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Please cite this article as: Silkina, A., Zacharof, M-P., Hery, G., Nouvel, T., Lovitt, R.W., Formulation and utilisation of spent anaerobic digestate fluids for the growth and product formation of single cell algal cultures in heterotrophic and autotrophic conditions, *Bioresource Technology* (2017), doi: http://dx.doi.org/10.1016/j.biortech.2017.05.133

Formulation and utilisation of spent anaerobic digestate fluids for the growth and product formation of single cell algal cultures in heterotrophic and autotrophic conditions

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Abstract

Spent anaerobically digested effluents of agricultural origin were collected and treated using membrane filtration to achieve three- large particle free- nutrient streams of N:P ratios of 16.53, 3.78 and 14.22. Three algal species were grown on these streams, achieving good levels of bioremediation of digester fluids simultaneously with biomass and associated end product formation. *Nannochloropsis oceanica* and *Scenedesmus quadricuada*, where proven highly effective in remediating the streams achieving ammonia and phosphate reduction over 60% while for *Schizochytrium limacinum SR21* these serve as an ideal production medium for lipids and biomass reaching 16.70 w/w% and 1.42 g L^{-1} correspondingly.

These processes thus provide treatment of sludge, avoiding the disposal problems by land spreading. The solid components are nutrient depleted but rich in organic matter as a soil enhancer, while the fluids rich in nutrients can be efficiently utilised for growth to generate high value materials of microalgae facilitating water reclamation.

Keywords: Anaerobic digestate, membrane filtration, nutrients, microalgae, phycoremediation

1.   Introduction

The heavy industrialisation of food production as well as the gradual uncoupling of carbon based energy generation has led to the development and steady increase of anaerobic digestion (AD) as a method of choice for waste treatment and for heat and electricity production (CHP) (Cave, 2013). Currently in the United Kingdom approximately 600 plants are operating, while in Europe several thousand digesters are fully functional, with Germany alone having 7215 units (Cave, 2013). However, when the process comes to end, a viscous sludge waste material that is complex in composition, nature and structure is produced. This is making its disposal problematic as it is rich in toxic materials and recalcitrant organic substances, metals, ammonia and phosphate (Tyagi & Lo, 2013). There is a strong possibility, of forming consolidated solid layers at the bottom of the digester inhibiting effective mixing and efficient functioning of the unit if not removed promptly of the digester (Zacharof & Lovitt, 2014 b). Land disposal of digestates is becoming increasingly expensive due to distance from the digester. It also becoming recognised that digestate sludges, if not handled correctly, can also result in losses to the environments as gases (NO_X) or nutrients dissolved in water (NH_3 and PO_4) and consequently in progressive degradation of air and water resources, causing simultaneously soil acidification and thereupon eutrophication of rivers and estuaries. It is therefore not surprising that such disposal of sludge is becoming more tightly regulated (Gerardo et al., 2013). Recently, strategies for adding value to AD processes are also focusing on the potential value of spend sludge (Tyagi & Lo, 2013; Zacharof & Lovitt 2014 a; 2016).

Numerous methods have been suggested for treating AD wastewater or sludge to make it safe for discharge to the open environment. These include biological processes based on bioremediation or additional physical (screening, settling, and flotation) and chemical treatments that demand costly plant processing using extensive amounts of energy (Tyagi & Lo, 2013). These treatments generally do not allow either the recovery or the reuse of chemicals, leading to the loss and dilution of important resources (Zacharof & Lovitt, 2014 a; 2016).

Previous research (Zacharof & Lovitt, 2014 b, Zacharof & Lovitt 2015) has demonstrated that membrane filtration as a method of treatment has been proposed and applied successfully, converting

the waste effluent sludge into particle free nutrient rich fluid and nutrient depleted solids stream. Such a strategy leads to a solids fraction with reduced nutrient content being disposed to land as an organic enhancer, while the soluble organic materials, ammonia and phosphate, can be concentrated and formulated into more useful materials and so valorising this route for the wastes. One such potential route is a source of nutrients and water for growth media, used for growing microbes, algae and plants (i.e. hydroponics and aquaponics). Low energy physical treatments such as dilution, sedimentation and filtration/diafiltration by pressure driven membrane technology have been proposed and can offer sustainable solutions. Membrane technology is still a developing technology and offers an economical option, as it is easily scalable with numerous arrangements and alternatives and easy to incorporate and integrate into waste treatment processes. It offers low operational cost compared to other competing technologies since there is no phase change required and minimal or no use of chemical additives (Zacharof & Lovitt, 2016). Using this technology, waste can be recycled back to the production systems, substituting newly manufactured materials. Particle separation -depending on the size of the matters of interest- can be achieved with a wide range of membranes technologies covering microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), while the substances of interest can be clarified, fractionated, and concentrated to produce high value streams at low cost (Zacharof & Lovitt, 2015).

These effluents, if used as nutrient media, can be highly profitable, especially when compared to the traditional synthetic media or those derived from food sources, such as crops. Filtration allows manipulation of the nutrient content, since it can be combined with leaching and acidification, using MF, or selective separation and concentration, using subsequent NF and RO processes. It also allows the successful removal of harmful bacterial and viral load, being safe for immediate use. These streams can then be blended, enabling the formulation of different concentrations of appropriate proportions (Gerardo et al.,2013) suitable for supplying the nutritional needs of microbial fermentations for the intensive production of biofuels, acids and other chemicals, such as lipids and enzymes (Li & Yu, 2011). Sustainability would be further promoted since the wastes are not released untreated to the environment causing phenomena of soil toxicity, eutrophication and microbial contamination (Hatti-Kaul et al., 2007). This strategy could greatly benefit current industry, lowering

substantially the cost of raw materials used for biochemical conversions, which is among the greatest challenges for the development of biorefineries. These are currently relying heavily on plant based material.

Several applications of this technology, namely the use of anaerobically digested effluents in aquaculture, regarding growth of microalgae within the scope of phycoremediation and commodities generation, have been effective in South East Asia and Mediterranean countries including Israel, Spain and Portugal (Rawat et al., 2011; Pereira et al., 2012; Silkina et al, 2017). Microalgae are highly adaptable organisms, resistant to the heavy metals and other toxins, able to use macro (nitrogen, phosphorus) and micro (vitamins, minerals) nutrients for successful growth. While algal biomass is mainly developed within the scope of biofuels, other applications of generation high value products are explored including feed and food products of which the regulatory aspects are developed (Yen et al., 2013).

Within this concept and to test its usefulness, two microalgae, autotrophic namely *Nannochloropsis oceanica* and *Scenedesmus quadricuada* and one thraustochytrid heterotrophic *Schizochytrium limacinum* SR21 were grown on treated digested agricultural wastewater of varying nutrient composition. *S. quadricuada* is a well-known freshwater species in bioremediation application and the biomass of this species is usually rich on proteins and lipids (Sharma & Chauhan, 2016; Silkina et al, 2017). *N. oceanica* on the other hand, similarly to *S. limacinum* can grow in marine and estuarial environments, including aquaculture wastewater (Jung & Lovitt, 2010). They are intensively investigated microorganisms (Mitra et al., 2015; 2016; Taisir et al., 2016). *S. quadricuada* species are frequently used in phycoremediation studies (Gonçalves et al.; Kim et al., 2014), *S. limacinum* is being investigated due to its key feature of heterotrophic production of lipids such as poly-unsaturated fatty acids (PUFA), particularly omega-3 fatty acids, including docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), that could be potentially used for nutrition, for example in aquaculture (Mitra et al., 2015 Taisir et al., 2016). *N. oceanica* is also suitable for aquaculture feeding and biodiesel production (Mitra et al., 2016).

Therefore this work reports on the use of formulated digested agricultural into growth media, suitable for growth of industrially significant microorganisms, associated with chemical and fuels production.

The effluents were physicochemically characterized, treated to produce balanced compositions of nitrogen and phosphate components for autorphic algae, while the medium was further enriched with carbohydrates and other agents in the case of *S. limacinum* SR21. These formulated media were then used for growth of the microalgae, in bench scale batch aerobic cultures. Comparative studies were done using common standardised growth media, the AD filtrates and the enriched AD filtrates. The growth of microorganisms was evaluated in term of growth rates and biomass productivities. The end products content of lipids and proteins was quantified in the varying culturing conditions as well as with regards to the reduction of ammonia content, investigating the potential of the microorganisms as a bioremediation agent.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Yeast extract, peptone, glucose, mannitol, glycerol, fructose, sodium acetate, sodium bicarbonate, potassium chloride, sodium chloride, calcium chloride, magnesium chloride, magnesium sulphate, trace elements and vitamins were obtained from Sigma-Aldrich (Gillingham, UK).

