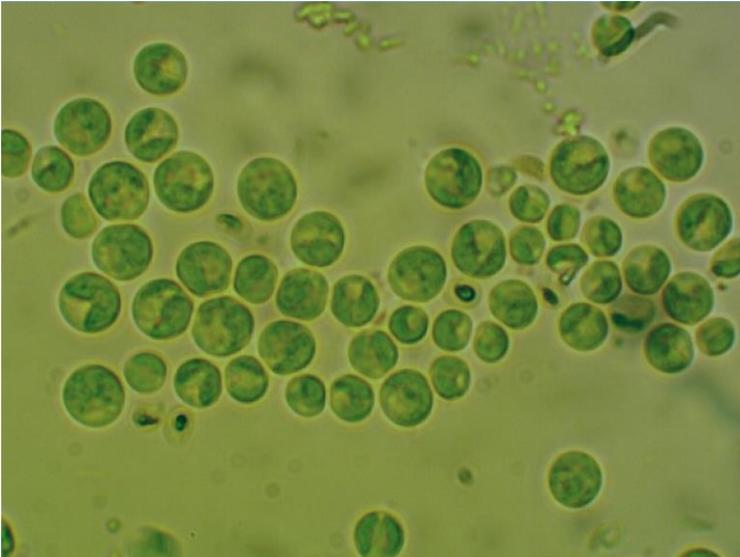


*Growth of microalgal biomass
on supernatant from biosolid
dewatering*



Francesca Alice Marazzi

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dewatering**



The research presented in this thesis was carried out at the Department of Earth and Environmental Sciences (DISAT), University of Milano-Bicocca, Milan, Italy

Cover image: Pictures of the microalgae grown on real wastewater kindly supplied by Istituto Lazzaro Spallanzani

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Cycle XXIX

GROWTH OF MICROALGAL BIOMASS ON SUPERNATANT FROM BIOSOLID DEWATERING

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1. Introduction

1.1 Scenario

The world population reached 7.3 billion as of mid-2015 and it is projected to increase by more than 11.2 billion by 2100 ¹. Consequently the world production of domestic wastewaters worldwide is increasing too and wastewater treatment plants are playing more and more a key role in quality improvement of treated effluents, which should not only be considered as a source of pollution but also as a source of energy, nutrients and water.

However, in the prospective of circular economy conventional wastewater treatment technology, including aerobic activated sludge processes or anaerobic digestion, still present serious technical–economic limitations caused by their high energy requirements ² and low possibilities of material recovery. For example, data from Germany and Italy show that electricity demand for wastewater treatment accounts for about 1% of total consumption of the country, which may be a good estimation for other European countries ³. Two critical points, in particular, contribute significantly to power consumption and loss: the need to remove nitrogen from the supernatant of sludge dewatering and the low biomethane production from under loaded anaerobic digesters.

The conventional wastewater treatment scheme includes water line and sludge line; the first one produces: a liquid effluent compatible for discharge to natural water bodies and a sludge rich in organic matter and nutrients as nitrogen and phosphorus. In large plants, the sludge generally undergoes anaerobic digestion, with biogas production, before being dewatered. The supernatant from sludge dewatering (called blackwater or

centrate if deriving from centrifuging) is rich in nitrogen and is normally sent back to the water line to be treated. The removal of nitrogen from supernatants (usually 10-20% of the total influent nitrogen load) involves significant energy costs due to the additional aeration. Generally, the consumption for aeration is between 0.18 and 0.8 kW h m⁻³. Aeration is an essential process in the majority of Waste Water Treatment Plant (WWTP) and accounts for the largest fraction of plant energy costs, ranging from 45 to 75% of the plant energy expenditure.

On the other hand, the efficiency of anaerobic digesters depends on the characteristics of sludge. To comply with the Nitrate Directive (91/676/ CE) and carry out efficient nitrogen removal, WWTPs operate with long sludge age so that the resulting excess sludge has a high degree of mineralization and provides a limited substrate for anaerobic digestion. So, many anaerobic digesters are now under loaded and could potentially co-digest other organic materials thus improving their biogas production. There are several studies about developing new technologies, that optimize WWTP and make WWTP economically viable. One of them is microalgal-bacterial process, attracting considerable attention because of the wide range of possibilities of material recovery from algal biomass.

In fact, algal biomass can be used as substrate for anaerobic digestion and biogas production, for the production of biofuels, fertilizers, plant biostimulating agents and biopolymers. The best solution seems to be the possibility to use microalgae grown on wastewaters for biogas production in anaerobic digestion, as demonstrated by several studies ^{4 5}.

Including algae-bacteria based processes within conventional wastewater sequence, feeding algae on centrate can has at least three main benefits:

- ✓ Removing nitrogen, the demand of electric power for nitrification decreases too ⁴;

- ✓ The algal/bacterial biomass can be fed to anaerobic digesters increasing their biogas production and reducing the need for first-generation biofuels for WWTP operation⁶;
- ✓ Photosynthesis uses CO₂ as carbon source, thus decreasing the carbon footprint of wastewater treatment.

1.2 Microalgae

Autotrophic microalgae are photosynthetic microorganisms with simple growing requirements that can produce lipids, proteins and carbohydrates in large amounts over short periods of time. Microalgae cultures grow continuously when all necessary nutrients, CO₂, and light energy are not limiting. For numerous aspects, microalgal cultivation is gradually increasing worldwide. Microalgae have been used extensively in environmental biotechnology, especially for bioremediation, biomonitoring and bioassay of environmental toxic pollutants; microalgal–bacterial systems have been extensively used in the treatment of nutrient rich wastewaters since 1950s⁷. However, their application has been limited to few, experimental cases, till the last decade, chiefly because of the poor knowledge of the processes and the limiting conditions and of the difficulties of standardization. Moreover, in the past, problems arising from energy constraints and greenhouse gas emissions were not as important as they are now and the existing biological processes seemed satisfactory for the purpose of removing organic matter and nutrients from wastewater.

The positive interactions between microalgae and bacteria are clear, as microalgae provide, through photosynthesis, the oxygen need for aerobic bacteria to biodegrade organic pollutants, consuming in turn the carbon dioxide released from

the bacterial activity. Studies often focused on the bioremediation potential of microalgae for various types of wastewaters such as municipal, industrial and agricultural wastewater especially in nutrient removal, based on their ability to uptake inorganic nitrogen (NH_4^+ , NO_3^- , NO_2^-) and phosphorus⁸. In particular, phosphorus resources are being depleted and the recovery of this vital element is a serious challenge for the next years.

Several studies have investigated the possibilities of using centrate as the sole nutrient source in microalgae production^{9 10 11}. The optimal centrate concentration that can be used as substrate for microalgae production has to be individually studied case by case.

However, the main constraints to include microalgae on wastewater treatment sequences are related to:

- Wastewaters, in particular supernatants from sludge dewatering, have high ammonia nitrogen content (normally $>100 \text{ mg L}^{-1}$, sometimes approaching 1000 mg L^{-1}), which could potentially be toxic to microalgae¹²;
- Supernatants have a high turbidity level, that reduces light penetration¹³.
- Supernatants are a very complex matrix and may contain compounds that inhibit microalgae growth such as, for instance, urea, organic acids, phenols and pesticides. So, before being fed to microalgal systems, a pre-treatment (by dilution, filtration or centrifugation) could be needed to partially remove solids¹⁴.
- To keep the process in the range of the low cost ones, avoiding artificial heating and illumination, the seasonality of the process must be taken into account, and the climatic conditions of the site may become a limiting factor.

- The anaerobic degradability of some algal species is sometimes long, due to the presence of resistant cell walls. The biodegradability can be evaluated case by case by testing their Biochemical Methane Potential (BMP). The results of BMP measurements reported in literature vary within a very wide range: 153 to 600 L CH₄/kgSV, according to Ward et al.(2014)⁵.
- Conventional microalgal harvesting methods are often considered as energy intensive, and their cost has been reported to contribute to more than 30% of the total biomass production cost ⁹.

1.3 Outline of the thesis

This thesis deals with the feasibility of including a mixed microalgal community in a real wastewater treatment sequence with the double aim of removing nitrogen and producing biomass to feed the anaerobic digesters. In particular, this thesis focuses on the identification of relevant waste streams from agricultural and municipal activities (like effluents, centrates, digestates, and mixtures of them) as substrates for microalgal culture.

Beside the introduction, the thesis contains six chapters. Chapter 2 is a critical review, which collects the current approaches to microalgal biomass production. In particular several studies using waste streams, including: wastewater, waste or CO₂-enriched gas (flue gas and biogas), waste organics (i.e., crude glycerol) and waste heat. Moreover, microalgal metabolic pathways supporting the biosynthesis of energy-rich molecules such as triacylglycerides (TAG) and starch are discussed.

The core of experimentation is described in Chapters 3-7 using centrate from urban settlings or agriculture activity as a substrate for microalgal culture; in all the tests mixed microalgal population dominated by *Chlorella spp.* and *Scenedesmus spp.*, previously adapted to grow on the centrate from anaerobic digester of one of Milan WWTPs, was used.

In particular, Chapter 3 shows the results of experimentation using municipal centrate to produce biomass to feed the anaerobic digesters removing at the same ammonia nitrogen.

Wastewaters from agricultural activities are the subject of Chapter 4, focusing, on the characterization of different kinds of agro-waste streams in view of their treatment with microalgae.

Chapter 4, as well as Chapter 5, reports the results obtained in lab-scale tests using the agro wastes, which had been shown to be more suitable for microalgae growth.

The encouraging lab scale results justify further pilot-scale experimentation, using agrowastes as the nutrient source for microalgae. The results of this experimentation are presented in Chapter 6. The objective of the tests is to investigate the use of microalgae to remove nitrogen from the same agro-digestate used in the previously lab-tests and to produce bioenergy (biogas) from the microalgal biomass by anaerobic digestion.

The search for the best biomass harvesting technique is the main objective of Chapter 7. The separation of microalgae from their growth substrate is a major challenge to produce microalgae at large scale ¹⁵, because the microalgal suspension produced in the reactors is substantially diluted (TSS concentration is generally 1-2 g L⁻¹). Harvesting is a necessary step before any use of algal biomass, including anaerobic digestion. Finally, the conclusions are reported in Chapter 8. The main outcome is that microalgae can grow on centrate in laboratory and natural conditions. Biomass growth rate and biomass volumetric productivity (VP) ranged between 0.14-0.36 d⁻¹ and 40-220 mg L⁻¹d⁻¹ respectively.

1.4 References

1. United Nations. *World population prospects. United Nations 1*, (2015).
2. Posadas, E., García-Encina, P., Soltau, A., Domínguez, A., Díaz, I. & Muñoz, R. Carbon and nutrient removal from centrates and domestic wastewater using algal – bacterial biofilm bioreactors. *Bioresour. Technol.* **139**, 50–58 (2013).
3. Longo, S., Mirko, B., Bongards, M., Chaparro, A., Cronrath, A., Fatone, F., Lema, J. M., Mauricio-Iglesias, M., Soares, A. & Hospido, A. Monitoring and diagnosis of energy consumption in wastewater treatment plants . A state of the art and proposals for improvement. *Appl. Energy* **179**, 1251–1268 (2016).
4. Ficara, E., Uslenghi, A., Basilico, D. & Mezzanotte, V. Growth of microalgal biomass on supernatant from biosolid dewatering. *Water Sci. Technol.* **69**, 896–902 (2014).
5. Ward, A. J., Lewis, D. M. & Green, F. B. Anaerobic digestion of algae biomass: A review. *Algal Res.* **5**, 204–214 (2014).
6. Monlau, F., Sambusiti, C., Ficara, E., Aboulkas, A., Barakat, A. & Carrère, H. New opportunities for agricultural digestate valorization: current situation and perspectives. *Energy Environ. Sci.* 2600–2621 (2015).
7. Ramanan, R., Kim, B. H., Cho, D. H., Oh, H. M. & Kim, H. S. Algae-bacteria interactions: Evolution, ecology and emerging applications. *Biotechnol. Adv.* **34**, 14–29 (2016).
8. Park, J., Jin, H.F., Lim, B.R., Park, K.Y. & Lee, K.

- Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. *Bioresour. Technol.* **101**, 8649–8657 (2010).
9. Ledda, C., Romero Villegas, G. I., Adani, F., Acién Fernández, F. G. & Molina Grima, E. Utilization of centrate from wastewater treatment for the outdoor production of *Nannochloropsis gaditana* biomass at pilot-scale. *Algal Res.* **12**, 17–25 (2015).
 10. Cabanelas, I. T. D., Ruiz, J., Arbib, Z., Chinalia, F. A., Garrido-Pérez, C., Rogalla, F., Nascimento, I. A. & Perales, J. A. Comparing the use of different domestic wastewaters for coupling microalgal production and nutrient removal. *Bioresour. Technol.* **131**, 429–436 (2013).
 11. Morales-Amaral, M. del M., Gómez-Serrano, C., Acién, F. G., Fernández-Sevilla, J. M. & Molina-Grima, E. Outdoor production of *Scenedesmus* sp. in thin-layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source. *Algal Res.* **12**, 99–108 (2015).
 12. Ugetti, E., Sialve, B., Eric, T. & Jean-Pierre, S. Integrating microalgae production with anaerobic digestion: a biorefinery approach. *Biofuels, Bioprod. Biorefining* **6**, 246–256 (2014).
 13. Ge, S. & Champagne, P. Nutrient removal, microalgal biomass growth, harvesting and lipid yield in response to centrate wastewater loadings. *Water Res.* **88**, 604–612 (2016).
 14. Vandamme, D., Foubert, I. & Muylaert, K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends Biotechnol.* **31**, 233–239 (2013).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

Abstract: Global increases in the generation of waste streams including liquid, gaseous, and solid waste have been posing serious challenges for waste management, as a result of their potential impacts on receiving environments and climate change. The conversion of waste streams into useful bioenergy, biofuels and bioproducts through recycling and/or recovery has been presented as a promising alternative. Coupling the bioremediation of waste streams with microalgae-based biofuel production, offers an alternative strategy to achieve waste-to-biofuel and bioenergy. A group of unicellular photosynthetic eukaryotes, microalgae require relatively simple nutrients and inorganic carbon sources to their support growth, while accumulating several biofuel precursors such as starch or storage lipids. This review summarizes the current approaches to microalgal biomass production using waste streams, including wastewater, waste or CO₂-enriched gas (flue gas and biogas), waste organics (i.e., crude glycerol) and waste heat, as well as the primary common operational challenges and corresponding mitigation strategies involved in cultivation approaches. Moreover, microalgal metabolic pathways supporting the biosynthesis of energy-rich molecules such as triacylglycerides (TAG) and starch are discussed. Metabolic constraints and potential approaches for the enhancement of microalgal TAG accumulation are systematically and critically analyzed.

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Keywords: microalgae; wastewater; flue gas; glycerol; triacylglyceride; biofuel

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2.1 Introduction

Compared to first and second generation biofuel feedstocks, green microalgae offer the advantages of high growth rates, without competing for agricultural or arable lands,¹ and can potentially produce 100-times more oils per acre of land than terrestrial plants such as coconut, palm oil, castor bean and sunflower seeds,² while sequestering significant amounts of carbon dioxide (CO₂). Microbial oil contents (Higher Heating Values (HHV): 30-45.9 MJ kg⁻¹)³ as high as 90% of dry cell mass have been reported under specific culture conditions, with up to 70% achieved during heterotrophic growth.^{4, 5} Most importantly, neutral lipids can be up to 79.5% of the total lipid content, with 88.7% present as oil triglycerides (TAG).⁶ However, biofuels are high-volume, low-value “C-neutral” products and this places a number of critical constraints on the use of microalgae for practical biofuel production.

Effective microalgae-based biofuel production depends on the photosynthetic efficiency of cultivation and the downstream biofuel refinement process. In high-rate microalgal biomass production systems, cultivation can account for up to 76.8% of the total cost in comparison 12.0% and 7.9% for harvesting and extraction, respectively.^{7, 8} Therefore, decreasing energy inputs for microalgal growth would significantly improve the economic viability of the entire biofuel process. Utilizing waste streams for microalgal growth have been demonstrated to be significant and transformative breakthroughs that positively impact the cost, sustainability and efficiency of microalgal biofuel systems.⁹ For example, large amounts of water, macronutrients (N, P), and micronutrients are necessary for microalgal cultivation; wastewater is an excellent source of energy-rich organic C-molecules, and macro- and

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micronutrients,¹⁰⁻¹² reducing the water footprint for microalgal culturing by up to 90%.¹³ Moreover, microalgal cultivation could be enhanced with CO₂ supplementation rather than air.¹⁴ Cement manufacturing facilities and coal-fired electrical power plants are considered significant stationary point sources of CO₂.¹⁵ Thus, CO₂ could be captured from flue gas with the added benefit of reducing greenhouse gas (GHG) emissions.¹⁶ In addition, mixotrophic systems with waste organics have been shown to provide higher growth productivities and lower harvesting costs.¹⁷ These regimes often exhibit high growth rates and cell densities, and some microalgae (*C. protothecoides*¹⁸ and *C. zofingiensis*⁶) have exhibited high lipid contents. Hence, all the findings noted above suggest that the integration of waste streams in microalgal cultivation will be critical in the evolution of a techno-economically viable for biofuel production process.

However, a multidisciplinary approach that integrates advances in fundamental biology, microalgal cultivation systems, bio-oil extraction and processing, and metabolic engineering of specific microalgal strains is required to uncover the full potential of microalgae as sustainable biofuel feedstock. Environmental stresses, particularly macronutrient (N, P) depletion, are well known triggers for the accumulation of highly reduced, energy-rich storage compounds such as starch and TAGs by microalgae.^{1, 17, 19, 20} However, TAG content and microalgal biomass productivity are often inversely correlated; *i.e.* nutrient limitation stimulates TAG accumulation at the expense of growth.^{21, 22} Improving microalgal strain performance for biofuel production will therefore require a much better understanding of C-allocation between the various, competing metabolic pathways, and the metabolic regulatory mechanisms that control this C-flux distribution, particularly during environmental stress.

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This critical review discusses current trends of the biotechnological application of microalgal cultivation with waste streams and the microalgal metabolic pathways supporting storage lipid biosynthesis for biofuel production. The feasibility and challenges of cultivation with wastewater, waste gas, waste organics, and waste heat, as well as metabolic pathways and lipid accumulation responses to changes in environmental conditions are discussed in detail.

2.2 Waste streams for microalgal cultivation

2.2.1 *Microalgae growth on Wastewater*

Microalgae have been used extensively in environmental biotechnology, especially for bioremediation, biomonitoring and bioassay of environmental toxic pollutants. Microalgal cultivation on wastewaters has been performed since the 1950s.²³ Studies often focused on the bioremediation potential of microalgae for various types of wastewaters such as municipal, industrial and agricultural wastewater especially in nutrient removal, based on their ability to uptake inorganic nitrogen (NH_4^+ , NO_3^- , NO_2^-) and phosphorus.^{24, 25} Effluent from anaerobic digesters (digestate, centrate),²⁶ has been another reported macronutrient source for microalgal growth; where the digestates were obtained from anaerobic digester systems fed on waste sludge at wastewater treatment plants (WWTPs) and/or agro-wastes in farm (**Table 2.1**). A variety of wastewater sources have been employed as substrates for microalgal cultivation and/or in nutrient removal and these are summarized in **Table 2.1**. Microalgal productivities have generally been found to be strongly linked to microalgal strains (*e.g.*, marine or freshwater species), climatic conditions (*e.g.*, light intensity, temperature) and the operational layout of the

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production systems (e.g., reactor design).²⁷ The strains most commonly used in studies are *Chlorella sp* and *Scenedesmus sp* for many reasons: they are a cosmopolitan genus commonly found in natural waterbodies, wastewaters and open pond systems; ²⁸⁻³⁰ they are usually able to adapt very well in different and extreme environmental conditions (such as high concentrations of nitrogen and/or organics).^{31, 32} They are two of the most resilient microalgae, because they are especially rapid in their responses to perturbations (e.g., pH, temperature, light, nutrient concentrations or salinity) ³³⁻³⁵ and they are ideal experimental materials due to their fast grow rate observed since 1950's.³⁶ Reported growth rates and biomass productivities range between 0.04-1.6 d⁻¹ and 0.037-1.13 g L⁻¹ d⁻¹, respectively. Moreover, microalgae have demonstrated high capacities for nutrient uptake and/or removal, with the uptake of ammonia being thermodynamically more favorable than nitrate uptake.³⁰ In general, >90% removal efficiencies have obtained, even under high ammonium loadings (>100 mg·L⁻¹), which could potentially be toxic to microalgae.³⁷ In addition, microalgae have been noted to remove the micro-contaminants, including: emerging organic contaminants, polycyclic aromatic hydrocarbons, biocides, surfactants and phenolic compounds from wastewater through biotic (e.g. microalgal uptake, biodegradation, metabolism), in addition to abiotic (volatilization, sorption, or photodegradation) treatment processes.³⁸⁻⁴¹ The removal efficiencies were found to be comparable to that reported for conventional activated sludge approaches.⁴²⁻⁴⁵ Microalgae are usually cultivated in open pond or closed (e.g. photobioreactors (PBRs)) systems. The choice of cultivation system depends on factors such as cost and required reliability.⁴⁶ The cost of PBR systems are almost one order of magnitude higher than those reported for open raceways.⁴⁷ Microalgae cultured in open raceways are more

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suitable for mass production than PBRs, even if the microalgae growth and maximum cell density is higher in PBR's than those reported for the open raceways.¹ The lower cell density in open raceway could be balanced by a high volume capacity. However, most of larger scale microalgal studies using wastewater for cultivation have been conducted in open pond systems.^{27, 46, 48, 49} A number of operational cultivation challenges must be considered in the design of microalgae-derived wastewater bioremediation systems. For example, light diffusion and nutrient toxicity were two important factors affecting microalgal growth, especially when digestate or centrate are employed, which contained high concentrations of total suspended solids (TSS) and ammonia nitrogen. This results in inherently high turbidities leading to shallow effective photosynthetic light penetration, and potentially toxic free ammonia concentrations, consequently reducing microalgal growth.²⁶ Therefore, appropriate pre-treatments to remove TSS, residual polymers, high concentrations of ammonia and other toxic compounds from wastewater were often necessary for optimal microalgal growth.^{50, 51} The most common forms of pretreatment include solid/liquid separation,⁵²⁻⁵⁴ macro or micronutrient adjustment,⁵⁵ filtration,⁵⁶ and dilution using tap water^{49, 57-60} or WWTP effluent.^{34, 46, 61-63} Additionally, the complexity of wastewater composition in terms of mixture of compounds can also negatively influence microalgal growth. The presence of pesticides,⁶⁴ pharmaceuticals (*e.g.* antibiotics),⁶⁴ inorganic micro-elements such as arsenic and cadmium in centrate,⁵⁰ and sodium (0.4 g L^{-1}) in digestate⁴⁶ can potentially inhibit microalgal growth. Lastly, a large number of microalgal cultivation studies using wastewaters have been performed under relatively controlled laboratory conditions (*e.g.* microalgal strain, loading rate, light, temperature, CO₂ supply

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and mixing, and synthetic and real wastewater).^{26, 30, 62, 65, 66} Some indoor pilot scales are also used to evaluate the feasibility of the microalgal process under some operational conditions that can be controlled more than if it was outside,^{52, 67, 68} as shown in **Table 2.1**. Relatively few studies have been conducted under outdoor pilot-scale conditions,^{37, 38, 46, 49, 50, 52, 57, 68, 69} where fluctuations in temperature and solar radiation intensity that would significantly influence microalgal growth in wastewater at different latitudes, have been systematically investigated.

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Table 2.1. Microalgae cultivation with wastewater a substrates.

