass with respect to the concentration of both nutrients in wastewater (error bars represent standard deviation, $n = 3$).

total soluble nitrogen was found to be up to 63% for fresh microalgae (around 95% of $N\text{-}NH_4^+$), while 59% was achieved for lyophilized and refrigerated microalgae. Similar results were obtained by De Alva *et al.* (2013), who used green microalgae (*Scenedesmus acutus*) repeatedly in various types of wastewater. They reported organic nitrogen and phosphorus removal from municipal wastewater at 94 and 66%, respectively. With regard to phosphorus removal, the final concentration in the effluent was around 3 mg/L, which was lower than the initial values observed in the influent. Afterwards, an increase in the concentration of phosphorus was observed as a consequence of the high hydraulic retention time in in the decanter. The percentage of nitrogen and phosphorus in the harvested microalgae mentioned previously is in accordance with their removal from wastewater and therefore a high ammount of phosphorus was uptaken from SWW. The presence of phosphorus-accumulating microorganisms in SWW makes it necessary to promote strict anoxic conditions. The difference between the removal of nitrogen and phosphorus might also be a consequence of the denitrification that takes places in parallel (elimination of nitrate and organic matter measured through the concentration of TOC) on the surface of the layers. This process could occur if denitrification and heterotrophic microorganisms are contained in wastewater. It is worth noting that the ratio among removed nitrogen and phosphorus has been found to be higher in those layers inoculated with fresh microalgae (11.49 for fresh microalgae; 9.26 for lyophilized microalgae and 9.39 for stored microalgae).

As an example, Figure 7 shows the variation in N and ammoniacal nitrogen in the influent and effluent of the TL prototype two weeks after inoculation. As can be noticed, a marked reduction was observed in both variables after the above-mentioned period of time, while phosphorus concentration remained virtually constant at low values.

With regard to the biomass productivity from the TL prototype used as tertiary treatment at the WWTP, the main results obtained showed that the production yield varied between 50 and 60 g TS/m^2 after a growth interval range of 2–4 weeks, depending on the colonisation of the layers, which varied depending on the lighting conditions and changes in the composition of wastewater influent.

Agronomic assessment of microalgae biomass as solid bio-based fertilizers

After harvesting the microalgae biomass, it was lyophilized to be stored and characterised. Table 5 shows the main characteristics of the microalgal biomass obtained from the prototype. As can be observed, microalgae are rich in nutrients, which might make it adequate for its use as a bio-based fertilizer. Microalgae had pH 6.1, high electrical conductivity, 4.9 dS/m, and a total solids content of 140 ± 5 g TS/kg with 90.7% volatile content. From an agronomical point of view, microalgae biomass presented 50.0% C, 5.5% N, 3.8% P, 1.6% K (w/w dry basis) and relevant humic substances content (58.9 mg/kg). The presence of microelements was also relevant (0.2% Ca, 0.28% Mg, 27.9 ppm of Fe and 91.8 mg/kg of Mn) as well as the presence of other salts, such as chlorides, nitrates, sulphates and phosphates (5.2, 1.3, 2.1 and 0.3 g/kg, respectively).

Regarding their biostimulant potential, it is important to note that more than 40% of the total nitrogen content was in protein form. Table 6 shows the composition and amount of amino acids in microalgae biomass. Moreover, they presented high β-glucosidase, phosphatase and urease activity (11.2, 50.9 and 9.8 μmol/g·h, respectively). In addition, the