2.1.2. Inoculum source

N. oceanica Suda & Miyashita, (Eustigmatophyceae), marine algae and *S. quadricauda* (Turpin) Brébisson fresh water algae were provided from the culture collection of the Centre for Sustainable Aquatic Research (CSAR) Swansea University, being originally outsourced in 2011 by the Hellenic Centre for Marine Research (HCMR) in Greece and Ingrepro Renewables B.V. in the Netherlands. The inocula for both species were maintained at constant room temperature (18°C) with light of 100 μmol photons m^{-2} s^{-1} light: dark cycle 16:8 hours. The f/2 standardised nutrient medium (Guillard & Ryther, 1962), (deionised water, $NaNO_3$ 0.075 g L^{-1}, $NaH_2PO_4.2H_2O$ 0.00565 g L^{-1}, trace elements stock solution 1 ml/L, vitamin mix stock solution 1 ml/L, with the trace elements solution comprised of Na_2EDTA 4.16 g L^{-1}, $FeCl_3.6H_2O$ 3.15 g L^{-1}, $CuSO_4.5H_2O$ 0.01 g L^{-1}, $ZnSO_4.7H_2O$ 0.022 g L^{-1}, $CoCl_2.6H_2O$ 0.01 g L^{-1}, $MnCl_2. 4H_2O$ 0.18 g L^{-1}, $NaMoO_4.2H_2O$ 0.18 g L^{-1}, and vitamin mix stock

solution made of cyanocobalamin (vitamin B_{12}) 0.0005 g L^{-1}, thiamine HCl (vitamin B_1) 0.1 g L^{-1}, biotin 0.0005 g L^{-1}), in 250 ml conical flasks was utilised on a weekly basis to maintain the master culture and the daily used inocula of this species. Both microalagae were grown for at least 10 generations on f/2 medium, offering optimised results regarding growth, and demonstrating adaptation of algae on this media.

S. limacinum SR21 was provided in a lyophilised form by ATCC (MYA-1381). The algae ware revived twice by inoculating the strain into 50 ml serum vials containing standardised nutrient media (artificial seawater enriched with yeast extract, peptone and glucose) and were incubated in a rotary shaker at 37°C (120 rpm, Innova 44, New Brunswick Scientific) for 24 hours. Stock culture solutions of each strain were made through the cryopreservation method. For constant use, the strain was regularly inoculated (on a weekly basis) into 30 ml serum vials containing standardised nutrient media (yeast extract 5 g L^{-1}, glucose 20 g L^{-1}, peptone 10 g L^{-1}, sodium chloride 26.3 g L^{-1}, potassium chloride 0.75 g L^{-1}, calcium chloride 1 g L^{-1}, magnesium chloride 6.10 g L^{-1}, and magnesium sulphate 3.95 g L^{-1}) and were preserved at 2 °C (Jung & Lovitt, 2010).

2.1.3. Waste Effluents

Waste effluent streams (agricultural wastewater derived from spent agricultural digested sludge, namely mixed waste of cattle slurry, vegetable waste and silage), running at over 30 day retention time, were taken off the output line of the anaerobic digester used for manure production, but before passing through the automatic coarse particle separator (>5mm) they were collected of Farm Renewable Environmental Energy Limited (Fre), Wrexham, United Kingdom, http://www.fre-energy.co.uk/.

2.2. Methods

2.2.1 AD Effluent Sludge to Nutrient Formulations Processing Scheme

These samples were pre-treated using dilution, mixing, sedimentation and sieving (Zacharof & Lovitt, 2014 b; 2015) in a 150 L capacity stainless steel vessel. The effluents of 3 varying concentrations of nutrients were formulated as follows; they were microfiltered through a pilot scale unit equipped with a ceramic membrane (Pall Membralox, 3.70 mm channels, 0.22 m^2 area with nominal pore size

<0.2μm) (Zacharof & Lovitt, 2014 b; 2015). The design of the unit has been described in extensive detail elsewhere (Zacharof & Lovitt, 2014 b; 2015). To achieve varying concentrations of nutrients, a scheme combining diafiltration with and without pre-acidification was applied (Zacharof & Lovitt, 2014 b; 2015). The process of continuous diafiltration was used without acidification, to achieve different nutrient concentrations (Fig.1). The batch process involved 3 sequential leaching steps which consisted of first concentration, and then dilution of the sludge with fresh tap water. Initially 30 L of the pre-treated sludge was collected, diluted 50:50 with tap water and placed in the feed vessel. When pre-acidification was used, 30 L of pre-treated sludge were acidified to pH 3 sludge and then placed in the feed vessel. They were processed through the membrane and then concentrated to 20 L; the permeate was then discarded. In the concentrated sludge, 20 L in the vessel, 10 L of tap water was added and then processed by the unit, to collect 10 L of permeate. This was repeated three more times. Of these, 10 L were collected each time and utilised for the culturing the algae. The permeate flow rate was manually recorded using a graduated vessel where the permeate fluid was collected. The difference in volume was recorded per minute using a stopwatch (Casio electronics, UK); on a two decimal point precision electronic scale (OHAUS I-10). The resulting, particle free, sterile effluents were used as nutrient media in this study.

2.2.2. Testing the ability of sludge to bind phosphate and ammonia

The ability of raw sludge to absorb and desorb nutrients in various pH conditions namely phosphate and nitrogen measured as ammonia was tested using the colorimetric vanadomolybdo-phosphoric acid (PO_4–P) and phenate (NH_3–N) methods respectively. These were determined according to Standard Methods for the Examination of Water and Wastewater published by APHA, AWWA and WPCF 20th Edition, 1998. Interference due to the pre-existing sludge colour was avoided by dilution of samples.

2.2.3. Physicochemical characterisation of the treated digested agricultural wastewater

Total solids (TS, g L^{-1}), total suspended solids (TSS, g L^{-1}), total dissolved solids (TDS), alkalinity, and optical density were determined according to Standard Methods for the Examination of Water and

Wastewater published by APHA, AWWA and WPCF 20th Edition, 1998. Each parameter was triplicated to obtain the average, offering highly significant results. Particle size distribution (PSD) of the sludge samples was determined by light scattering technique using Mastersizer 2000 (Malvern, UK), the zeta potential was determined by the Zetasizer (Malvern, UK) and the conductivity and salinity of the samples were measured using a conductivity meter (Russell systems, UK) calibrated with a standard solution of 0.1M of KCl.

2.2.4. Growth of *N. oceanica* and *S. quadricauda* formulated nutrient media from spent AD sludge

The two algal species were cultured, in fresh and sea water conditions with addition of treated digested and f/2 standardised medium (pH 8.0). The calculation of the amount of nutrients in the digestate was based on initial N and P concentrations (Table 1). The treated and reformulated digestate was diluted 10 times with deionised water to become suitable for the algal growth. The experiment was set-up at 18.0 ± 3 °C (controlled temperature room) on bench scale at 250 mL flasks externally illuminated on one side by a twin florescent tube (natural daylight Osram tube, Munich, Germany), at an 18:6 light cycle, at 200 µmol photons m^2 s^{-1}. Each flask was sealed using a nitrile rubber stopper and two separate ports created, with two glass tubes inserted; for aeration and sampling and exhausting gas. The flasks were continuously sparged with ambient air at 0.1 VVM with the addition of 0.03% v/v CO_2 during the light cycle. During the growth of the culture the pH was maintained by the addition of 10 mg L^{-1} sodium bicarbonate and after a few days of growth the algal culture provided buffering capacity.

2.2.5. Growth of *S. limacinum* on formulated nutrient media from spent AD sludge

The specified quantities of powdered materials for the medium were weighed on an electronic balance (Sartorius, CP4202S, JENCONS-PLS, Germany) and they were added into an Erlenmeyer flask containing 1L of distilled water. Once mixed, the medium (pH 7.8) was decanted into 250 ml

Erlenmeyer flasks in 100 ml aliquots, which were inoculated with a 10% v/v inoculum size at late exponential growth phase.

The treated digested agricultural wastewater was decanted into 250 ml Erlenmeyer flasks, in 100 ml aliquots. The medium, having passed through a microfiltration membrane, was considered sterile. The treated agricultural wastewater used as nutrient medium was then enriched with various carbohydrate sources (2% w/v glucose, mannitol, fructose solutions added in a 1:1 ratio and 2% v/v glycerol solution), nitrogen sources (2% w/v yeast extract solution added in a 1:1 ratio) and buffering agents (1% w/v sodium acetate and sodium bicarbonate solutions added in a 1:1 ratio). The flasks, then sealed and secured with cotton and aluminium foil, were gently mixed using a vortex mixer and inoculated with 10 ml of inoculum. They were then incubated in a rotary shaker at 37 ℃ (Thermo Scientific Incubator, UK) at 37 °C.

2.2.6. Measurement of cellular growth and biomass

The cellular growth was measured into a UV–Visible UNICAM UV300 dual beam spectrophotometer at 550 nm for *S. limacinum*. The tube had a 1 cm light path. Maximum specific growth rates (μ_{max}, h^{-1}) of the microbial strain were determined in under after a 10 hour cycle of static incubation at 37 °C (Thermo Scientific Series 6000 Incubator, USA). Dry cell weight (gL^{-1} as DCW) measurements were made directly by collecting 10 ml cultured broths, filtering the sample through Whatman No. 2 filter paper, rinsing thrice with distilled water to remove salts and drying the papers for 4 h at 105 °C. DCW was determined by a weight difference (Sartorius, CP4202S, JENCONS-PLS, Germany) between the blank filter paper and the dried sample. For *N.oceanica* and *S. quadricauda* daily 15 mL samples were taken from the cultures at the end of the light cycle. Samples were immediately measured for their pH and then cell concentration and cellular volume assessed by Coulter Counter Multisizer 4 (Becker, London, United Kingdom).