Strains	BP	GR (d ⁻¹)	Scale/ Reactor	WW type	N removal	P removal	Pre-treatment	Inlet N-NH ₄ (mg L ⁻¹)	Ref.
<i>Chlorella sorokiniana</i>	5 g L ⁻¹	-	Lab scale In	Synthetic medium, urine	60-100 %	20-100%	-	235-540	43
<i>Chlorella sp., Scenedesmus subpicatus, Pseudokirchneriella subcapitata</i>	0.5 g L ⁻¹ d ⁻¹	0.3	Inr Lab scale	Secondary MWW	100 %	100%	-	20	70
<i>Stigeoclonium sp., Diatoms, Chlorella sp., Monoraphidium sp.</i>	6-24 gTSSm ⁻² d ⁻¹	-	Out Open pond	MWW	90-99 %	-	-	20-80 (Raw influent)	38
<i>Scenedesmus sp</i>	45.8 g m ⁻² d ⁻¹ 0.2 g L ⁻¹ d ⁻¹	-	Out Open pond	MWW	38 mgN L ⁻¹ d ⁻¹	3.9 mgP L ⁻¹ d ⁻¹	Dilution with synthetic medium	122-406	37

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Table 2.1. (continued)

Strains	BP	GR (d ⁻¹)	Scale/ Reactor	WW type	N removal	P removal	Pre-treatment	Inlet N-NH ₄ (mg L ⁻¹)	Ref.
<i>Muriellopsis</i> sp.	1.13 ±0.05 g·L ⁻¹ ·d ⁻¹	0.3	Lab scale In	MWW, centrate	47.5 mgN·L ⁻¹ ·d ⁻¹	3.8 mgP L ⁻¹ d ⁻¹	Dilution medium	50-166 (Raw centrate, 406)	71
<i>Pseudokirchneriella subcapitata</i>	1.02 ±0.05 g·L ⁻¹ ·d ⁻¹				27.5 mgN·L ⁻¹ ·d ⁻¹	2.7 mgP L ⁻¹ d ⁻¹			
Mixed <i>Chlorella</i> sp <i>Scenedesmus</i> sp	1.6 g L ⁻¹	0.04-0.06	Lab scale In	MWW, centrate	77-82 %.	-	Dilution with secondary effluent	120 (Raw centrate, 250)	72
<i>Nannochloropsis gaditana</i>	0.15 g L ⁻¹ d ⁻¹	0.33	Out PBR	MWW, centrate	32 mg N L ⁻¹ d ⁻¹	1.3-2.6 mg P L ⁻¹ d ⁻¹	Dilution with seawater	145 (Raw centrate, 650)	48
	0.04 g L ⁻¹ d ⁻¹	0.2	Out Open pond		14 mg N L ⁻¹ d ⁻¹	0.8-1.4 mg P L ⁻¹ d ⁻¹			
<i>Chlorella</i> sp	34.6 g·m ⁻² ·d ⁻¹	0.53	Out PBR	MWW, centrate	19.5 %	58 %	-	180 (Raw centrate)	50
<i>Chlorella luteoviridis</i> <i>Parachlorella hussii</i>	-	-	OutOpen pond	MWW, centrate	>80 %	>90 %	Dilution with secondary effluent	97-120 (Raw centrate, 180±12)	46

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

Table 2.1. (continued)

Strains	BP	GR (d ⁻¹)	Scale/ Reactor	WW type	N removal	P removal	Pre-treatment	Inlet N-NH ₄ (mg L ⁻¹)	Ref.
Mixed consortia (<i>Chlorella is dominant</i>)	5.2 g m ⁻² d ⁻¹	-	In Open Pond	MWW, centrate	74%	73 %	Filtration	200-250	56
Microalgal consortium collected from a pilot HRAP treating	2.9 g m ⁻² d ⁻¹	-	Out PBR	MWW, centrate	0.4-1.7 g N m ⁻² d ⁻¹ 100%	0.34 g P m ⁻² d ⁻¹	Dilution with water	20-100 (Raw centrate, 646 ± 29)	57
Mixed microalgal cultures: <i>Spirulina platensis</i> , <i>Phoridium sp.</i> , <i>Oocystis sp.</i> , <i>Microspora sp</i>	1.04±0.07 g L ⁻¹	0.18	Out Open pond	MWW, centrate	-	-	Dilution with water	515 ± 200 (Raw centrate, 3800)	49
Mixed microalgal cultures dominated by <i>Scenedesmus sp</i>	0.1-2.6 g _{TSS} L ⁻¹	0.04-0.9	Lab scale In	MWW, centrate	-	-	Dilution with effluent	50-260 (Raw centrate, 950)	73

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

Table 2.1. (continued)

Strains	BP	GR (d ⁻¹)	Scale/ Reactor	WW type	N removal	P removal	Pre-treatment	Inlet N-NH ₄ (mg L ⁻¹)	Ref.
<i>Chlorella sp.</i> ADE5	3.01 g L ⁻¹	-	InLab scale	MWW, centrate	133 mg N g ⁻¹ biomass, Removal >90%	>90%	Dilution with effluent	150	63
<i>Chlorella sp.</i>	0.16 g L ⁻¹ d ⁻¹	0.32	Out PBR	Raw dairy WW	7 mg L ⁻¹ d ⁻¹	3 mg P L ⁻¹ d ⁻¹	Settled and filtered	180 Raw influent	68
	0.26 g L ⁻¹ d ⁻¹	0.2	In Lab scale		38 mg L ⁻¹ d ⁻¹	2 mg P L ⁻¹ d ⁻¹			
Dominant <i>Scenedesmus</i> spp	0.142 g L ⁻¹ d ⁻¹	0.6	Lab scale In	Swine digestate	21.2 ± 1.2 N-NH ₃ L ⁻¹ d ⁻¹	2.5 mg P L ⁻¹ d ⁻¹	Dilution with water	Raw centrate, 900 120 N-NH ₃	58
<i>Scenedesmus spp.</i>	0.045 g _{dw} L ⁻¹ d ⁻¹	0.89	Lab scale In	Sludge digestate	-	-	Dilution with effluent	1360 Raw centrate	34
<i>Nannochloris spp</i>	0.078 g _{dw} L ⁻¹ d ⁻¹	1.01							
<i>Chlorella vulgaris</i> <i>Scenedesmus obliquus</i>	0.057 g _{vss} L ⁻¹ d ⁻¹	-	In open pond	DSS Fresh	84% 23 mg L ⁻¹ d ⁻¹	-	Centrifugation and dilution	300-600	52
	0.037 g _{vss} L ⁻¹ d ⁻¹			DSS anaerobically	92% 53 mg L ⁻¹ d ⁻¹				

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

Table 2.1. (continued)

Strains	BP	GR (d ⁻¹)	Scale/ Reactor	WW type	N removal	P removal	Pre-treatment	Inlet N-NH ₄ (mg L ⁻¹)	Ref.
<i>Chlorella sp.</i>	-	0.28-0.41	Lab scale In	Diary manures	100% (in 21 days)	62-75%	Dilution and filtration	~80-180 (Raw centrate 2232)	53
<i>Neochloris oleoabundans</i>	0.20-0.24 g L ⁻¹ d ⁻¹	0.26-0.37	Lab scale In	AZD	3.5-6.9 mg L ⁻¹ d ⁻¹	0.09-0.3 mg L ⁻¹ d ⁻¹	Dilution and centrifugation	1680 Raw digestate	54
<i>Chlorella vulgaris</i>	0.21-0.26 g L ⁻¹ d ⁻¹	0.23-0.49			3.8-7.4 mg L ⁻¹ d ⁻¹	0.12-0.28 mg L ⁻¹ d ⁻¹			
<i>Scenedesmus obliquus</i>	0.22-0.26 g L ⁻¹ d ⁻¹	0.49-0.64			3.0-7.8 mg L ⁻¹ d ⁻¹	0.16-0.36 mg L ⁻¹ d ⁻¹			
<i>Scenedesmus sp. AMDD</i>	0.42 g L ⁻¹	1.62	Lab scale In	SWD algal biomass	99%	92%	Dilution with deionized water	1.58 (mol N-NH ₃ L ⁻¹)	59
	0.14 g L ⁻¹	1.34		SM	57 %	45%		1.50(mol N-NH ₃ L ⁻²)	
	0.11 g L ⁻¹	1.38		Cow manure	47%	69%		1.48(mol N-NH ₃ L ⁻²)	
	0.09-0.42 g L ⁻¹	0.85-1.59		SM/other nutrients	99-23%	13-97%		1.40-1.64 (mol N-NH ₃ L ⁻¹)	

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Table 2.1. (continued)

Strains	BP	GR (d ⁻¹)	Scale/ Reactor	WW type	N removal	P removal	Pre-treatment	Inlet N-NH ₄ (mg L ⁻¹)	Ref.
<i>Chlorella sp</i> , <i>Scenedesmus sp</i>	5.88×10 ⁷ cells L ⁻¹ d ⁻¹	-	Lab scale In	AZD	8.5 mgN L ⁻¹ d ⁻¹	-	Dilution and addition of micro- nutrients	(raw digestate= 3500a)	55
Mixed: <i>Phormidium</i> , <i>Oocystis</i> , <i>Microspora</i>	-	-	Ind Lab scale	AIR potato	19-60 %	80-89%	Dilution with water	(>600mgN- NH ₄ L ⁻¹ raw influent) 9-565	60
				AIR fish	40-64 %	N.A.			
				AIR animal feed	62-80 %	57-83%			
				AIR coffee	8-80 %	8%			
				AIR yeast	23-38 %	N.A			

Out=outdoor; In=indoor; GR= growth rate, BP=biomass productivity;PBR=Photobioreactor; DW=Dried Weight, TSS=Total Suspended Solid; MWW: municipal wastewater; AZD= Agro-zootechnical digestate; VSS=Volatile Suspended Solid; AIR= agro-industrial residual; SM= swine manure; DSS= digested swine slurry.

2.2.2 Microalgae growth on waste or CO₂-enriched gas

Compared to terrestrial plants that capture and utilize atmospheric CO₂ for photosynthesis, microalgae have a greater capacity to fix CO₂ because chlorophyll is present within a single cell permitting a more rapid biomass generation.⁷⁴ Growth and lipid production have been studied using strains of *B. braunii*,¹⁴ *C. vulgaris*,⁷⁵ and *C. reinhardtii*,⁷⁶ under moderate CO₂ concentrations (2-20%). Lipid contents increased by up to 30% or 2.7-fold with increasing CO₂ up to 10%. These findings suggest that the use of high CO₂ concentrations to cultivate microalgae for bio-oil production could be beneficial, while partially addressing growing concerns of global warming due to anthropogenic CO₂ emissions. Therefore, the availability of CO₂-rich gas sources could play an important role in mitigating the cost of microalgae cultivation. In municipal and/or agricultural areas, methane-dominant biogas from the anaerobic digestion of wastewaters, biosolids, or agricultural wastes (e.g. livestock manure) is also rich in CO₂ (30-45%, v/v). In industrial areas, CO₂ levels in exhaust emitted by combined heat and power (CHP) processes, flue gas and gas flaring, are also relatively high (3-30%).