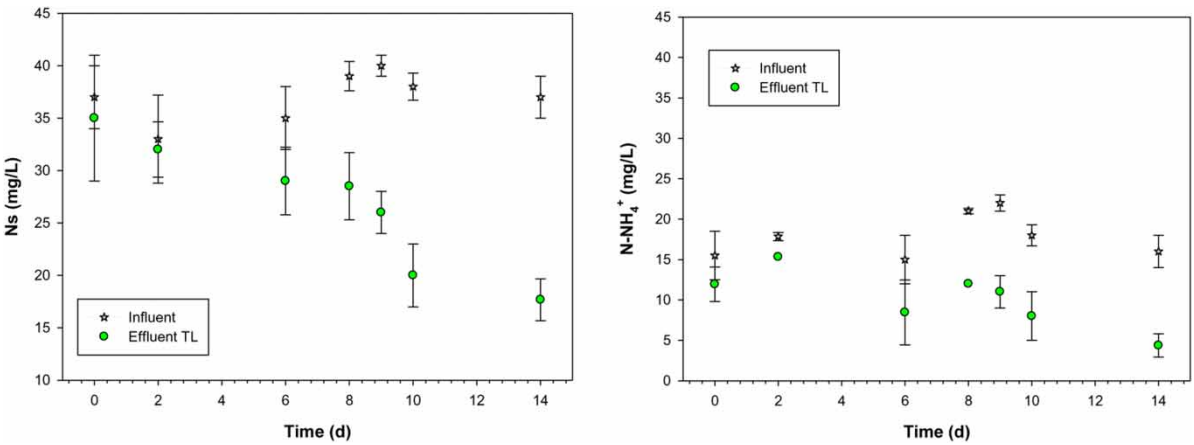


Figure 7 | Variation in N and ammoniacal nitrogen with time. TL prototype (error bars represent standard deviation, $n = 3$).

Table 5 | Characterisation of the harvested microalgae and compost and the best solid state formulation

Parameters	Lyophilized microalgae	Compost	Compost + microalgae (2%)
Dry matter (%)	97.30	67.20	68.30
pH	6.10	9.10	8.70
EC (dS/m)	4.90	3.74	3.90
Total Carbon (g/100 g)	50.00	23.50	24.90
Total Organic Carbon (OC) (g/100 g)	49.50	20.40	18.20
Water soluble C (g/100 g)	9.80	0.60	0.90
Water soluble nitrogen (g/100 g)	1.80	0.18	0.20
Volatile solids (%)	92.50	38.90	41.90
NTK (g/100 g)	5.54	2.30	2.40
Total phosphorus (g/100 g)	3.84	0.90	0.86
Total potassium (g/100 g)	1.60	3.10	2.90
Calcium (g/100 g)	0.20	10.20	8.90
Magnesium (g/100 g)	0.28	0.95	0.88
Iron (mg/kg)	279	8,594	7,497
Manganese (mg/kg)	91.80	283.50	262.30
Cadmium (mg/kg)	0.00	0.34	0.36
Chromium (mg/kg)	0.90	23.20	20.40
Copper (mg/kg)	20.50	52.70	36.40
Lead (mg/kg)	0.67	20.30	16.67
Nickel (mg/kg)	0.33	9.40	9.66
Zinc (mg/kg)	79.50	139.60	125.80
Chlorides (mg/kg)	5,154	6,792	7,077
Nitrates (mg/kg)	1,277	2,829	2,231
Sulphates (mg/kg)	2,065	2,617	2,817
Phosphates (mg/kg)	303	90	375
Bromides (mg/kg)	2.41	2.62	2.80
Nitrites (mg/kg)	0.00	0.00	0.00
Salmonella spp. (MET-MI-Salmonella)	Absence	Absence	Absence
E. coli (β-D-glucoronidase positive)	<10 cfu	<10 cfu	<10 cfu
Carbohydrates (mg C-Gluc/kg)	142.00	41.70	84.60
Humic substances C (mg C/kg)	58.90	55.00	57.10
β-glucosidase activity (μmol PNF/g·h^{-1})	11.267	1,827	1,271
Phosphatase activity (μmol PNF/g·h^{-1})	50.960	14.378	13.110
Urease activity (μmol N-NH_4^+/g· h^{-1})	9.799	0.09	0.15

Table 6 | Concentration of free and total amino acids in the harvested microalgae