2.2.7. End Product Analysis and Bioremediation Measurements

Post growth and biomass removal, analysis of the treated digested agricultural wastewater was performed using the standard methods from APHA, AWWA and WPCF 20th Edition, 1998. Samples for measurements of total nitrogen and phosphorus analyses were taken every 24 h and centrifuged for

15 minutes under 3,000 rpm to obtain the supernatant. Supernatant samples were then passed through GF/F Whatman filters. Supernatant samples were frozen in a -20 °C freezer for 1 week prior to the analysis. Total nitrogen (or total soluble, if the sample is filtered) was assayed by oxidation with alkaline persulphate to nitrate, followed by analysis using the hydrazine reduction technique. Total phosphorus was determined using the colorimetric vanadomolybdo-phosphoric acid (PO_4–P).

End products generation was measured in case of *S. limacinum* total lipids, according to the following method; the culture liquid was removed from the samples by centrifugation and then the pellets were washed with distilled water twice. Washed pellets were dried at 105 °C for 4 h. Lipids were extracted from dried biomass with chloroform: methanol mixture in a 2:1 ratio until the colour of the solvent layer disappeared. The amounts of lipids (w/w %) produced were determined by the weight difference between the blank flask and the flask containing the extracted oil after the solvent evaporation under vacuum.

Analysis of biomass was done using the C:N SerCon GSL elemental analyser (1000 C combustion temperature). The protein content determined by multiplying the nitrogen content of dried biomass measured by a factor of 6.28 (Safi et al., 2013). This approach is analogous to that based upon Kjeldahl digestion (Safi et al., 2013) for the case of *N. oceanica* and *S. quadricauda.*

2.2.8. Numerical Analysis of the Experimental Data

Each differential parameter was triplicated to obtain the average data. The data were statistically analysed for accuracy and precision calculating standard deviation, standard error, experimental error, regression factor and reading error (Microsoft Excel software Version 2007). All the numerical data were proven to be highly accurate and reproducible having a mean standard deviation of below 5% and experimental error below 5%, offering highly significant results.

3. Results and Discussion

3.1. Evaluation of the ability of sludge to absorb nutrients

Sludge has been reported (Gerardo et al., 2013) to have the ability to absorb further nutrients and to release them when leached either *in vitro* or *in vivo* (rainwater, floods, soil erosion). pH is an important factor in the case of phosphate, contributing to the amount released in the environment

(Bauer et al., 2009) since it is susceptible to changes of state when exposed to pH changes from H_3PO_4 in acidic environment and to PO_4^{3-} in alkaline conditions. Raw, untreated sludge was found containing 2026 mg of PO_4-P L^{-1} and 2208 mg NH_3-N L^{-1} (Table 1), a considerable amount of nutrients that, if released in large amounts untreated, could potentially harm the environment. Leaching treatment combined with acidification to pH 3 was tested. It was found that 36% (731 mg) of the phosphate is removed during the fisrt leaching, while 64% (1298 mg) of phosphate remained attached on the solids despite two further leaching processes. On the other hand, 89% (1965 mg) of ammonia is dissolved in the first leaching, with only 10% (222 mg) of ammonia remaining bound to the solids.When alkaline conditions are maintained (sludge original pH 8.23), 20% (407 mg) phosphate is removed at the first leaching, with the remaining 80% (1622 mg) being attached on the solids. Ammonia is removed by 63% (1392 mg), with a further 15% (332 mg) removed in the second and third leaching and the remaining percentage is bound to the solids. It can be assumed that acidic conditions favor the release of phophate and ammonia to the environment, however a large amount of nutrient remains atached to the solids, calling for the development of different methods for their recovery.

3.2. Physicochemical Characteristics of Treated Agricultural Waste Effluent Streams

After 24 h sedimentation, the wastewater effluent was filtered through the ceramic cross-flow microfiltration unit at a TMP of 10 psi, achieving a flux of 87.48 L m^{-2} h^{-1}, using diafiltration with and without acidification. The purpose of diafiltration was to investigate the effects of removing the soluble components of the sludge, such as inorganic ions like calcium, phosphorus and metals. Phosphorus is often found in sludge in the form of struvite. Struvite is a phosphate mineral comprising magnesium, ammonia and phosphate, therefore acidification and leaching are of great importance to dissolve these substances into the resulting solutions. During filtration, the majority of solids and insoluble organic matter were retained by the membrane filter (Table 1). Interestingly, the cross flow arrangement of the filtration unit allowed the continuous circulation of the processing fluid in the

system. This enabled the continuous disengagement of nutrients retained in a compressible permeable cake, formed by the deposition of solids in the membrane channels (Zacharof & Lovitt 2014 b).

Three different compositions were formulated in order to investigate the suitability of AD sludge as growth and high value end products generation media. The nutrients were transferred to the permeates, allowing the formulation of three complex but large particle free solutions to a molar N: P ratio of 16.53, 3.78 and 14.22 (Table 1). Each solution has different physicochemical characteristics with strong variations to the nutritive content and metal ions that can be used as growth stimulants (nitrogen, carbon and hydrogen intake). This is expected to generate different responses from the microorganisms, favouring either biomass or lipids formation. The solid matter content, the conductivity and the ions related indicate a solution rich in mineral salts that may be taken up during the microbial metabolism. In certain cases, these might help the growth of, for example, algal microorganisms, while in others may hinder intensive growth due to toxicity. For bacteria, algae or fungi, though, with metabolic products such as acids or biofuels like ethanol, biodiesel or lipids, these effluents can be used safely.

Further to the above, sequestration of carbon and nitrogen is being tested, aiming to develop a low cost, two step remediation process of AD effluents, combining mechanical (membrane processing) and biological treatment (algal growth in which N are P are accumulated in potentially useful products). This strategy is scalable and can be applicable to an on-site or to a centralised processing setting.

3.3. Assessment of treated digested agricultural wastewater as nutrient medium

The formulated waste effluents (Table 1) were then used to assess the performance of the autotrophic and heterotrophic algae, in bench scale batch aerobic cultures. In the case of *N. oceanica* and *S. quadricuda* the waste effluents were diluted (factor of 10) (see Section 2.2.4 of Materials and Methods). The autotrophic cultivation was studied with two microalgae *N. oceanica* (Eustigmatophyta) and *S. quadricuda* (Chlorophyta). In this study both species showed comparable growth (Fig. 2, 3) with the standardised growth media (f/2), a great potential for the high value end products accumulation growing on waste based reformulated media.

The microalgal cultures were successfully acclimated to the experimental conditions and showed great growth performance. Both species had a very short lag adaptation growth phase (from day 0 to day 2), when grown on the modified anaerobically digested sludge (Fig. 2, 3). The exponential growth for *N. oculata* was from day 2 to day 9, with maximum growth rate of 0.6 d^{-1} for 16.53, and 14.22 solutions and control conditions, while when grown on the 3.78 composition a 0.45 d^{-1} growth rate was developed (Table 2).

 S. quadricuada (Fig. 3) grew slightly slower in comparison with *N. oculata*, (Fig. 2); the average daily growth rate when grown on the waste solutions was approximately 0.3 d^{-1}. These results were comparable to other studies (Tan et al., 2016), showing great adaptation and stabilisation of culture growing on waste base nutrients.

N. oceanica biomass was high on lipids content growing on 3.78 media. Slightly similar results were obtained growing on the 16.53 media formulation (Table 2). The accumulation of lipids for this species was previously investigated by other researchers, growing these algae on waste water (Gong & Jiang, 2011). The limitation of nitrogen or phosphate sources shifting the physiological way of storage materials (lipids) in algal metabolism (Vitova et al., 2015) was demonstrated in the results.

Biomass of *S. quadricuada* was not high in lipids accumulation in comparison to *N. oceanica* and *S. limacinum.* However, this species shows high concentration of proteins in comparison with other species. Particularly under 14.22 media, the protein accumulation was 0.13 DWC g L^{-1} (Table 3). These results were comparable to Pancha et al. (2014)(Pancha et al., 2014), showing the great functional feed application of this species.

S. limacinum adaptability to several physicochemical conditions including aeration, pH, temperature and its ability to metabolize a wide range of carbohydrates and other sources made this micro algae an ideal candidate for bioconversion of waste to high value end products (Ethier et al., 2011; Fenton & Ó hUallacháin, 2012). Comparative studies were conducted among the formulated waste based media; enriched waste based media and in vitro standardised media. Comparable growth (Table 4) to synthetic growth media was achieved by the microorganism when using waste based media; with optimum results being achieved when the strain was grown on the 16.53 ratio effluent (Fig. 4a). The

other two media compositions did not offer high growth or productivity results, possibly due to the dependence of *S. limacinum* on nitrogen and carbon sources rather than solely phosphate (Table 4).