2.2.2.1 Anaerobic digestion-derived biogas as a C-source

Biogas has long been considered an environmental-friendly and cost-effective biofuel as it is often generated from residual biomass and waste. As such, biogas production through anaerobic digestion has been adopted worldwide and supported by local legislative tools to increase biogas production in various economic sectors.⁴⁷ In general, 50–70% methane (CH₄, v/v), the most important gas particularly in combustion processes,

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

predominate crude biogas composition with 30-45% CO₂ and 2% other constituents including nitrogen and hydrogen sulfide (H₂S).⁷⁸ However, the presence of incombustible constituents such as CO₂, H₂S, and H₂O reduces the calorific value of crude biogas and, hence, its efficient use,⁷⁹ which must therefore be upgraded to meet the requirement of efficient combustion (i.e., CH₄ concentration > 90%, v/v).⁷⁸

Microalgae can be used to purify biogas while biogas contributes to cultivation. A number of studies have presented microalgal biogas upgrading approaches (**Table 2.2**). These were shown to have the potential to simultaneously upgrade biogas, yield microalgal biomass and reduce CO₂ emissions. Prandini *et al.*⁵⁸ employed swine wastewater-derived biogas to growth native microalgal strains and found that CO₂ was completely removed from raw biogas at 219.4±4.8 mg L⁻¹ d⁻¹, with a biomass yield of 1.1±0.2 g L⁻¹ and growth rate of 141.8±3.5 mg L⁻¹ d⁻¹. Similar studies also demonstrated that microalgal-based wastewater treatment processes could benefit from *in situ* CO₂-rich biogas.⁷⁹⁻⁸² However, the photosynthetic production of oxygen could potentially lead to the explosion of the gas mixture and would therefore necessitate oxygen separation.⁸³⁻⁸⁵ An alternative would involve the separation of the biogas upgrading process (CO₂ removal) from oxygen desorption in a two-stage process. Meier *et al.*⁸³ showed that a two-stage process, a PBR connected with a gas/liquid mass transfer unit, was an efficient way to simultaneously remove CO₂, while maintaining low oxygen concentrations in the biogas. Alternatively, low oxygen concentrations in the upgraded biogas could be achieved in the co-cultivation of microalgae with heterotrophic, aerobic bacteria.^{49,86}

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Table 2.2. Microalgae cultivation with biogas as a C-source

Strains	Gas resource	Biogas components (% , v/v)			CO ₂ removal (%)	Ref
		CO ₂	CH ₄	H ₂ S		
<i>Chlorella</i> sp.	Synthetic biogas	50	50	---	89.3%	79
<i>Uncultured Scenedesmus</i> clone	Raw biogas	20-25	65-73	1800-3100 ppmv	~100	58
<i>Scenedesmus obliquus</i>	Desulfurized biogas	37.54±2.93	58.67±3.45	<50 ppm	54.26–73.81	80
<i>Scenedesmus obliquus</i> , <i>Chlorella</i> sp., <i>Selenastrum bibrainum</i>	Desulfurized biogas	35.28±1.86	61.7±5	<50 ppm	---	81
<i>Nannochloropsis gaditana</i>	Raw biogas	28±2	72±2	--	95	83
<i>Chlorella vulgaris</i>	Raw biogas	55-71	44-48	<0.1	74-95	87
<i>Mutant Chlorella</i> sp.	Desulfurized biogas	~70	~20	<50 ppm	~70	88
<i>Chlorella vulgaris</i> , <i>Scenedesmus obliquus</i> , <i>Neochloris oleoabundans</i>	Synthetic biogas	25-55	44.5-74.5	<50 ppm	74.11–80.57	89
<i>Chlorella vulgaris</i> , <i>Scenedesmus obliquus</i> , <i>Neochloris oleoabundans</i>	Desulfurized biogas	34.45±3.48	61.32±5.74	<0.005	40.25-61.89	90

2.2.2.2 Flue gas as a C-source

Flue gas has been reported to be an excellent C-source for microalgae growth. A number of studies, summarized in **Table 2.3**, have explored the feasibility of microalgae cultivation sparged with different sources of flue gas, while providing simultaneous gas purification.

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One of the main drawbacks associated with the use of flue gas for large-scale microalgae cultivation are the low CO₂ mass transfer rate and utilization efficiency.⁹¹ This is, in part, due to the acidic pH conditions generated in the cultivation media with the introduction of flue gas, including SO_x and NO_x, which was not suitable for the microalgae growth and CO₂ sequestration. Some studies reported that SO_x and NO_x can be almost completely removed by washing with an alkaline solution (e.g. CaO⁹² CaCO₃⁹³ and limestone powder⁹⁴) and/or SO_x biological treatment.⁹² However, the consumption and recycling of these chemicals, and their sludges, should be considered in reducing process cost.⁹⁴ Strategies such as intermittent aeration controlled by pH feedback,^{94, 95} decreasing aeration rate and CO₂ concentration⁹⁶ were found to reduce the amount of alkaline chemicals required to neutralize the cultivation media. In addition, the CaSO₄ precipitate could be easily separated by centrifugation, with little influence on microalgal growth and the quality of microalgal product. Furthermore, the addition of plant growth stimulator, such as Triaccontanol, to the culture medium could offer an efficient and cost effective approach to enhancing microalgal tolerance to acidic flue gas pollutants.⁹⁷ Alternatively, acidic-tolerant or SO_x-tolerant microalgae strains could be isolated and selected for the process. For instance, acidophilic microalgae can grow at a pH as low as 0.05.^{98, 99} Several studies have indicated that high concentrations of NO in flue gas could completely or partly inhibit microalgal growth depending on the microalgal species and operating conditions.^{100, 101} Such inhibition was attributed to either direct NO gas inhibition or lower pH caused by NO. Therefore, the flue gas pretreatment could enhance the utilization of NO_x (especially the primary component NO) as nitrogen sources for microalgal growth in the downstream cultivation. Wet scrubbing-based NO removal with the addition of Fe(II)EDTA

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was proposed to increase microalgal NO uptake, but the efficiency was low due to the very low solubility and reactivity of NO.¹⁰² More recently, Eynde *et al.*¹⁰¹ employed photocatalytic air pretreatment to transform NO into NO₂, with subsequent conversion to nitrite and nitrate, which is then biologically available for microalgal conversion into ammonia (via nitrate reductase) and its subsequent assimilation into organic form. This provides a viable alternative for the flue gas pretreatment, but the environmental impact and cost effectiveness need further investigation. As a result, the development of novel and efficient flue gas pretreatment technologies and the potentially toxic effects of NO_x in flue gas are important considerations in the scalability of utilizing flue gas in microalgal cultivation.

The presence of heavy metals in flue gas (*e.g.* from coal combustion¹⁰³) should also be seriously considered in the utilization of flue gas for microalgal cultivation.¹⁰⁴ As previously noted for heavy metals in wastewaters above, the most important impact is related to their presence in biodiesel. Hence, heavy metal concentrations in the final biofuel product and by-products must be quantified in future studies to provide a more accurate assessment regarding the full impact of integrating of flue gas supplementation with microalgae cultivation.¹⁰⁵ The large-scale production of microalgae with flue gas would best be conducted using strains adapted for the operational conditions imposed by the use of flue gas including and toxic pollutant concentrations, and high temperature.

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Table 2.3. Microalgae cultivation with flue gas as a C-source

Strains	Gas resources	Biogas components (%, v/v)			CO ₂ removal (%)	Ref
		CO ₂	SO _x	NO _x		
<i>Nannochloropsis</i> sp. <i>Phaeodactylum</i> sp.	Boiler-based flue gas	14.1	185 ppm	125 ppm	54	106
<i>Monoraphidium minutum</i>	Simulated flue gas	13.6	0.02	0.015	---	107
<i>Scenedesmus dimorphus</i>	Simulated flue gas	10-20	100-400 ppm	100-500 ppm	75.61	94
<i>Chlorella</i> sp.	Coal-fired flue gas	13.3	--	6.9 ppm	6	108
<i>Thalassiosira weissflogii</i>	Simulated flue gas	1	--	50 ppm NO	~100	101
<i>Spirulina</i> sp. LEB 18 <i>Chlorella fusca</i> LEB 111	Coal-fired flue gas	9.4	500 ppm	400 ppm	42.8 20.5	109
<i>S. obliquus</i>	Coke-fired flue gas	18	200 ppm	150 ppm	67	110
<i>Chlorella</i> sp. MTF-15	Flue gas from steel plant	24	25-30	15-20	25-50	111
<i>Chlorella vulgaris</i>	Flue gas from incinerator	10-13	0.0045 ppm	0.18 ppm	4.4 g CO ₂ L ⁻¹ d ⁻¹	112
<i>Dunaliella parva</i> ; <i>Dunaliella tertiolecta</i>	Flue gas from power plant	8	--	20 ppm	82.3s 50.1c	113

S=sunny; c=cloudy

2.2.3 Waste glycerol to grow microalgae

Many microalgae that can grow under mixotrophic regimes photosynthesize and fix CO₂ by day and use heterotrophic metabolism of fixed carbon during both diurnal and dark respiration.¹⁷ Hence, mixotrophic microalgae can be used advantageously as large amounts of carbon can be incorporated

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into biofuel, while cell biomass is increased at the same time through the addition of organic C-sources dissolved in the cultivation media.¹¹⁴⁻¹¹⁶ In comparing cultivation regimes for *Spirulina sp.*, mixotrophic cultures were found to reduce photoinhibition and improve growth rates over autotrophic and heterotrophic cultures.^{117, 118} Mixotrophic metabolism has also been reported in *Botryococcus*,¹¹⁹ *Chlamydomonas*,⁷⁶ *Chlorella*,^{18, 30, 120} and *Scenedesmus*^{121, 122} strains. Thus, mixotrophic cultivation likely offers the most applicable growth regime for sustainable biofuel production from microalgae.

Mixotrophic growth can be achieved using a variety of organic C-sources including inexpensive waste sources. One of the potential C-sources for producing bio-oil is glycerol, an inexpensive and abundant by-product of the biodiesel fuel production that is generated in large quantities (up to 1 kg per 10 kg of microalgal oil) during the transesterification of lipids such as animal fats, vegetable oil, and microbial lipids.¹²³ Crude glycerol can be used as a feedstock for the cultivation of lipid producing microalgae and the lipid produced can then be reused for biodiesel production. Growth and lipid production studies using glycerol as an organic C-source have been conducted using *C. protothecoides*,¹²⁴⁻¹²⁶ *C. vulgaris*,^{28, 123, 125, 127} *S. limacinum*,¹²⁸ *N. oculata*,¹²⁷ *B. braunii*,¹²⁵ *Haematococcus sp.*,¹²⁹ *Nannochloris sp.*,¹²⁹ and *Scenedesmus sp.*^{125, 129} to demonstrate the potential for recycling crude glycerol generated during biodiesel production in the cultivation process. Perez-García *et al.*¹³⁰ observed that enzymes related to glycerol consumption (such as glycerol kinase; **Figure 2.1**) were upregulated in microalgal cultures using glycerol as a C-source, while nitrate transporter proteins were downregulated.

While the potential for using waste or crude glycerol as a C-source does exist, there are practical challenges that should also be considered when using crude glycerol to supplement

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cultivation media with mixotrophic microalgae. Firstly, the impurities contained in the crude glycerol such as methanol (6.2-12.6% w/w), soap (20.5-31.4% w/w),¹³¹ free fatty acids, unreacted and partially reacted fats, and catalyst residues could influence microalgal biomass growth, as well as lipid production and composition. For example, soap-containing media resulted in lower biomass yields in comparison to soap-free medium.¹²⁸ Similarly, the presence of methanol decreased microalgal growth yields at higher concentrations of glycerol.¹³² Secondly, the nutrient utilization efficiencies, gas mass transfer (O_2/CO_2) and light penetration may be affected by the inherent viscosity of glycerol. Thirdly, the optimum crude glycerol concentration should be determined as a function of microalgal species genotype, cell physiology, and variations in operational conditions (e.g. inorganic sources, temperatures). Moreover, micro- and/or macronutrient supplementation may be required to sustain microalgal growth, due to the generally low content of these nutrients and their potential chemical interactions with other constituents in crude glycerol. For instance, only 57 ± 18 mg L^{-1} of magnesium is present in crude glycerol,¹³³ and the presence of soaps could react with essential divalent metal cations (Mg^{2+} or Ca^{2+}), rendering them biologically inaccessible.¹²³

2.2.4 Waste heat to grow microalgae

Microalgal physiological and metabolic functions are temperature sensitive and with each species exhibiting optimal growth, lipid yields or nutrient assimilation within a certain temperature range. However, spatial, seasonal and daily fluctuations in temperature and light intensity make difficult to