	Free amino acids (%, w/w)	Total amino acids (%, w/w)
Moisture	4.760	4.760
4-Hidroxiproline	<0.001	<0.001
Aspartic ac + asparagine	0.100	4.050
Glutamic acid + glutamine	0.662	5.350
Alanine	0.237	5.470
Arginine	0.428	3.940
Cysteine	<0.001	<0.001
Cystine	0.003	0.344
Phenylalanine	0.016	2.680
Glycine	<0.001	1.700
Hystidine	0.018	0.642
Isoleucine	0.010	1.020
Leucine	0.029	4.220
Lysine	0.270	3.170
Methionine	0.010	0.966
Proline	0.211	3.690
Serine	0.421	2.220
Tyrosine	0.015	1.270
Threonine	0.116	4.420
Tryptophan	0.003	0.003
Valine	0.030	1.310
Total	**2.600**	**46.600**

microalgae biomass produced was in line with the stringent requirements established in FPR (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019, which laid down rules on the making of the EU fertilising products available in the market, amended Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealed Regulation (EC) No 2003/2003. In particular, microalgae biomass was in line with the product functional category PFC 6 '*Plant biostimulants*', which presented low levels of heavy metals (less than 1 ppm of Cd, Hg, Cr, Ni, and Pb; 79 ppm of zinc and 20.5 ppm of copper) and no detectable pathogens (salmonella and *E. coli*).

In a preliminary evaluation, a germination test was carried out with barley seeds. In general, enhancements were observed during the first stages of barley crop when lyophilized microalgae were supplied at different concentrations both in terms of germination index and root length. After 5 days, the most significant results showed that the improvement of the barley seeds' germination index was

proportional to the increase of microalgae concentration, being highest for 1/5 extract, which corresponds to a microalgae concentration of 0.075%. In this case, the root length was 8.3 cm, which is 1.6 cm more than the control test (6.7 cm). This fact indicates that microalgae might be an adequate substrate that produces positive effects on crops. However, further studies were required. In this context, additional evaluation was carried out at a higher scale.

At the greenhouse scale, the main results obtained were expressed as the fresh and dry weight of ryegrass (a) and barley yield (b) under different treatments in Figure 8. When microalgae was supplied to ryegrass as an additive