The ability of *S. limacinum* strain to metabolise various carbohydrates and other sources was investigated (Table 5). Of the numerous carbohydrate sources used, fructose offered optimised results. It was found that the addition of fructose elevated significantly the growth rate, offering high biomass productivity and lipids content, an increase of 76% and 51% respectively when compared to the treated digested agricultural wastewater (Fig. 4b). The addition of glucose offers favourable results, as it increased lipids content by 20%, while glycerol is proven ineffective slightly raising the growth rate when compared with the AD derived media or the standardised media (Table 5). Mannitol, on the other hand, affects negatively algal growth, reducing the growth rate by 45.5%., possibly due to the carbohydrate being slightly more complex and larger than glucose and fructoseand consequently has low uptake. Yeast extract was added to test the effect of supplementing further, nitrogen growth rate was improved by 36.4% but not the end product productivity or the biomass produced.

Several studies (White et al., 2013) have indicated the effect of bicarbonate and acetate as carbon sources and buffering agents, boosting the growth of algae. In this study though, buffering agents such as sodium bicarbonate have an inhibitory effect on *S. limacinum* growth, reducing μ_{max} by 54.5%. While sodium bicarbonate is the most commonly used non-toxic buffer in traditional culture media and reagents, in order to be effective in improving the pH control on the media and maintain it within physiological, the cultures should be incubated in microaerophilic conditions. This was not the case for *S.limacinum* possibly causing hindrance on growth, since carbonate ions were not utilised. Sodium acetate though did have a positive effect on growth and lipids production when compared with the standardised media, but no significant effect when compared with the treated solution of 16.53 composition (Table 5).

Carbon based agents such as powdered or liquid carbohydrates i.e. glucose powder or fructose syrup, although very important supplements for microorganisms growth are costly additives, with a current bulk value of USD $400 per ton and USD $825 per ton respectively. They could be replaced by confectionary, dairy and sugar processing waste, for example corn syrup, whey or molasses. That would be advantageous since this type of waste has high nutrient content, dairy whey for example has

been estimated to contain 48 g L^{-1} of lactose and 10 g L^{-1} of protein (Bridgwater et al., 2015). They require extensive treatment before disposal, their processing and treatment is expensive especially for small and medium producing industries.

3.4. Assessment of autotrophic and heterotrophic algae as bioremediation agents

N. oceanica and *S. quadricuada* remediation ability was tested. Regarding *N. oceanica* for N:P 16.53 and N:P 3.78 waste based media both species remediated the nitrogen by the 6th day of cultivation (20 mg L^{-1} to 1 mg L^{-1}) but on the N:P 14.22 composition nitrogen was assimilated in a slower rate (Fig. 5). The phosphorus uptake was even quicker; 95% (1.8 mg L^{-1} 1 to 0.2 mg L^{-1}) was remediated by second day of cultivation period. The cultivation of *S. quadricuada* on N:P 16.53 and N:P 3.78 reformulated media completely accumulated (100%) nitrogen by day 7, achieving similar results to the f/2 media (Fig.6). During the algal growth on the N:P 14.22 media, consumption was slower with 50% of total nitrogen uptaken by day 5 (20 mg L^{-1} to 10 mg L^{-1}) (Fig. 6). The slow remediation was followed until the end of experiment. On the other hand, phosphorus was consumed very quickly by day 3 (1.8 mg L^{-1} to 0.1 mg L^{-1}) the total amount was consumed. Further to the above, both species growth reduced the colour of by 90% (from 0.10 to 0.01) and pH by 24.72% for *N. oceanica* and 17.97% for *S. quadricuada*.

Both microalgal species have shown active waste nutrients uptake, resulting in growth and end products generation. The growth of *N. oculata* (μ=0.40d^{-1}) was higher than *S. quadricuada,* (μ=0.38 d^{-1}) (p>0.05), demonstrating adjustability of the species to the formulated media. Both species successfully reduced colour and, while their growth was not hindered by media pH, that was maintained during growth (Mitra et al, 2016; Rawat, 2011). *N. oculata* and *S. quadricuada* have a potential to be used as bioremediation agents. The large scale cultivation of both species would substantially help to reduce waste nutrients. Their ability as autotrophic algae to reduce nitrogen and phosphorus content in waste before it would discharge to the natural aquatic bodies is of great interest to the industry (Gonçalves et al., 2016). The use of algal species for bioremediation could possibly replace high cost nitrification and denitrification chemical methods with environmentally friendly approaches (Renuka et al., 2013).

The effect of *S. limacinum* growth on ammonia, phosphate, conductivity, pH and colour was tested. In all the samples there was a reduction in the parameters varying from 1.50% to 91.6%. Optimum results were achieved in the treated digested agricultural wastewater enriched with fructose, by the end of the culturing time (48 h, late exponential phase). Ammonia was reduced by 91.6% (33 mg L^{-1} 1 to 17.22 mg L^{-1}), phosphate by 15.3% (4.07 mg L^{-1} to 3.53 mg L^{-1}), conductivity by 50.7% (17.33 mS/cm to 11.5 mS/cm), pH by 13.3% (9.37 to 8.27) and colour by 66.6% (0.10 to 0.033), indicating a reduction of organic matter in the AD sludge.

According to the experimental results presented, it can be concluded that treated digested agricultural wastewater can be successfully used to grow *S. limacinum* SR21. The effluents supported the production of lipids to a satisfactory level and, if enriched, could achieve high productivity yields in terms of biomass and lipids content. *S. limacinum* could potentially serve as a bioremediation agent, since during its growth it reduces significantly the organic and inorganic content of the waste effluents.

A medium size anaerobic digester is able to treat 11000 - 15000 m^3 of organic waste (cattle manure, chicken manure, vegetative waste) per year, within the scope of manure production and biogas generation, generating a considerable amount of excess untreated sludge (Morley &Bartlett, 2008). Removing the surplus digestate ensures the continuous function of the digester, benefiting financially the industry by avoiding the disruption of digester's function due to cleaning. It could be removed by pumping to a locally sited pre-treatment tank and then be transported elsewhere to a local level for treatment. Having treated the digested waste effluents with a combination of dilution, sedimentation and microfiltration has reduced their ammonia, phosphate, salts and organic matter content. This treatment can possibly satisfy the newly introduced specifications (BSI PAS 110, 2014) regarding the use of digestates as raw material, rather than being treated as waste. However, due to the continuously stringent regulations regarding wastewater disposal regulations, further treatment could be possibly requested. The bioremediation of waste water by autotrophic algae has been previously described (Zhu et al., 2013; Gómez-Serrano et al., 2015) and is of great interest to the public and private waste treatment sectors, especially as a secondary treatment.

The wider application of this technology is relying on its practical and cost effective application to the formulation of nutrient media, tailored in compositions to the nutritional needs of the microorganisms of interest (www.biogas-info.co.uk; Appels et al., 2008). However, a new resource would have been created, lowering the impact of digested effluents to the environment, valorising them since they can be used for the production of valuable products. The cost of treating the excess digestate by microfiltration in order to formulate it into nutrient media, in other words the production cost, was calculated as $USD per kg of treated effluents, as 0.0033 $USD per kg (Zacharof & Lovitt, 2015), significantly lower when compared to the cost per kilogram of common standardised nutrient media; for example for clostridia the cost has been calculated as 0.0094 $USD per kg. The effluents could possibly be used into large quantities as growth media, due to the low cost of production.

Another advantage of this approach -the use of treated waste effluents as biobased production of chemicals, media- is the minimization of the use of yeast extract. Yeast extract is a protein and nitrogen rich, up to 85% composition, microbial growth supplement. It is traditionally produced by virgin yeast cells grown on beet or cane molasses on a batch or fed batch mode (Bekatorou et al., 2006). Despite containing carbohydrates up to a 75%, molasses do not fully support yeast growth, therefore vitamins (biotin, thiamine, pantothenic acid) and magnesium and potassium salts are added (Gómez-Pastor et al., 2011). The high cost of raw materials as well as the sophisticated techniques used for the downstream processing (membrane filtration, spray drying) have elevated the cost of yeast extract up to $3000 USD per ton (Rahimpour et al., 2014). Substituting the use of yeast extract with a nitrogen rich solution derived from waste would be highly profitable as well as beneficial for the environment, since yeast industry wastewater is characterised by high chemical oxygen demand (COD) often above 25,000 mg L^{-1}, dark colour, and high concentrations of total nitrogen and non-biodegradable organic pollutants, demanding extensive treatment prior to discharge (In et al., 2005; Bekatorou et al., 2006).

Although there are several studies of growth of algae on anaerobically digested wastewater of various sources (swine manure, domestic, municipal wastewater) (Munoz & Guieysse, 2006;Cai et al., 2013) they have been mainly focused on filamentous cyanobacteria (blue-green algae) (Markou,&., Georgakakis, 2011) and species such as *Chlorella* (Singh et al., 2011). The main interest of these

studies was remediation of waste and in certain cases biofuel production (Park et a .,2011) . None of these studies though has been conducted in formulated, standardised waste based media which could be used to support high value end products generation.