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control or maintain constant microalgal cultivation conditions, which represent an operational challenge particularly in open pond systems. Studies examining the effects of temperature on microalgae production in outdoor cultivation systems demonstrated that heated pond systems performed better (with higher growth yields) than unheated systems (up to strain specific maximum temperatures).¹³⁴⁻¹³⁶ Higher temperatures generally increase microalgal growth rates and extend their growing season in temperate and cold climates, such as in Canada.¹³⁷ As such, microalgal cultivation using waste heat from the cooling waters of fossil fuel powered electricity-generating plants could be beneficial. This could reduce requirements of fossil fuel inputs to microalgal cultivation systems, such as electricity consumption, and natural gas used to dry the microalgae.¹³⁸ For example, in the winter environmental testing of its hybrid microalgae production facility in Montana, Green Star Product, Inc. passed exhaust heat from its generator into the microalgae cultivation system and simultaneously, the exhaust CO₂ was also fed into the microalgal system. Moreover, growing microalgae in the waste heated water maximized microalgae growth when lower solar insolation limited microalgal growth in winter. Another advantage of growing microalgae at higher (>40°C) temperatures that was noted, was the reduction in the consumption of the microalgal crop by invertebrates since few invertebrate grazers could survive effectively at these temperatures.¹³⁷

2.3 Metabolic constraints to microalgal TAG accumulation

Photosynthetic organisms usually store energy in two types of highly reduced, energy-rich fuel macromolecules: TAGs (lipids) and starch (a glucose polysaccharide).^{130, 139} The metabolic pathways involved in microalgal fatty acid (FA) and TAG biosynthesis appear to be directly analogous to those more widely described in vascular plants, particularly developing oilseeds.¹⁴⁰⁻¹⁴³ FA synthesis occurs in plastids (chloroplast or leucoplast in the case of non-green oilseeds). The first committed step in long chain FA synthesis is the carboxylation of acetyl-CoA into malonyl-CoA, catalyzed by acetyl-CoA carboxylase (ACC) (**Figure 2.1**). FAs produced in the plastid are exported as fatty acyl-CoA esters to the endoplasmic reticulum where they are sequentially bonded onto a glycerol backbone, resulting in TAG.¹⁴⁰⁻¹⁴³ Carbohydrates produced in chloroplasts via photosynthetic CO₂ fixation can also be transiently stored as starch or directed into various metabolic pathways, including parallel plastidic and cytosolic glycolytic pathways (**Figure 2.1**). A major rate-limiting factor limiting C-flux into plastidic FA synthesis appears to be how rapidly glycolysis produces C-skeletons (pyruvate), as well as the chemical energy (ATP) and reducing power (NAD(P)H) needed for FA synthesis.¹⁴⁰⁻¹⁴³ In heterotrophic or mixotrophic green microalgal cultures, a major route of C-flux to FAs appears to be the production of phosphoenolpyruvate (PEP) by cytosolic glycolysis.¹⁴⁴ PEP is transported into the plastid by a PEP translocator, where it is subsequently converted into pyruvate and then acetyl-CoA by the plastidic isozymes of pyruvate kinase and the pyruvate dehydrogenase complex¹⁴⁰⁻¹⁴³ (**Figure 2.1**). However, in plants and green microalgae there are multiple points of C-flux into the plastid that are balanced by the

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competing needs for C-skeletons (*e.g.* pyruvate), ATP, and NAD(P)H.^{140, 141, 145} For example, owing to the action of NADP-dependent malic enzyme (ME), plant/microalgal plastids can use malate as an alternative substrate for plastidic acetyl-CoA, NADPH, and FA synthesis (**Figure 2.1**).^{140, 141} The levels of plastid and cytosolic glycolytic enzymes may, therefore, represent a major rate-limiting factor for microalgal FA and TAG synthesis. Plant/microalgal glycolysis is known to be controlled from the 'bottom up' with primary and secondary regulation exerted at the levels of PEP and fructose-6-P utilization, respectively.^{144, 145} In this way phosphofructokinase, pyruvate kinase, and PEP carboxylase (PEPC) play a central role in the overall regulation of plant respiration since the control of their activities ultimately dictates the rate of photosynthate mobilization of for provision of: (i) respiratory substrates (pyruvate, malate) needed for mitochondrial ATP production via oxidative phosphorylation, and (ii) pyruvate, malate, ATP, and NAD(P)H needed for plastidic FA synthesis. Plant and microalgal PK and PEPC are tightly regulated, allosteric enzymes belonging to a small multi-gene family that catalyze irreversible reactions *in vivo*.^{144, 145}

Once TAGs are synthesized, they are stored as lipid droplets (LD) located in the cytoplasm. LDs consist of a TAG core surrounded by a phospholipid monolayer, and there is evidence for structural proteins (*i.e.* major lipid droplet protein, MLDP) that extending from the LD surface to the TAG core.¹⁴⁶⁻¹⁴⁸ To facilitate oil extraction the size of the LD can be manipulated by modulating MLDP expression; *e.g.*, downregulation of this protein results in larger LDs.¹⁴⁷ TAG accumulation and LD size are also enhanced when microalgae are subjected to N or P deprivation.^{139, 149} However, the molecular mechanisms by which FA and TAG biosynthesis or MLDP activity are influenced by N or P nutrition remain poorly understood.

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Manipulation of metabolic pathways can redirect cellular function for the enhanced biosynthesis of preferred end-products such as TAG. As previously discussed, one method involves employing specific nutrient regimes (*e.g.*, N or P limitation) to induce the desired changes in metabolic fluxes. However, when microalgae divert energy into accumulating oil, they usually don't grow very well, whereas when they devote energy into growth they don't tend to make much oil. As an alternative to manipulating cultivation conditions, metabolic engineering is a very promising technology that involves the targeted modification of specific metabolic pathways via mutagenesis or the introduction of transgenes. The overall goal is to manipulate genes encoding specific enzymes so that microalgal metabolic pathways are tricked into producing abundant amounts of FAs and hence TAG, even when the microalgae are not undergoing stress. However, to date very little work has been conducted to identify regulatory properties of key enzymes that control the provision of C-skeletons (*e.g.*, pyruvate, malate, acetyl-CoA), ATP, and reducing power (NAD(P)H) needed for long-chain FA synthesis in green microalgae cultivated under conditions that maximize oil accumulation.^{142, 143} Attempts to boost oil levels by overexpression of ACC and other enzymes of the FA biosynthetic pathway have been disappointing.¹⁴¹⁻¹⁴³ In particular, the origins and fate of acetyl-CoA, ATP, and reductant are crucial points of control for the FA synthesis pathway. Similarly, very little is known about the genetic origin and molecular, kinetic/regulatory properties of key enzymes such as glycerol kinase that mediate incorporation exogenous C-compounds such as crude glycerol into microalgal metabolism (**Figure 2.1**). Furthermore, some microalgal strains preferentially store excess C as starch rather than TAG by diverting C-flux into the starch biosynthetic pathway.^{150, 151}

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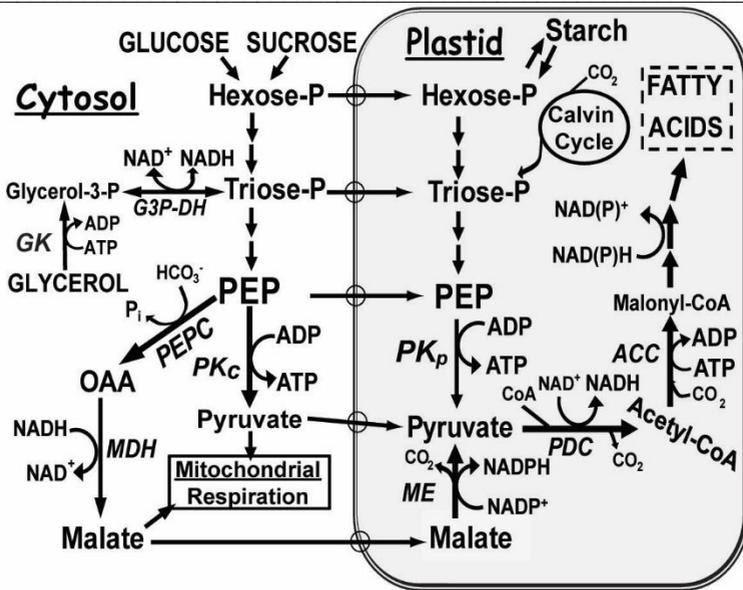


Figure 2.1 Alternative metabolic route for production of fatty acids in vascular plants and green microalgae

2.4 Lipids synthesis pathway and lipid accumulation in response to environmental condition

The prospect of producing sustainable replacements to petroleum-derived fuel and chemical building blocks has led to the investigation of a number of biological routes for the production or extraction of molecules that could act as substitutes for their fossil-derived counterpart. Genetic engineering and synthetic biology are playing important roles in the manipulation of C-fluxes into metabolic pathways that result

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in the production of chemically desirable end-products such as ethylene,¹⁵² bioplastics monomers,¹⁵³ and fuels, in the form of fatty acids methyl esters (FAMES) and alkanes.¹⁵⁴

The biosynthesis of fatty acid containing lipids is a characteristic feature of all living organisms, as they represent the major component of cellular membranes. The phospholipid bilayer membrane requires a fine regulation of the chemical structure of its fatty acids, with respect to chain length and degree of unsaturation, since it is directly involved in cell homeostasis and responses to environmental fluctuations. Lower temperature, for instance, induces the synthesis of phospholipids with an increased proportion of unsaturated fatty acids to maintain an optimal semi-fluidity and, hence, cellular membrane function.¹⁵⁵

However, these lipids represent only a small fraction of the cell biomass, in contrast to oleaginous microorganisms, in which lipids stored as TAGs for energy and carbon reserves can account for more than 50% of the cell dry weight.¹⁵⁶ Of course, this difference is also observed in complex eukaryotes, where some tissues are specialized for TAG accumulation (e.g. adipose tissue in mammals, oilseeds in plants).^{156, 157} Exploring the differential transcriptional pattern of different tissues in a single organism (mice), Qiao *et al.*¹⁵⁷ identified possible genes that could deregulate FA synthesis. Metabolic pathways that support algal fatty acid synthesis are illustrated in **Figures 2.1** and **2.2**. One rate-limiting step is the conversion of acetyl-CoA into malonyl-CoA, by acetyl-CoA carboxylase (ACC); however, other levels of metabolic control are present and need to be considered to ensure high lipid accumulation. Examining the transcriptome of fat accumulating cells such as mammalian adipocytes, liver, and skeletal muscle cells, Qiao *et al.*¹⁵⁷ noted that in addition to ACC, stearoyl-CoA desaturase (SCD) was highly overexpressed compared to profiles obtained from heart, lungs, brain and other non-lipid-accumulating cells. The fatty

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acid biosynthesis route in plants is initiated in the chloroplast, producing saturated C₁₆ and C₁₈ fatty acids (palmitic acid and stearic acid) covalently linked to Coenzyme A (**Figure 2.2**). The SCD catalyzes the desaturation of recently elongated fatty acid, producing palmitoleic acid (16:1 n-7) and oleic acid (18:1 n-9). ACC is under allosteric regulation, being inhibited by the saturated C₁₆ and C₁₈; hence, when these fatty acids are desaturated by SCD, the negative feedback on ACC is decreased and it can resume function in the fatty acid rate-limiting step: conversion of acetyl-CoA into malonyl-CoA. However, SCD is also regulated by its products, and the accumulation of palmitoleic acid and oleic acid decreases SCD activity. The deregulation of lipid biosynthesis was successfully accomplished in yeast through the overexpression of the TAG-assembling enzyme diacylglyceride-acyl-transferase (DGA1). Qiao *et al.*¹⁵⁷ showed that the overexpression of ACC, SCD and DGA1 in the yeast *Yarrowia lipolytica* was sufficient to relieve negative feedback and increase lipid productivity up to 11.5 fold, reaching 22 g L⁻¹ d⁻¹.¹⁵⁷ The extensive metabolic engineering tools available for yeast and bacteria allowed researchers to explore different control mechanisms in FA synthesis.¹⁵⁷ Although different organisms, it is believed that similar mechanisms are present in oleaginous algae, and the approach employed in *Yarrowia lipolytica* could likely show similar results if DGA1 were cloned and overexpressed in the algal chloroplast.¹⁵⁷⁻¹⁵⁹ However, the development of molecular tools for microalgae is still in its infancy. Microalgal species are widely spread in the tree of life and techniques developed for one species will likely require adaptation or redesign prior to its application in other species. The methods used for *Chlamydomonas reinhardtii* cloning is ineffective to other Chlorophyte as *Chlorella vulgaris* or *Scenedesmus obliquus*.¹⁶⁰ The few reports of new transformation methods for green

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microalgae announced in recent years were unstable or are still to be replicated by other groups.¹⁶¹⁻¹⁶³ Diatoms, for example, are classified within the *Chromista Kingdom* and a few strains have an established method for transformation based on the biolistic technique.¹⁵⁸ Using these tools, Trentacoste *et al.*¹⁵⁹ explored a different approach to increase lipid productivity in the diatom *Thalassiosira pseudonana*: knocking down the lipases active during silicon starvation. This strategy led to an increase in the overall lipid productivity without disrupting cell growth. Silicon starvation induced lipid accumulation in the stationary phase, an established physiological approach for diatoms,¹⁶⁴ and the simple disruption of the lipid catabolism led to a 4.1-fold increase in the cellular lipid content. That being said, there remain a number of avenues to explore within the FA regulatory system.