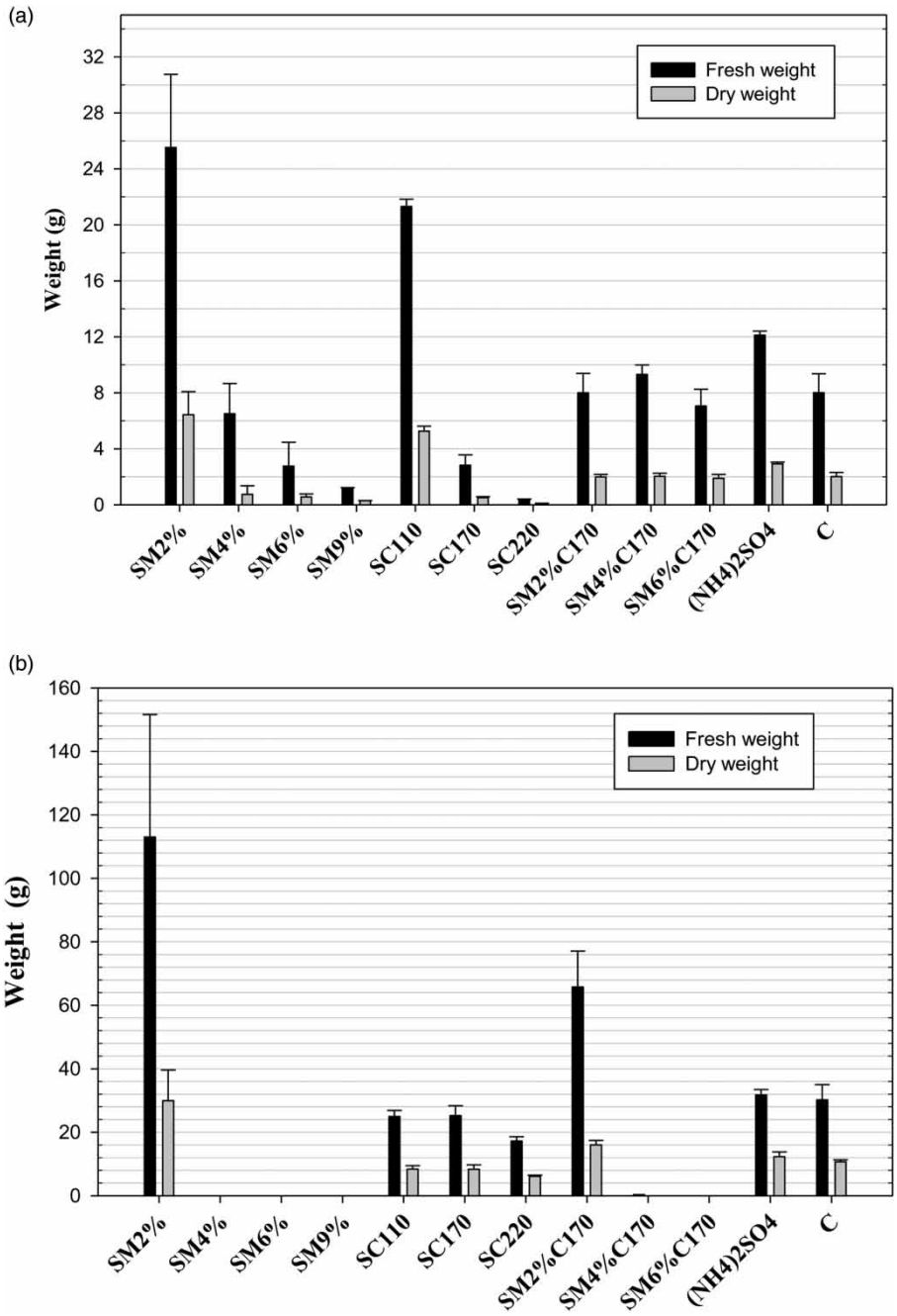


Figure 8 | (a) Ryegrass and (b) barley yields using microalgae alone or as an additive of vegetable compost at greenhouse scale. Cultivation time – 45 days (error bars represent standard deviation, $n = 3$).

in solid state formulations at a concentration of 2%, the weight of ryegrass after 45 days was 6.4 ± 1.7 g TS. This value greatly exceeded the ryegrass weight without treatment, 2.0 ± 0.3 g TS, and the weight of the ryegrass treated with ammonium sulphate (2.9 ± 0.1 g TS). In this regard, the ryegrass yield increased by 220% when compared to the ryegrass yield without treatment and 120% when compared to the ryegrass that used ammonium sulphate as a fertilizing product. Nevertheless, microalgae concentrations higher than 2% had clear negative effects on ryegrass yields. The reduction in production yield at increasing concentrations of algae might be a consequence of an excess of nitrogen and salts supply.

In parallel, positive impacts were also observed when vegetable compost (110 kg N/ha) was supplied to ryegrass. The weight of ryegrass was 5.26 ± 0.4 g TS, which is an increase by 160% when compared to control tests and 80% when compared to tests that used ammonium sulphate as a fertilizing product.

A higher dose of compost (170 and 220 kg N/ha), supplied alone or in combination with microalgae, resulted in no enhancements in the ryegrass yields derived from an excess of nitrogen and salts supply.

Similar results were observed when barley was treated with microalgae alone or as an additive of vegetable compost. Significant improvements were observed when barley was treated with microalgae in a concentration of 2% (29.9 ± 9.7 g TS against 10.7 ± 0.6 g TS of barley without treatment, resulting in 180% of enhancement, and 12.3 ± 1.5 g TS of barley treated with ammonium sulphate, resulting in 143% of enhancement). As observed in raygrass, improvements were also visible when barley was treated with microalgae (2%) as an additive of vegetable compost (110 kg N/ha).

In summary, the best performance was observed during the agronomical assessment that corresponded to the formulation of 2% of microalgae supplied alone or together with vegetable compost (110 kg N/ha). Positive impacts related to plant growth were derived from a sum of effects of amino acids, phytohormonal substances, as well as carbon pools contained in the supplied microalgae, which have the potential to enhance nutrition efficiency, abiotic stress tolerance and crop quality traits and increase the availability of confined nutrients in the soil or rhizosphere (Du Jardin 2015).