The cultivation of autotrophic and heterotrophic species produced different results. The autotrophic grown species were more effective on waste nutrients remediation, but low on biomass productivity and lipids accumulations. The heterotrophic cultivation provided less effective remediation potential but high accumulation of lipids and high productivity

The autotrophic species used in this project had successfully uptaken inorganic nitrogen and phosphorus (80-100% in 4-6 days) and used them in their metabolism for effective growth and formation of biomass. The algal biomass can be used as a fertilizer, source of energy in a biorefinery, as enrichment agents in animal feed and in other applications (Zhu et al, 2013).

It is worth commenting on the potential impact on using ammonia from wastes as a source of nitrogen, compared to the use of fertilizers. In these, ammonium and nitrate content are manufactured with a significant carbon footprint as methane is used as energy and hydrogen source to reduce atmospheric nitrogen to ammonia. It is noted that for the production of nitrates using the best available technology and practise, 2.2 tons CO_2 per tonne of nitrogen as ammonia or 3.5 tons of CO_2 as ammonium nitrate are generated (www.yara.co.uk).

The average elemental composition for microalgae, given by Oswald (1988) is $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, giving composition of 53% C w/w and 9.3% N w/w%. It is therefore possible to calculate the net CO_2 fixed by the algae as the CO_2 fixed by algae minus that CO_2 produced in the synthesis of ammonia and ammonium nitrate; i.e. 53 g carbon require 9.3 g N or 193g CO_2 fixed minus 18.6 g CO_2 produced in ammonia manufacture. This gives about 10% net reduction in net CO_2 fixed, if ammonia from fertiliser had been used. In addition, if ammonium nitrate is used then this process would produce 32.55g of CO_2, with the waste nutrient representing almost 17% reduction in CO_2 net consumed and this would be even higher in less efficient fertilizer manufacturing plant. It also has a significant impact on the energy balance within algae biorefinery, where the N used exported as protein rather than the N being recycled through AD of residual biomass in algal biofuels production systems.

A further benefit is the removal and recovery of ammonia is that alternative bioprocessing, i.e. in nitrification and denitrification, for the removal of nitrogen (ammonia) from the waste is slow and requires a lot of energy for aeration of the fermentation i.e. about 4 kWh per kg (Mauer at al., 2003) and equivalent of about 1000g CO_2 per kWh using coal. It can be concluded that the effluents can be used effectively as biotechnological growth and productivity stimulating agents for algal species. Although microfiltration is proven valid in removing pollutants from surplus digestate, further treatment might be necessary, due to the continuously stringent regulations regarding disposal.

4.0. Conclusions

Summarizing,

- Anaerobically digested effluents can effectively be used as nutrient support, being an economically viable option to the synthetic nutrient media.

- *N. oceanica* and *S. quadricauda* grew successfully on the waste streams with high uptake rate of inorganic nitrogen and phosphorus, comparable to the alternative methods of waste treatment.

- Good growth rates, biomass productivities and end product (lipids) generation were found regarding *S. limacinum*, especially when growth stimulating agents were introduced to the waste based media.

- Autotrophic cultivation of algae provided a biomass rich on proteins, the heterotrophic cultivation helped accumulate high level of lipids

Acknowledgements

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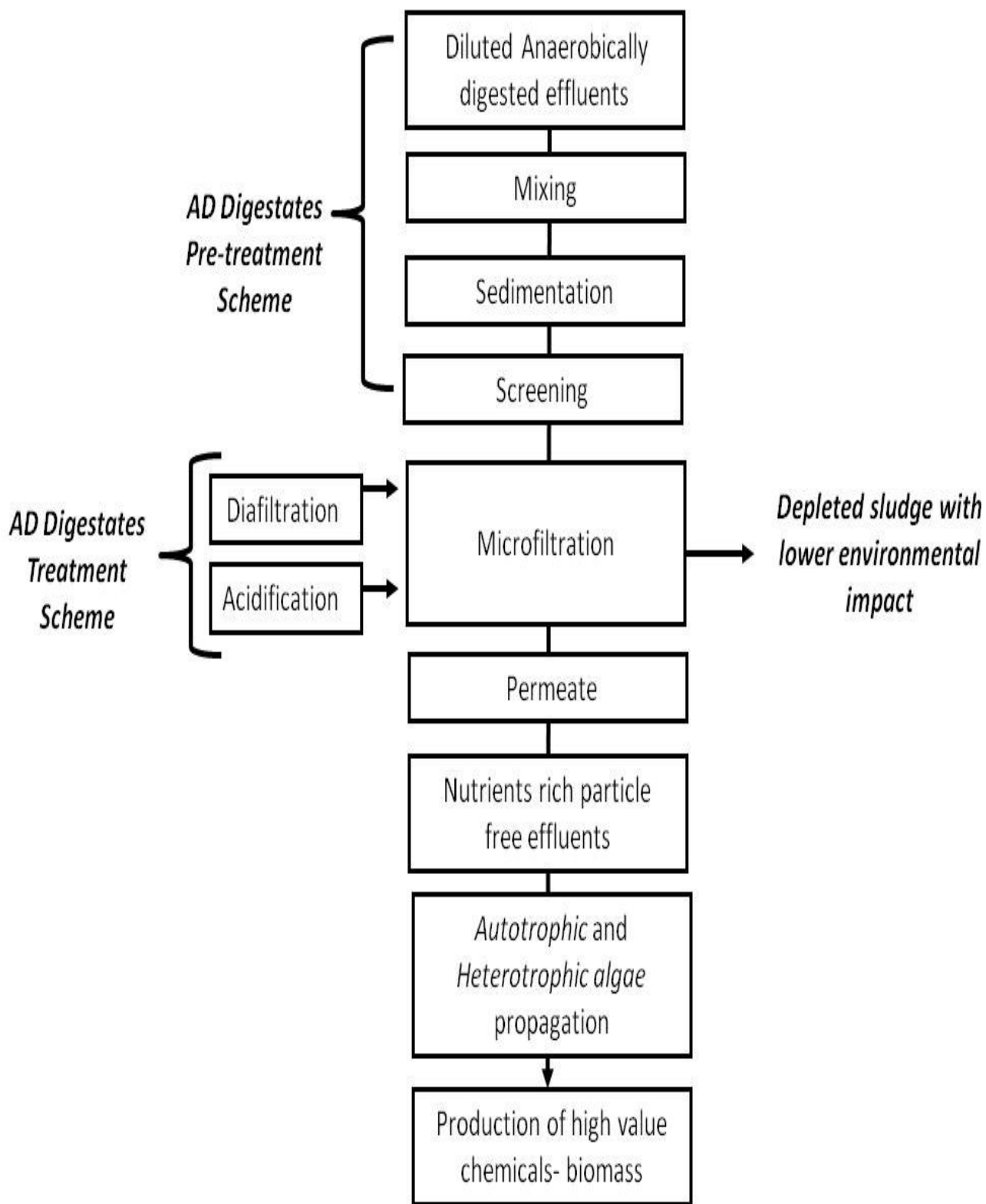
Fig 1. Schematic diagram of processing scheme of complex waste effluents and their use for intensive production of platform chemicals
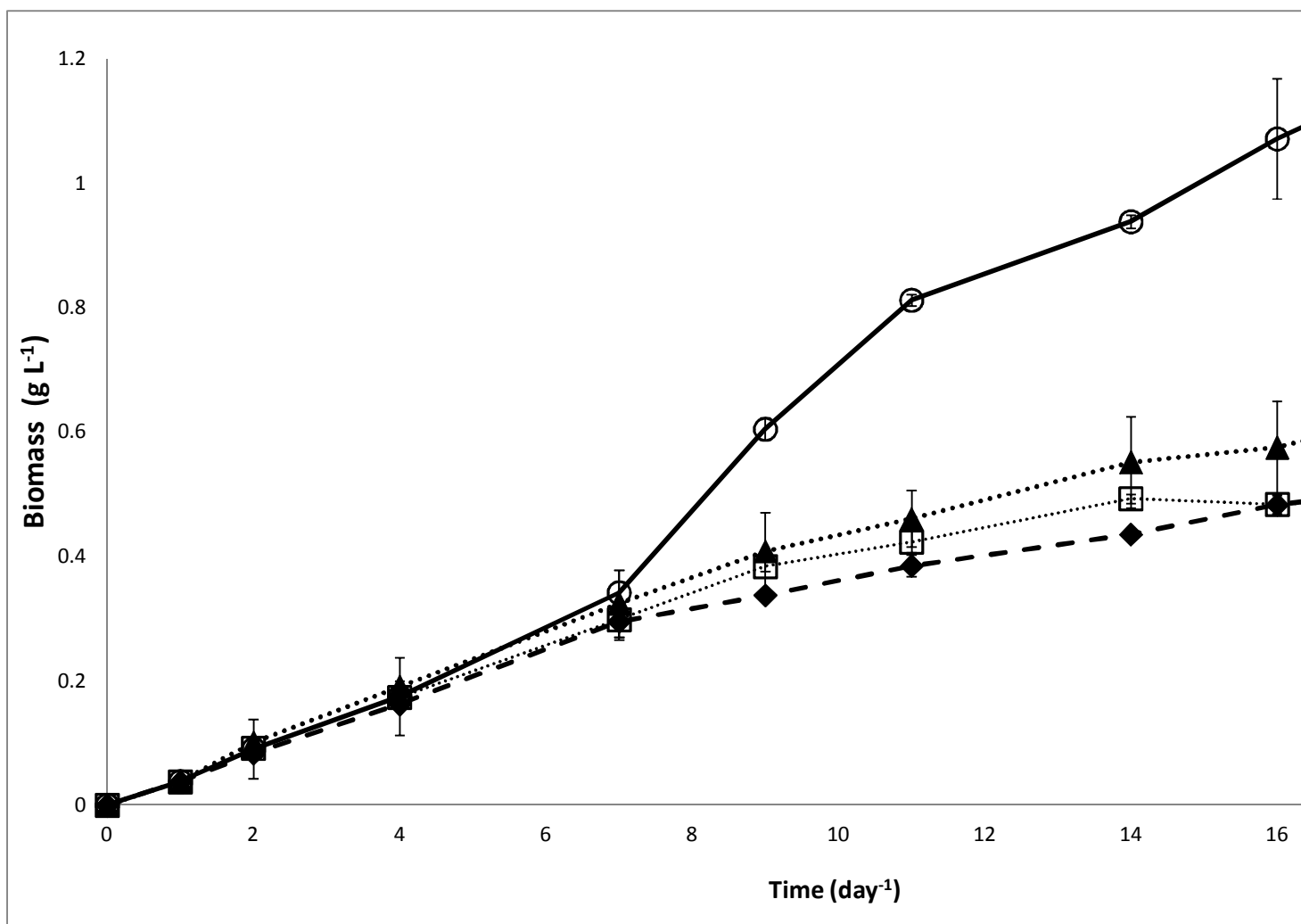
Fig. 2. Growth of *N.oceanica* treated digested agricultural wastewater N:P 14.22 (balanced) (♦),treated digested agricultural wastew
(☐),treated digested agricultural wastewater N:P 3.78 (high phosphate) (▲)in vitro standardised liquid media (f/2) (O).