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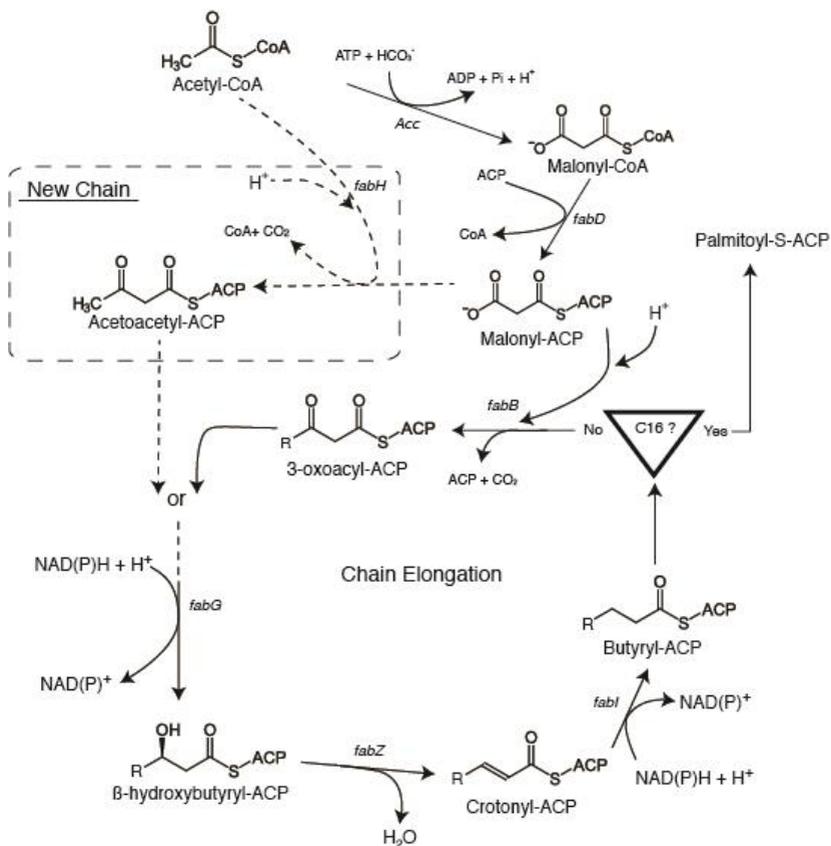


Figure 2.2 The fatty acid biosynthesis route in bacteria and plants

However, one of the limitations of genetically modified organisms is that they are typically adapted for a specific set of conditions and may now be amenable to successful cultivation under a wide range of scenarios. Lipid productivity can be enhanced through manipulation of nutritional state and environmental conditions. The most widely used mechanism is nitrogen starvation, which will generally limit microalgal

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growth and divert C-flux for energy reserve, in most cases, TAGs. The lipid content can easily be doubled, reaching 50% of the biomass dry cell weight (DCW) in some cases.^{19, 165} Phosphorus starvation has been shown to be less effective, increasing the lipid content from 22% to 33% DCW, but smaller than the 52% DCW observed in the same cells under nitrogen depletion condition.²⁰ Other stress conditions that have been reported include iron deficiency for *Chlorella vulgaris* and sodium chloride excess for *Dunaliella tertiolecta*.^{166, 167}

One disadvantage of this strategy is the two-stage cultivation requirement because the lipid accumulation process is linked to a growth arrest resulting from an induced physiological stress.¹⁶⁸ Cells are grown to a desirable cell density under optimal conditions, then cultures are subjected to an artificial physiological stress in the second stage, which will slow cell growth but allow for lipid accumulation as a carbon reserve.²² From an operational, this transition can take several days, which can leave the culture vulnerable for grazing organisms or simply prolonging retention time.¹⁶⁹ One alternative is the use of xylose. This pentose has been shown to induce lipid accumulation up to four-fold in 6-12 hours,¹⁷⁰ reducing the cultivation time required for the same or higher lipid accumulation compared to that reported for nitrogen starvation approach.

Different cultivation strategies demand different solutions. A wastewater treatment plant cultivating microalgae in open ponds and releasing the effluent to a surface receiver would not likely use a genetically modified organism. However, closed cultivation system aiming for high-efficiency of CO₂ conversion into microalgal biomass could certainly benefit from strains genetically tailored for lipid accumulation.

2.5 Conclusions

The integration of wastewater, waste or CO₂-enriched gas, waste glycerol and waste heat with microalgal cultivation system offer an economically feasible and environmentally sustainable strategy for waste treatment and biofuel production. But the corresponding microalgal species and operational parameters should be identified and optimized case by case, particularly for large scale application. In addition, a detailed understanding of metabolic regulatory networks controlling carbohydrate partitioning between FA and storage TAG synthesis and alternative storage end-products (i.e., starch, protein) during microalgal cultivation will therefore be essential, especially for the rational application of metabolic engineering to maximize microalgal bio-oil production without sacrificing biomass yield under different environmental stresses.

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2.7 References

1. Zhu L, Microalgal culture strategies for biofuel production: a review. *Biofuels Bioprod Biorefin* **9**: 801-814 (2015).
2. Singh A, Nigam, PS, Murphy, JD, Mechanism and challenges in commercialisation of algal biofuels. *Bioresour Technol* **102**: 26-34 (2011).
3. Mutanda T, Ramesh, D, Karthikeyan, S, Kumari, S, Anandraj, A, Bux, F, Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresour Technol* **102**: 57-70 (2011).
4. Subramaniam R, Dufreche, S, Zappi, M, Bajpai, R, Microbial lipids from renewable resources: production and characterization. *J Ind Microbiol Biot* **37**: 1271-1287 (2010).
5. Chisti Y, Biodiesel from microalgae. *Biotechnol Adv* **25**: 294-306 (2007).
6. Liu J, Huang, J, Sun, Z, Zhong, Y, Jiang, Y, Chen, F, Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic *Chlorella zofingiensis*: assessment of algal oils for biodiesel production. *Bioresour Technol* **102**: 106-110 (2011).
7. Beal CM, Hebner, RE, Webber, ME, Ruoff, RS, Seibert, AF, King, CW, Comprehensive evaluation of algal biofuel production: experimental and target results. *Energies* **5**: 1943-1981 (2012).
8. Ge S, Agbakpe, M, Zhang, W, Kuang, L, Heteroaggregation between PEI-Coated Magnetic Nanoparticles and Algae: Effect of Particle Size on Algal Harvesting Efficiency. *ACS Appl. Mater. Interfaces* **7**: 6102-6108 (2015).
9. Packer M, Algal capture of carbon dioxide; biomass generation as a tool for greenhouse gas mitigation with reference

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

to New Zealand energy strategy and policy. *Energ Policy* **37**: 3428-3437 (2009).

10. Ge S, Wang, S, Yang, X, Qiu, S, Li, B, Peng, Y, Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: A review. *Chemosphere* **140**: 85-98 (2015).

11. Van Den Hende S, Carré, E, Cocaud, E, Beelen, V, Boon, N, Vervaeren, H, Treatment of industrial wastewaters by microalgal bacterial flocs in sequencing batch reactors. *Bioresour Technol* **161**: 245-254 (2014).

12. Ge S, Peng, Y, Wang, S, Guo, J, Ma, B, Zhang, L, Cao, X, Enhanced nutrient removal in a modified step feed process treating municipal wastewater with different inflow distribution ratios and nutrient ratios. *Bioresour Technol* **101**: 9012-9019 (2010).

13. Yang J, Xu, M, Zhang, X, Hu, Q, Sommerfeld, M, Chen, Y, Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. *Bioresour Technol* **102**: 159-165 (2011).

14. Ge Y, Liu, J, Tian, G, Growth characteristics of *Botryococcus braunii* 765 under high CO₂ concentration in photobioreactor. *Bioresour Technol* **102**: 130-134 (2011).

15. Canada E Canada's Greenhouse Gas <https://www.ec.gc.ca/ges-g/default.asp?lang=En&n=83A34A7A-1>. (accessed April 17).

16. Chisti Y, Biodiesel from microalgae beats bioethanol. *Trends Biotechnol* **26**: 126-131 (2008).

17. Brennan L, Owende, P, Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renew Sust Energ Rev* **14**: 557-577 (2010).

18. Miao X, Wu, Q, Biodiesel production from heterotrophic microalgal oil. *Bioresour Technol* **97**: 841-846 (2006).

19. Abdelaziz AE, Leite, GB, Hallenbeck, PC, Addressing the challenges for sustainable production of algal biofuels: I. Algal strains and nutrient supply. *Environ Technol* **34**: 1783-1805 (2013).
20. Li Y, Han, F, Xu, H, Mu, J, Chen, D, Feng, B, Zeng, H, Potential lipid accumulation and growth characteristic of the green alga *Chlorella* with combination cultivation mode of nitrogen (N) and phosphorus (P). *Bioresour Technol* **174**: 24-32 (2014).
21. Sharma YC, Singh, B, Korstad, J, A critical review on recent methods used for economically viable and eco-friendly development of microalgae as a potential feedstock for synthesis of biodiesel. *Green Chem* **13**: 2993-3006 (2011).
22. Van Vooren G, Le Grand, F, Legrand, J, Cuiné, S, Peltier, G, Pruvost, J, Investigation of fatty acids accumulation in *Nannochloropsis oculata* for biodiesel application. *Bioresour Technol* **124**: 421-432 (2012).
23. Hoffmann JP, Wastewater treatment with suspended and nonsuspended algae. *J Phycol* **34**: 757-763 (1998).
24. Park J, Jin, H-F, Lim, B-R, Park, K-Y, Lee, K, Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. *Bioresour Technol* **101**: 8649-8657 (2010).
25. Powell N, Shilton, AN, Pratt, S, Chisti, Y, Factors influencing luxury uptake of phosphorus by microalgae in waste stabilization ponds. *Environ Sci Technol* **42**: 5958-5962 (2008).
26. Monlau F, Sambusiti, C, Ficara, E, Aboulkas, A, Barakat, A, Carrère, H, New opportunities for agricultural digestate valorization: current situation and perspectives. *Energy Environ Sci* **8**: 2600-2621 (2015).
27. Slegers P, Lösing, M, Wijffels, R, van Straten, G, van Boxtel, A, Scenario evaluation of open pond microalgae production. *Algal Res* **2**: 358-368 (2013).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

28. Liang Y, Sarkany, N, Cui, Y, Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol Lett* **31**: 1043-1049 (2009).
29. Palmer C, Algae in american sewage stabilization's ponds. *Rev. Microbiol.(S-Paulo)* **5**: 75-80 (1974).
30. Ge S, Champagne, P, Nutrient removal, microalgal biomass growth, harvesting and lipid yield in response to centrate wastewater loadings. *Water Res* **88**: 604-612 (2016).
31. Collos Y, Harrison, PJ, Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Mar Pollut Bull* **80**: 8-23 (2014).
32. Pittman JK, Dean, AP, Osundeko, O, The potential of sustainable algal biofuel production using wastewater resources. *Bioresour Technol* **102**: 17-25 (2011).
33. Steiner CF, Long, ZT, Krumins, JA, Morin, PJ, Population and community resilience in multitrophic communities. *Ecology* **87**: 996-1007 (2006).
34. Fouilland E, Vasseur, C, Leboulanger, C, Le Floc'h, E, Carré, C, Marty, B, Steyer, J-P, Sialve, B, Coupling algal biomass production and anaerobic digestion: Production assessment of some native temperate and tropical microalgae. *Biomass Bioenerg* **70**: 564-569 (2014).
35. Kim J, Lingaraju, BP, Rheume, R, Lee, J-Y, Siddiqui, KF, Removal of ammonia from wastewater effluent by *Chlorella vulgaris*. *Tsinghua Science & Technology* **15**: 391-396 (2010).
36. Andersen RA, *Algal Culturing Techniques*. 2005; Vol. 53, p 578.
37. del Mar Morales-Amaral M, Gómez-Serrano, C, Acién, FG, Fernández-Sevilla, JM, Molina-Grima, E, Outdoor production of *Scenedesmus* sp. in thin-layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source. *Algal Res* **12**: 99-108 (2015).