The organic fertilizers market will be regulated under the new regulation on fertilizer products, *FPR (EU) 2019/1009*, which will be implemented on July 2022. In this regard, the proposed formulations must meet the technical requirements in terms of composition and the presence of the toxic elements established in the Product Functional Categories (Annex I) and Component Material Categories (Annex II). In this regard, selected solid state formulations could fit the definition of the Product Functional Category 7, '*Fertilising product blend*', where it is specified that each component material has to meet the technical requirements regarding origin, composition and processing activities defined for each component: PFC 3 '*Soil improvers*' in the case of vegetable compost and PFC 6 '*Plant biostimulants*' for lyophilized microalgae.

CONCLUSION

The cultivation of fresh *Scenedesmus* sp. was found to be an interesting option to improve the quality of the effluents derived from secondary treatments in small size WWTP. TL might help reduce eutrophication and the use of chemical reagents for N and P removal with markedly high removal percentages, especially for ammoniacal nitrogen (95%). Furthermore, the harvested microalgal biomass met the requirements to be used as a solid bio-based fertilizer, although the microalgae proportions in the blends of over 2% of microalgae led to negative effects in the formulation of solid state bio-based fertilizers under the study's conditions. The results obtained also indicate that valorising microalgae would reduce the dependence of intensive agriculture on inorganic fertilizers and allow the closing of the N and P biogeochemical cycles, which are deeply affected by anthropogenic activities with consequent social and environmental benefits.

ACKNOWLEDGEMENTS

The authors thank the financial support provided by the LIFE Project LIFE13 ENV/ES/000800 TL-BIOFER, which is partially supported by the EU programme LIFE+ 2013.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Acién, F. G., Gómez-Serrano, C., Morales-Amaral, M. M., Fernández-Sevilla, J. M. & Molina-Grima, E. 2016 Wastewater treatment using microalgae: how realistic a contribution might it be to significant urban wastewater treatment? *Applied Microbiology and Biotechnology* 9013–9022. https://doi.org/10.1007/s00253-016-7835-7.

American Public Health Association, Water Pollution Control Federation (APHA) 1989 *Standard Methods for the Examination of Water and Wastewater*, 17.

Bligh, E. D. & Dyer, W. J. 1959 A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917. https://doi.org/10.1139/y59-099.

Cortez, S., Teixeira, P., Oliveira, R. & Mota, M. 2008 Rotating biological contactors: a review on main factors affecting performance. *Reviews in Environmental Science and Biotechnology* **7**, 155–172. https://doi.org/10.1007/s11157-008-9127-x.

De Alva, M. S., Luna-Pabello, V. M., Caden, E. & Ortíz, E. 2013 Green microalgae *Scenedesmus acutus* grown on municipal wastewater to couple nutrient removal with lipid accumulation for biodiesel. *Bioresource Technology* **146**, 744–748. https://doi.org/10.1016/j.biortech.2013.07.061.

Directive 91/271/EEC of 21 May 1991 Concerning urban waste-water treatment.

Doria, E., Longoni, P., Scibilia, L., Iazzi, N., Cella, R. & Nielsen, E. 2012 Isolation and characterization of a *Scenedesmus acutus* strain to be used for bioremediation of urban wastewater. *Journal of Applied Phycology* **24**, 375–383. https://doi.org/10.1007/s10811-011-9759-z.

Du Jardin, P. 2015 Plant biostimulants: definition, concept and categories. *Scientia Horticulturae* **196**, 3–14. https://doi.org/10.1016/j.scienta.2015.09.021.

Eivazi, F. & Tabatabai, M. A. 1988 Glucosidases and galactosidase in soils. *Soil Biology and Biochemistry* **20** (5), 601–606. https://doi.org/10.1016/0038-0717(88)90141-1.

Frostegard, A., Tunlid, A. & Baath, E. 1993 Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. *Soil Biology and Biochemistry* **26**, 723–730. https://doi.org/10.1016/0038-0717(93)90113-P.

Hernández, T. & García, C. 2003 *Estimación de respiración microbiana del suelo (Estimation of Soil Microbial Respiration)*. Técnicas de Análisis de Parámetros Bioquímicos en Suelos. Mundi-Prensa, Madrid, pp. 311–346.

ISO 7251:2005 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique.