Fig. 3. Growth of *S. quadricuada* treated digested agricultural wastewater N:P 14.22 (balanced) (◆),treated digested agricultural was
(□),treated digested agricultural wastewater N:P 3.78 (high phosphate) (▲) in vitro standardised liquid media (f/2) (O).
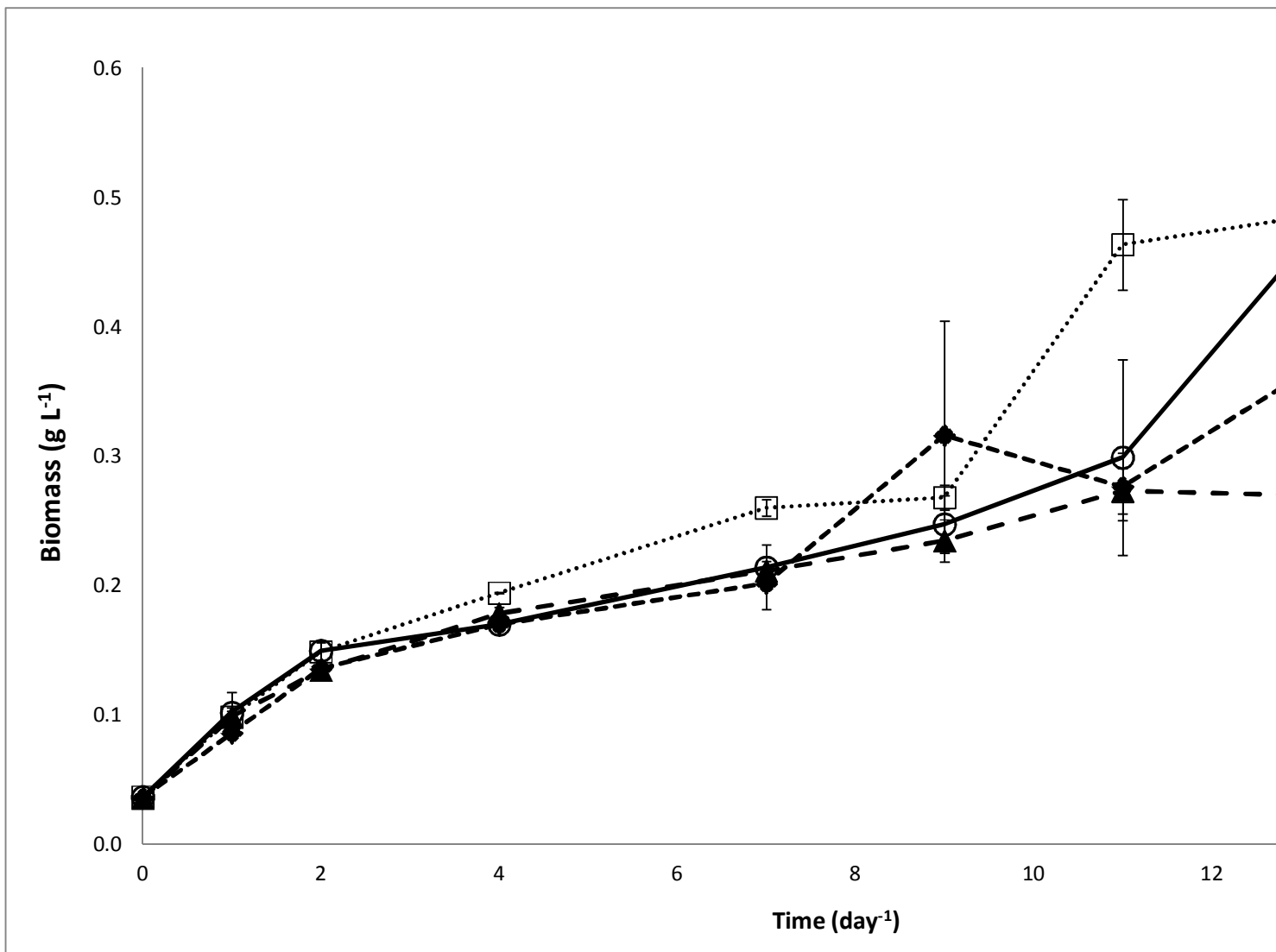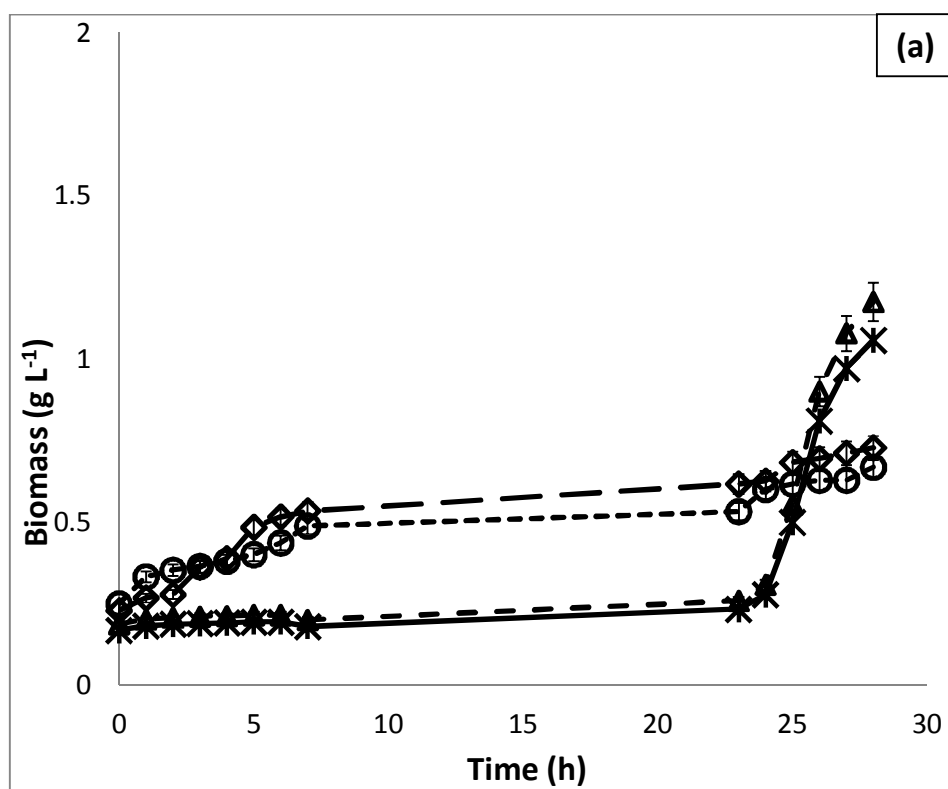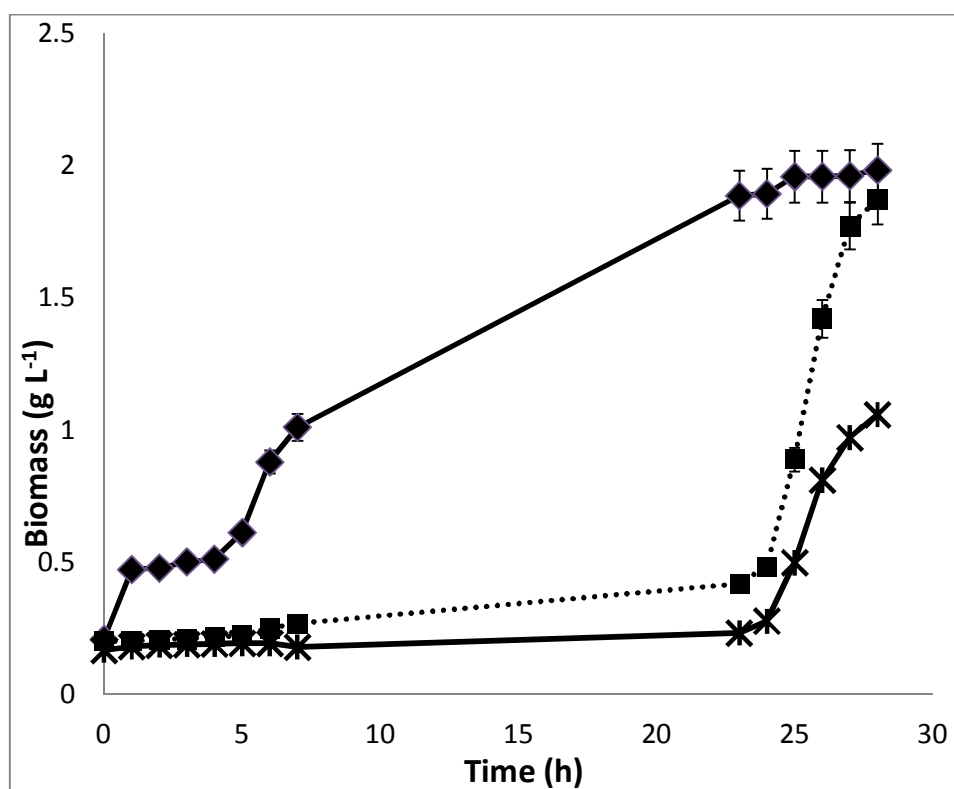
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Fig. 4 (a,b). Growth of *S.limacinum* SR21 treated digested agricultural wastewater N:P 14.22(○),treated digested agricultu... treated digested agricultural wastewater N:P 3.78(◇) and in vitro standardised liquid media (*) treated digested agricultu...),treated digested agricultural wastewater & yeast extract (■) and in standardised liquid media (*)