38. Matamoros V, Gutiérrez, R, Ferrer, I, García, J, Bayona, JM, Capability of microalgae-based wastewater treatment systems to remove emerging organic contaminants: A pilot-scale study. *J Hazard Mater* **288**: 34-42 (2015).
39. Abargues M, Ferrer, J, Bouzas, A, Seco, A, Removal and fate of endocrine disruptors chemicals under lab-scale posttreatment stage. Removal assessment using light, oxygen and microalgae. *Bioresour Technol* **149**: 142-148 (2013).
40. Haritash A, Kaushik, C, Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J Hazard Mater* **169**: 1-15 (2009).
41. de-Bashan LE, Bashan, Y, Immobilized microalgae for removing pollutants: review of practical aspects. *Bioresour Technol* **101**: 1611-1627 (2010).
42. González C, Marciniak, J, Villaverde, S, García-Encina, PA, Muñoz, R, Microalgae-based processes for the biodegradation of pretreated piggery wastewaters. *Appl Microbiol Biot* **80**: 891-898 (2008).
43. de Wilt A, Butkovskiy, A, Tuantet, K, Leal, LH, Fernandes, TV, Langenhoff, A, Zeeman, G, Micropollutant removal in an algal treatment system fed with source separated wastewater streams. *J Hazard Mater* **304**: 84-92 (2016).
44. Abargues MR, Ferrer, J, Bouzas, A, Seco, A, Removal and fate of endocrine disruptors chemicals under lab-scale posttreatment stage. Removal assessment using light, oxygen and microalgae. *Bioresour Technol* **149**: 142-148 (2013).
45. Norvill ZN, Shilton, A, Guieysse, B, Emerging contaminant degradation and removal in algal wastewater treatment ponds: Identifying the research gaps. *J Hazard Mater* **313**: 291-309 (2016).
46. Osundeko O, Pittman, JK, Implications of sludge liquor addition for wastewater-based open pond cultivation of

microalgae for biofuel generation and pollutant remediation. *Bioresour Technol* **152**: 355-363 (2014).

47. Collet P, Hélias, A, Lardon, L, Ras, M, Goy, R-A, Steyer, J-P, Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresour Technol* **102**: 207-214 (2011).

48. Ledda C, Villegas, GR, Adani, F, Fernández, FA, Grima, EM, Utilization of centrate from wastewater treatment for the outdoor production of *Nannochloropsis gaditana* biomass at pilot-scale. *Algal Res* **12**: 17-25 (2015).

49. Bahr M, Díaz, I, Dominguez, A, González Sánchez, A, Muñoz, R, Microalgal-Biotechnology As a Platform for an Integral Biogas Upgrading and Nutrient Removal from Anaerobic Effluents. *Environ Sci Technol* **48**: 573-581 (2014).

50. Min M, Wang, L, Li, Y, Mohr, MJ, Hu, B, Zhou, W, Chen, P, Ruan, R, Cultivating *Chlorella* sp. in a pilot-scale photobioreactor using centrate wastewater for microalgae biomass production and wastewater nutrient removal. *Appl Biochem Biotech* **165**: 123-137 (2011).

51. Acién FG, Fernández, JM, Magán, JJ, Molina, E, Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnol Adv* **30**: 1344-1353 (2012).

52. González-Fernández C, Molinuevo-Salces, B, García-González, MC, Nitrogen transformations under different conditions in open ponds by means of microalgae–bacteria consortium treating pig slurry. *Bioresour Technol* **102**: 960-966 (2011).

53. Wang L, Li, Y, Chen, P, Min, M, Chen, Y, Zhu, J, Ruan, RR, Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. *Bioresour Technol* **101**: 2623-2628 (2010).

54. Franchino M, Comino, E, Bona, F, Riggio, VA, Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. *Chemosphere* **92**: 738-744 (2013).

55. Marcilhac C, Sialve, B, Pourcher, A-M, Ziebal, C, Bernet, N, Béline, F, Digestate color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem. *Water Res* **64**: 278-287 (2014).
56. Halfhide T, Dalrymple, OK, Wilkie, AC, Trimmer, J, Gillie, B, Udom, I, Zhang, Q, Ergas, SJ, Growth of an Indigenous Algal Consortium on Anaerobically Digested Municipal Sludge Centrate: Photobioreactor Performance and Modeling. *BioEnergy Res* **8**: 249-258 (2015).
57. Posadas E, García-Encina, P-A, Soltau, A, Domínguez, A, Díaz, I, Muñoz, R, Carbon and nutrient removal from concentrates and domestic wastewater using algal–bacterial biofilm bioreactors. *Bioresour Technol* **139**: 50-58 (2013).
58. Prandini JM, da Silva, MLB, Mezzari, MP, Pirolli, M, Michelon, W, Soares, HM, Enhancement of nutrient removal from swine wastewater digestate coupled to biogas purification by microalgae *Scenedesmus* spp. *Bioresour Technol* **202**: 67-75 (2016).
59. Bjornsson WJ, Nicol, RW, Dickinson, KE, McGinn, PJ, Anaerobic digestates are useful nutrient sources for microalgae cultivation: functional coupling of energy and biomass production. *J Appl Phycol* **25**: 1523-1528 (2013).
60. Posadas E, Bochon, S, Coca, M, García-González, M, García-Encina, P, Muñoz, R, Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability. *J Appl Phycol* **26**: 2335-2345 (2014).
61. Ficara E, Uslenghi, A, Basilico, D, Mezzanotte, V, Growth of microalgal biomass on supernatant from biosolid dewatering. *Water Sci Technol* **69**: 896-902 (2014).
62. Uggetti E, Sialve, B, Latrille, E, Steyer, J-P, Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. *Bioresour Technol* **152**: 437-443 (2014).

63. Cho S, Lee, N, Park, S, Yu, J, Luong, TT, Oh, Y-K, Lee, T, Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. *Bioresour Technol* **131**: 515-520 (2013).
64. Chu W-L, Biotechnological applications of microalgae. *JeJSME* **6**: S24-S37 (2012).
65. Caporgno MP, Taleb, A, Olkiewicz, M, Font, J, Pruvost, J, Legrand, J, Bengoa, C, Microalgae cultivation in urban wastewater: Nutrient removal and biomass production for biodiesel and methane. *Algal Res* **10**: 232-239 (2015).
66. Ji M-K, Abou-Shanab, RAI, Kim, S-H, Salama, E-S, Lee, S-H, Kabra, AN, Lee, Y-S, Hong, S, Jeon, B-H, Cultivation of microalgae species in tertiary municipal wastewater supplemented with CO₂ for nutrient removal and biomass production. *Ecol Eng* **58**: 142-148 (2013).
67. Halfhide T, Dalrymple, OK, Wilkie, AC, Trimmer, J, Gillie, B, Udom, I, Zhang, Q, Ergas, SJ, Growth of an Indigenous Algal Consortium on Anaerobically Digested Municipal Sludge Centrate: Photobioreactor Performance and Modeling. *BioEnergy Res* 1-10 (2014).
68. WeiDong L, ZhongMing, W, XueWei, W, ZhenHong, Y, Cultivation of *Chlorella* sp. using raw dairy wastewater for nutrient removal and biodiesel production: characteristics comparison of indoor bench-scale and outdoor pilot-scale cultures. *Bioresour Technol* **192**: 382-388 (2015).
69. Zimmo OR, van der Steen, NP, Gijzen, HJ, Nitrogen mass balance across pilot-scale algae and duckweed-based wastewater stabilisation ponds. *Water Res* **38**: 913-920 (2004).
70. Gómez-Serrano C, Morales-Amaral, M, Ación, F, Escudero, R, Fernández-Sevilla, J, Molina-Grima, E, Utilization of secondary-treated wastewater for the production of freshwater microalgae. *Appl Microbiol Biot* **99**: 6931-6944 (2015).

71. del Mar Morales-Amaral M, Gómez-Serrano, C, Acién, FG, Fernández-Sevilla, JM, Molina-Grima, E, Production of microalgae using centrate from anaerobic digestion as the nutrient source. *Algal Res* **9**: 297-305 (2015).
72. Ficara E, Uslenghi, A, Basilio, D, Mezzanotte, V, Growth of microalgal biomass on supernatant from biosolid dewatering. *Water Science & Technology* **69**: (2014).
73. Uggetti E, Sialve, B, Latrille, E, Steyer, J-P, Anaerobic digestate as substrate for microalgae culture: the role of ammonium concentration on the microalgae productivity. *Bioresour Technol* **152**: 437-443 (2014).
74. Bhola V, Swalaha, F, Ranjith Kumar, R, Singh, M, Bux, F, Overview of the potential of microalgae for CO₂ sequestration. *Int J Environ Sci Technol* **11**: 2103-2118 (2014).
75. Widjaja A, Chien, C-C, Ju, Y-H, Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *J Taiwan Inst Chem Eng* **40**: 13-20 (2009).
76. Kong Q-x, Li, L, Martinez, B, Chen, P, Ruan, R, Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. *Appl Biochem Biotech* **160**: 9-18 (2010).
77. Muradyan E, Klyachko-Gurvich, G, Tsoglin, L, Sergeyenko, T, Pronina, N, Changes in lipid metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO₂ concentration. *Russ J Plant Physiol+* **51**: 53-62 (2004).
78. Ryckebosch E, Drouillon, M, Vervaeren, H, Techniques for transformation of biogas to biomethane. *Biomass Bioenerg* **35**: 1633-1645 (2011).
79. Tongprawhan W, Srinuanpan, S, Cheirsilp, B, Biocapture of CO₂ from biogas by oleaginous microalgae for improving methane content and simultaneously producing lipid. *Bioresour Technol* **170**: 90-99 (2014).