Kandeler, E., Stemmer, M., Palli, S. & Gerzabek, M. H. 1999 Xylanase, invertase and urease activity in particle-size fractions of soils. In: *Effect of Mineral-Organic-Microorganism Interactions on Soil and Freshwater Environments* (J. Berthelin, P. M. Huang, J. M. Bollag & F. Andreux, eds), Vol. 1. Springer, Boston, MA, pp. 275–286. doi:10.1007/978-1-4615-4683-2_30.

McFadden, G. I. & Melkonian, M. 1986 Use of HEPES buffer for microalgal culture media and fixation for electron-microscopy. *Phycologia* **25** (4), 551–557. https://doi.org/10.2216/i0031-8884-25-4-551.1.

Mehta, C. M., Khunjar, W. O., Nguyen, V., Tait, S. & Batstone, D. J. 2015 Technologies to recover nutrients from waste streams: a critical review. *Critical Reviews in Environmental Science and Technology* **45** (4), 385–427. https://doi.org/10.1080/10643389.2013.866621.

Naumann, T., Çebi, Z., Podola, B. & Melkonian, M. 2013 Growing microalgae as aquaculture feeds on twin-layers: a novel solid-state photobioreactor. *Journal of Applied Phycology* **25** (5), 1413–1420. https://doi.org/10.1007/s10811-012-9962-6.

Podola, B., Li, T. & Melkonian, M. 2017 Porous substrate bioreactors: a paradigm shift in microalgal biotechnology? *Trends in Biotechnology* **35** (2), 121–132. https://doi.org/10.1016/j.tibtech.2016.06.004.

Regulation (EU) 2019/1009 *of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003*/2003 (Text with EEA relevance).

Royal Decree 509/1996, of 15 March, which develops the Royal Decree-Law 11/1995, of 28 December, and establishes the applicable rules on treatment of urban waste-waters (BOE no. 77, of 29 March 1996).

Royal Decree-Law 11/1995, of 28 December, which sets up rules for treatment of urban waste-waters (BOE no. 312, of 30 December 1995).

Schultze, L. K., Simon, M. V., Li, T., Langenbach, D., Podola, B. & Melkonian, M. 2015 High light and carbon dioxide optimize surface productivity in a twin-layer biofilm photobioreactor. *Algal Research* **8**, 37–44. https://doi.org/10.1016/j.algal.2015.01.007.

2016 Spanish Environmental Profile 2017. Directorate General of Environmental and Natural Environment Quality and Assessment. NIPO: 013-17-180-1. Publications Catalog of the General State Administration. Available from: http://publicacionesoficiales.boe.es (accessed 27 January 2020).

Stephens, E., Ross, I. L., King, Z., Mussgnug, J. H., Kruse, O., Posten, C., Borowitzka, M. A. & Hankamer, B. 2010 An economic and technical evaluation of microalgal biofuels. *Nature Biotechnology* **28** (2), 126. https://doi.org/10.1038/nbt0210-126.

Sturm, B. S. & Lamer, S. L. 2011 An energy evaluation of coupling nutrient removal from wastewater with algal biomass production. *Applied Energy* **88** (10), 3499–3506. https://doi.org/10.1016/j.apenergy.2010.12.056.

Tabatabai, M. A. 1982 Soil enzymes. In: *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties*, 2nd edn (A. L. Page, ed.). Soil Science Society of America, Madison, pp. 903–947.

Tabatabai, M. A. & Bremmer, J. M. 1969 Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology*

and Biochemistry **1**, 301–307. https://doi.org/10.1016/0038-0717(69)90012-1.

Thakur, I. S. & Medhi, K. 2019 Nitrification and denitrification processes for mitigation of nitrous oxide from wastewater treatment plants for biovalorization: chalenges and opportunities. *Bioresource Technology* **282**, 502–513. https://doi.org/10.1016/j.biortech.2019.03.069.

Thompson, W. H., Leege, P. B., Millner, P. & Watson, M. E. 2002 *Test Methods for the Examination of Composting and Compost*. US Composting Council. https://doi.org/10.1007/978-94-011-5068-2_22.

Zucconi, F. & de Bertoldi, M. 1987 Organic waste stabilization throughout composting and its compatibility with agricultural uses.

First received 3 February 2020; accepted in revised form 21 July 2020. Available online 10 August 2020