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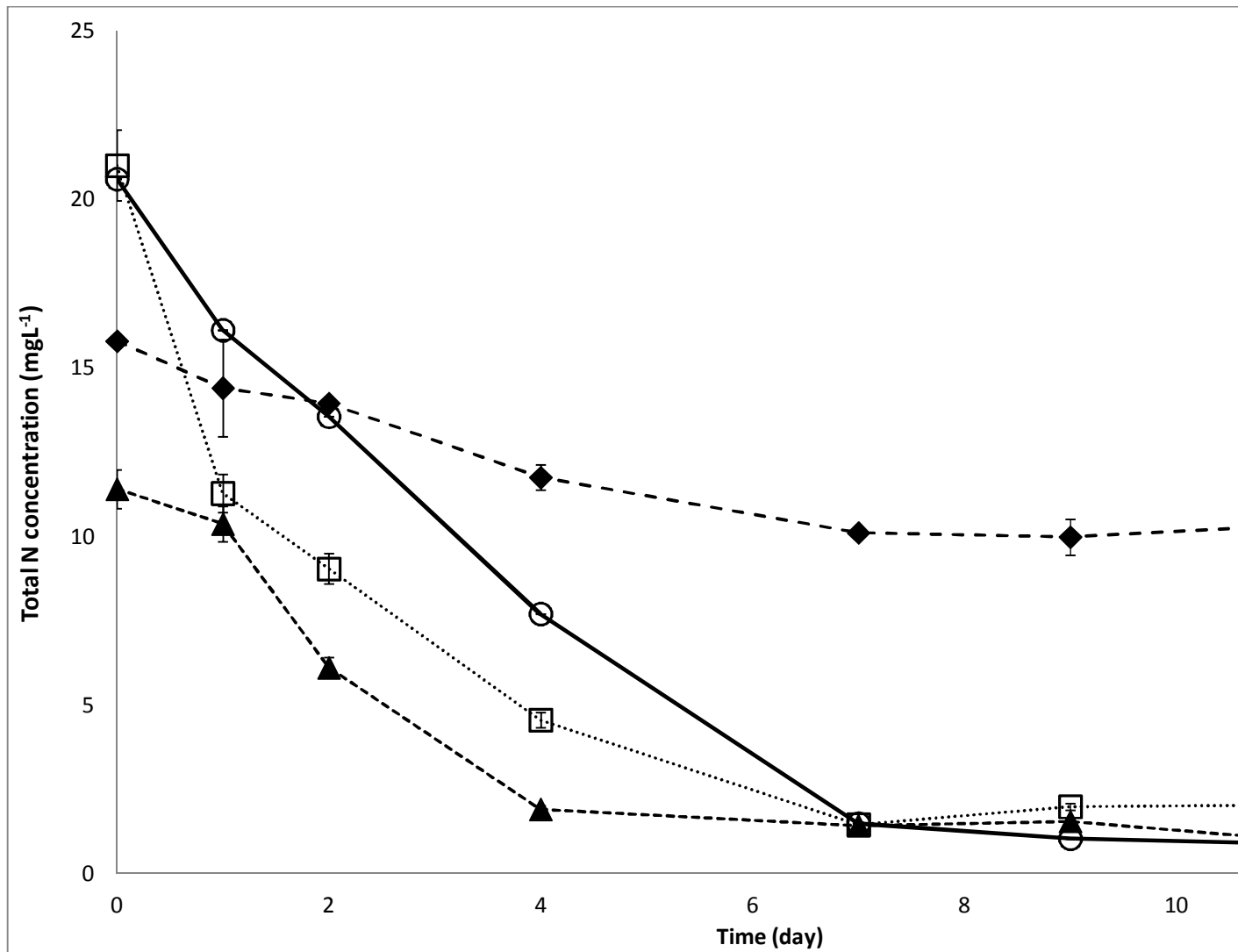
Fig. 5. Total N (TDN, TON and Nitrates) consumption by *Nannochloropsis oceanica* when grown on treated digested agricultura
(balanced) (◆),treated digested agricultural wastewater N:P 16.53 (high ammonia) (□),treated digested agricultural wastewa
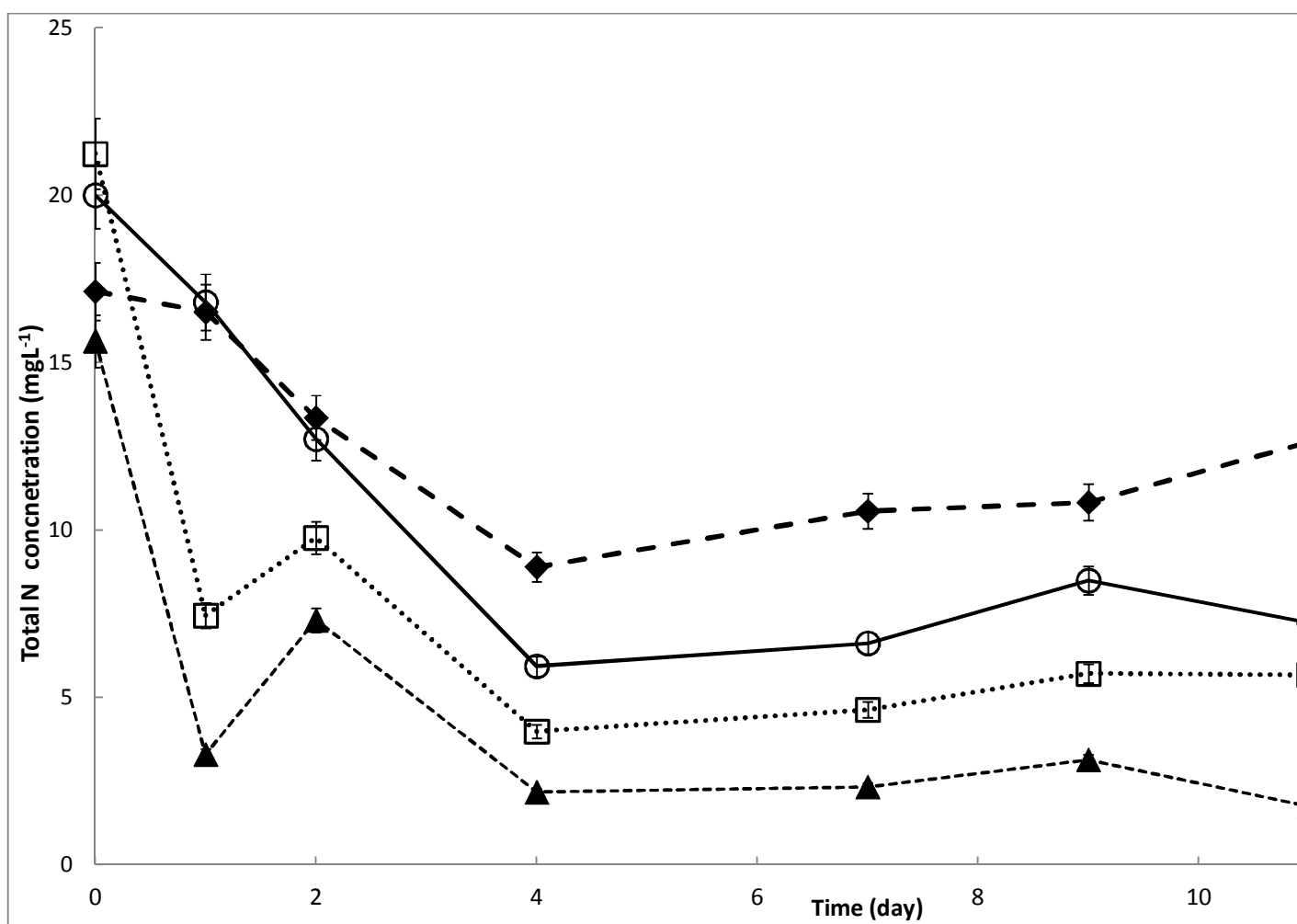(▲)in vitro standardised liquid media (f/2) (O).

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Fig. 6. Total N (TDN, TON and Nitrates) consumption by *S. quadricuada* when grown on treated digested agricultural wastewat),treated digested agricultural wastewater N:P 16.53 (high ammonia) (□),treated digested agricultural wastewater N:P 3.78 (I standardised liquid media (f/2) (O).

Parameters	Untreated raw sludge	Treated digested agricultural wastewater microfiltered (0.2 µm) permeate used as growth media		
	N:P 1.09			
		N:P 16.53	N:P 3.78	N:P 14.22
Total Solids (TS, g L^{-1})	27.21	6.04	9.7	10.2
Total Suspended Solids (TSS, g L^{-1})	1.71	0.19	0.20	0.21
Total Dissolved Solids (TDS, g L^{-1})	11.54	4.25	4.89	4.38
Conductivity (mS cm^{-1})	14.40	5.30	3.41	5.16
Alkalinity (g CaCO3 L^{-1})	14	2.287	2.288	2.50
Optical Density (580 nm)	0.8	0.10	0.09	0.11
pH	8.23	8.25	8.56	8.50
Zeta potential (mV)	-36.85	-24.2	-29.43	-29.60
Sizing (µm)	37.69	2.93	4.21	5.70
		Concentration g L^{-1}		
Acetic Acid	4.43	1.27	0.32	0.64
Butyric Acid	4.88	1.39	0.35	0.70
Metal ions (Ca,Cu, Co,Fe, Pb, Mg, Mn, Zn,K, As)	3.10	0.88	0.68	0.59
Ammonia	2.21	0.69	0.27	0.36
Phosphate	2.03	0.04	0.07	0.03

Table 1: Physical characteristics and chemical composition of the untreated and pretreated anaerobically digested agricultural sludge

(Gerardo et al., 2013;Zacharof &Lovitt, 2014 ,2016;)

Algal strain	Growth media	Dry weight (g L^{-1})	Growth rate (μ_{max}, D^{-1})	Algal Carbon (C, DWC g L^{-1})	Algal Nitrogen (N, DWC g L^{-1})
	Standardised growth media (f/2)	1.1	0.45	0.35	0.03
	Treated digested agricultural wastewater				
	N:P 16.53	0.57	0.42	0.27	0.013
N. oceanica	N:P 3.78	0.47	0.40	0.28	0.010
	N:P 14.22	0.48	0.41	0.15	0.012

Table 2: Comparison of the effect on *N.oceanica* growth biomass composition and production on waste based media and on s

Algal strain	Growth media	Dry weight (g L^{-1})	Growth rate (μ_{max}, D^{-1})	Algal Carbon (C, DWC g L^{-1})	Algal Nitrogen(N, DWC g L^{-1})
	Standardised growth media (f/2)	0.58	0.38	0.05	0.008
	Treated digested agricultural wastewater				
	N:P 16.53	0.41	0.39	0.05	0.010
S. quadricuada	**N:P 3.78**	0.24	0.37	0.06	0.010
	N:P 14.22	0.37	0.39	0.05	0.021

Table 3: Comparison of the effect on *S. quadricuada* growth, biomass composition and production on waste based media and

Algal strain	Growth media	Growth rate (μ_{max}, h^{-1})	Bi
	Standardised growth media	0.11	1.
S.limacinum SR21	*Treated digested agricultural wastewater*		

N:P 16.53		0.12	1.
N:P 3.78		0.09	1.
N:P 14.22		0.06	1.

Table 4: Comparison of the effect on *S.limacinum* SR21 growth and lipids production on waste based media, enriched waste r
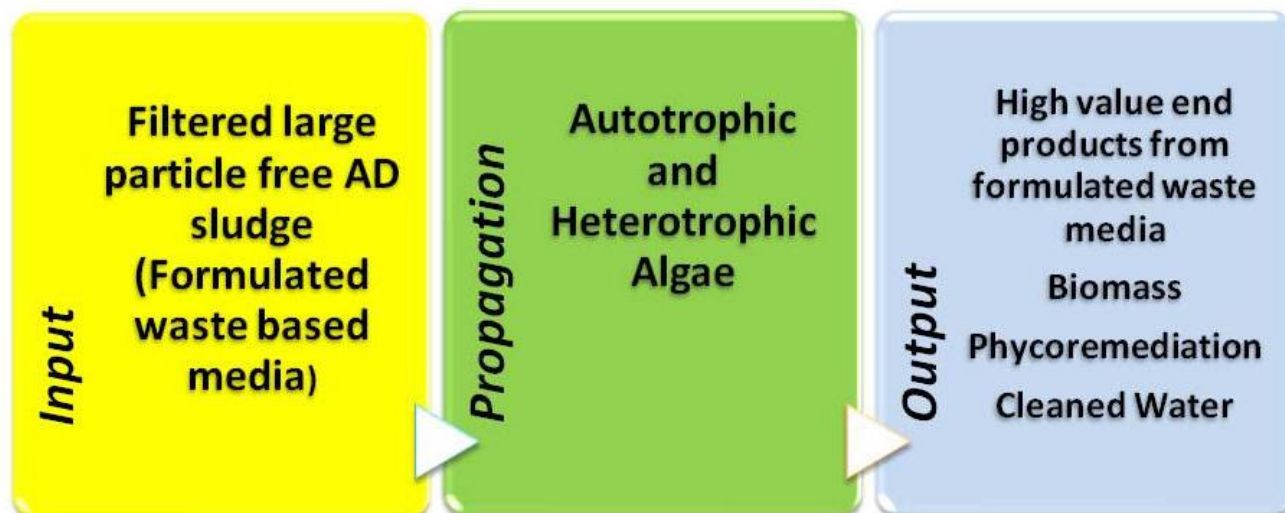
Algal strain	Growth media	Growth rate (μ_{max}, h^{-1})	Bioma
	Standardised growth media	0.11	1.38
	Treated digested agricultural wastewater N:P 16.53	0.12	1.42
	Enriched Treated digested agricultural wastewater **N:P 16.53** *with carbon and nitrogen sources*		
S.limacinum SR21	**Glucose**	0.11	1.85
	Mannitol	0.06	1.02
	Fructose	0.18	2.15

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	0.13	1.56
Glycerol	0.13	1.56
Yeast extract	0.15	1.32
Sodium bicarbonate	0.04	0.90
Sodium acetate	0.12	1.18

Table 5: Comparison of the effect on *S.limacinum* SR21 growth and lipids production on enriched waste media and

Highlights

- *N. oceanica, S. quadricauda* & *S.limacinum* were grown on treated spent digestates.

- 3 streams of N:P ratios of 16.53, 3.78 &14.22 were formulated using simple physical processes.

- Autotrophic growth of *N. oceanica* & *S. quadricauda* reduced N & P over 60% .

- Heterophic growth of *S.limacinum* produced lipids & biomass of 16.70 w/w% & 1.42 g L^{-1} .

- These low impact media gave comparable performance with the synthetic commercial media.

Input: Filtered large particle free AD sludge (Formulated waste based media)

Propagation: Autotrophic and Heterotrophic Algae

Output: High value end products from formulated waste media — Biomass, Phycoremediation, Cleaned Water