80. Xu J, Zhao, Y, Zhao, G, Zhang, H, Nutrient removal and biogas upgrading by integrating freshwater algae cultivation with piggery anaerobic digestate liquid treatment. *Appl Microbiol Biot* **99**: 6493-6501 (2015).
81. Yan O, Zhao, Y, Sun, S, Hu, C, Ping, L, Effect of light intensity on the capability of different microalgae species for simultaneous biogas upgrading and biogas slurry nutrient reduction. *Int Biodeter Biodegr* **104**: 157-163 (2015).
82. Ho S-H, Chen, C-Y, Lee, D-J, Chang, J-S, Perspectives on microalgal CO₂-emission mitigation systems — A review. *Biotechnol Adv* **29**: 189-198 (2011).
83. Meier L, Pérez, R, Azócar, L, Rivas, M, Jeison, D, Photosynthetic CO₂ uptake by microalgae: An attractive tool for biogas upgrading. *Biomass Bioenerg* **73**: 102-109 (2015).
84. Mann G, Schlegel, M, Schumann, R, Sakalauskas, A, Biogas-conditioning with microalgae. *Agron Res* **7**: 33-38 (2009).
85. Xia A, Herrmann, C, Murphy, JD, How do we optimize third-generation algal biofuels? *Biofuels Bioprod Biorefin* **9**: 358-367 (2015).
86. Serejo ML, Posadas, E, Boncz, MA, Blanco, S, García-Encina, P, Muñoz, R, Influence of Biogas Flow Rate on Biomass Composition During the Optimization of Biogas Upgrading in Microalgal-Bacterial Processes. *Environ Sci Technol* **49**: 3228-3236 (2015).
87. Conde JL, Moro, LE, Travieso, L, Sanchez, EP, Leiva, A, Dupeirón, R, Escobedo, R, Biogas purification process using intensive microalgae cultures. *Biotechnol Lett* **15**: 317-320 (1993).
88. Kao C-Y, Chiu, S-Y, Huang, T-T, Dai, L, Hsu, L-K, Lin, C-S, Ability of a mutant strain of the microalga *Chlorella* sp. to capture carbon dioxide for biogas upgrading. *Appl Energ* **93**: 176-183 (2012).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

89. Sun S, Ge, Z, Zhao, Y, Hu, C, Zhang, H, Ping, L, Performance of CO₂ concentrations on nutrient removal and biogas upgrading by integrating microalgal strains cultivation with activated sludge. *Energy* **97**: 229-237 (2016).
90. Zhao Y, Sun, S, Hu, C, Zhang, H, Xu, J, Ping, L, Performance of three microalgal strains in biogas slurry purification and biogas upgrade in response to various mixed light-emitting diode light wavelengths. *Bioresour Technol* **187**: 338-345 (2015).
91. Huang G, Chen, F, Kuang, Y, He, H, Qin, A, Current Techniques of Growing Algae Using Flue Gas from Exhaust Gas Industry: a Review. *Appl Biochem Biotech* 1-19 (2015).
92. Teong LK, Bhatia, S, Mohamed, AR, Preparation and characterization of adsorbents prepared from ash (waste material) for sulfur dioxide (SO₂) removal. *J Mater Cycles Waste* **7**: 8 (2005).
93. Maeda K, Owada, M, Kimura, N, Omata, K, Karube, I, CO₂ fixation from the flue gas on coal-fired thermal power plant by microalgae. *Energ Convers Manage* **36**: 717-720 (1995).
94. Jiang Y, Zhang, W, Wang, J, Chen, Y, Shen, S, Liu, T, Utilization of simulated flue gas for cultivation of *Scenedesmus dimorphus*. *Bioresour Technol* **128**: 359-364 (2013).
95. Chiu S-Y, Kao, C-Y, Huang, T-T, Lin, C-J, Ong, S-C, Chen, C-D, Chang, J-S, Lin, C-S, Microalgal biomass production and on-site bioremediation of carbon dioxide, nitrogen oxide and sulfur dioxide from flue gas using *Chlorella* sp. cultures. *Bioresour Technol* **102**: 9135-9142 (2011).
96. Ryu HJ, Oh, KK, Kim, YS, Optimization of the influential factors for the improvement of CO₂ utilization efficiency and CO₂ mass transfer rate. *J Ind Eng Chem* **15**: 471-475 (2009).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

97. Taştan BE, Duygu, E, Atakol, O, Dönmez, G, SO₂ and NO₂ tolerance of microalgae with the help of some growth stimulators. *Energ Convers Manage* **64**: 28-34 (2012).
98. Gross W, Ecophysiology of algae living in highly acidic environments. *Hydrobiologia* **433**: 31-37 (2000).
99. Das BK, Roy, A, Koschorreck, M, Mandal, SM, Wendt-Potthoff, K, Bhattacharya, J, Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. *Water Res* **43**: 883-894 (2009).
100. Yen HW, Ho, SH, Chen, CY, Chang, JS, CO₂, NO_x and SO_x removal from flue gas via microalgae cultivation: A critical review. *Biotechnol J* **10**: 829-839 (2015).
101. Eynde EV, Lenaerts, B, Tytgat, T, Blust, R, Lenaerts, S, Valorization of Flue Gas by Combining Photocatalytic Gas Pretreatment with Microalgae Production. *Environ Sci Technol* **50**: 2538-2545 (2016).
102. Xia A, Cheng, J, Murphy, JD, Innovation in biological production and upgrading of methane and hydrogen for use as gaseous transport biofuel. *Biotechnol Adv* doi:10.1016/j.biotechadv.2015.12.009: (2016).
103. Council NR, *Managing Coal Combustion Residues in Mines*. National Academies Press: Washington, D.C., 2006.
104. Jakob A, Stucki, S, Kuhn, P, Evaporation of Heavy Metals during the Heat Treatment of Municipal Solid Waste Incinerator Fly Ash. *Environ Sci Technol* **29**: 2429-2436 (1995).
105. Napan K, Teng, L, Quinn, JC, Wood, BD, Impact of heavy metals from flue gas integration with microalgae production. *Algal Res* **8**: 83-88 (2015).
106. Matsumoto H, Shioji, N, Hamasaki, A, Ikuta, Y, Fukuda, Y, Sato, M, Endo, N, Tsukamoto, T, Carbon dioxide fixation by microalgae photosynthesis using actual flue gas discharged from a boiler. *Appl Biochem Biotech* **51**: 681-692 (1995).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

107. Brown LM, Uptake of carbon dioxide from flue gas by microalgae. *Energ Convers Manage* **37**: 1363-1367 (1996).
108. Praveenkumar R, Kim, B, Choi, E, Lee, K, Park, J-Y, Lee, J-S, Lee, Y-C, Oh, Y-K, Improved biomass and lipid production in a mixotrophic culture of *Chlorella* sp. KR-1 with addition of coal-fired flue-gas. *Bioresour Technol* **171**: 500-505 (2014).
109. Vaz B, Costa, JAV, Morais, MG, CO₂ Biofixation by the *Cyanobacterium Spirulina* sp. LEB 18 and the Green Alga *Chlorella fusca* LEB 111 Grown Using Gas Effluents and Solid Residues of Thermoelectric Origin. *Appl Biochem Biotech* **178**: 418-429 (2015).
110. Li F-F, Yang, Z-H, Zeng, R, Yang, G, Chang, X, Yan, J-B, Hou, Y-L, Microalgae Capture of CO₂ from Actual Flue Gas Discharged from a Combustion Chamber. *Ind Eng Chem Res* **50**: 6496-6502 (2011).
111. Kao C-Y, Chen, T-Y, Chang, Y-B, Chiu, T-W, Lin, H-Y, Chen, C-D, Chang, J-S, Lin, C-S, Utilization of carbon dioxide in industrial flue gases for the cultivation of microalga *Chlorella* sp. *Bioresour Technol* **166**: 485-493 (2014).
112. Douskova I, Doucha, J, Livansky, K, Machat, J, Novak, P, Umysova, D, Zachleder, V, Vitova, M, Simultaneous flue gas bioremediation and reduction of microalgal biomass production costs. *Appl Microbiol Biot* **82**: 179-185 (2008).
113. Vunjak-Novakovic G, Kim, Y, Wu, X, Berzin, I, Merchuk, JC, Air-Lift Bioreactors for Algal Growth on Flue Gas: Mathematical Modeling and Pilot-Plant Studies. *Ind Eng Chem Res* **44**: 6154-6163 (2005).
114. Bhatnagar A, Chinnasamy, S, Singh, M, Das, KC, Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. *Appl Energy* **88**: 3425-3431 (2011).

115. Das P, Aziz, SS, Obbard, JP, Two phase microalgae growth in the open system for enhanced lipid productivity. *Renew Energ* **36**: 2524-2528 (2011).
116. Das P, Lei, W, Aziz, SS, Obbard, JP, Enhanced algae growth in both phototrophic and mixotrophic culture under blue light. *Bioresour Technol* **102**: 3883-3887 (2011).
117. Chojnacka K, Noworyta, A, Evaluation of *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. *Enzyme Microb Tech* **34**: 461-465 (2004).
118. Abreu AP, Fernandes, B, Vicente, AA, Teixeira, J, Dragone, G, Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy waste as organic carbon source. *Bioresour Technol* **118**: 61-66 (2012).
119. Rawat I, Ranjith Kumar, R, Mutanda, T, Bux, F, Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Appl Energ* **88**: 3411-3424 (2011).
120. Feng Y, Li, C, Zhang, D, Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. *Bioresour Technol* **102**: 101-105 (2011).
121. Mandal S, Mallick, N, Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Appl Microbiol Biot* **84**: 281-291 (2009).
122. Girard J-M, Roy, M-L, Hafsa, MB, Gagnon, J, Fauchoux, N, Heitz, M, Tremblay, R, Deschênes, J-S, Mixotrophic cultivation of green microalgae *Scenedesmus obliquus* on cheese whey permeate for biodiesel production. *Algal Res* **5**: 241-248 (2014).
123. Sarma SJ, Das, RK, Brar, SK, Le Bihan, Y, Buelna, G, Verma, M, Soccol, CR, Application of magnesium sulfate and its nanoparticles for enhanced lipid production by mixotrophic cultivation of algae using biodiesel waste. *Energy* **78**: 16-22 (2014).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

124. Chen Y-H, Walker, TH, Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. *Biotechnol Lett* **33**: 1973-1983 (2011).
125. O'Grady J, Morgan, JA, Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. *Bioprocess Biosyst Eng* **34**: 121-125 (2011).
126. Feng X, Walker, TH, Bridges, WC, Thornton, C, Gopalakrishnan, K, Biomass and lipid production of *Chlorella protothecoides* under heterotrophic cultivation on a mixed waste substrate of brewer fermentation and crude glycerol. *Bioresour Technol* **166**: 17-23 (2014).
127. Gupta PL, Choi, HJ, Lee, S-M, Enhanced nutrient removal from municipal wastewater assisted by mixotrophic microalgal cultivation using glycerol. *Environ Sci Pollut R* 1-10 (2016).
128. Pyle DJ, Garcia, RA, Wen, Z, Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. *J Agr Food Chem* **56**: 3933-3939 (2008).
129. Andruleviciute V, Makareviciene, V, Skorupskaite, V, Gumbyte, M, Biomass and oil content of *Chlorella sp.*, *Haematococcus sp.*, *Nannochloris sp.* and *Scenedesmus sp.* under mixotrophic growth conditions in the presence of technical glycerol. *J Appl Phycol* **26**: 83-90 (2013).
130. Perez-Garcia O, Escalante, F, de-Bashan, L, Bashan, Y, Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res* **45**: 11 - 36 (2011).
131. Hu S, Luo, X, Wan, C, Li, Y, Characterization of crude glycerol from biodiesel plants. *J Agr Food Chem* **60**: 5915-5921 (2012).

132. Liang Y, Sarkany, N, Cui, Y, Blackburn, JW, Batch stage study of lipid production from crude glycerol derived from yellow grease or animal fats through microalgal fermentation. *Bioresour Technol* **101**: 6745-6750 (2010).
133. Sarma SJ, Brar, SK, Le Bihan, Y, Buelna, G, Soccol, CR, Hydrogen production from meat processing and restaurant waste derived crude glycerol by anaerobic fermentation and utilization of the spent broth. *J Chem Technol Biot* **88**: 2264-2271 (2013).
134. Béchet Q, Shilton, A, Guieysse, B, Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation. *Biotechnol Adv* **31**: 1648-1663 (2013).
135. Ras M, Steyer, J-P, Bernard, O, Temperature effect on microalgae: a crucial factor for outdoor production. *Rev Environ Sci Biotechnol* **12**: 153-164 (2013).
136. Béchet Q, Muñoz, R, Shilton, A, Guieysse, B, Outdoor cultivation of temperature-tolerant *Chlorella sorokiniana* in a column photobioreactor under low power-input. *Biotechnol Bioeng* **110**: 118-126 (2013).
137. Wilde EW, Benemann, JR, Weissman, JC, Tillett, DM, Cultivation of algae and nutrient removal in a waste heat utilization process. *J Appl Phycol* **3**: 159-167 (1991).
138. Slade R, Bauen, A, Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. *Biomass Bioenerg* **53**: 29-38 (2013).
139. Příbyl P, Cepák, V, Zachleder, V, Production of lipids and formation and mobilization of lipid bodies in *Chlorella vulgaris*. *J Appl Phycol* **25**: 545-553 (2013).
140. Hu Q, Sommerfeld, M, Jarvis, E, Ghirardi, M, Posewitz, M, Seibert, M, Darzins, A, Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J* **54**: 621-639 (2008).

141. Baud S, Lepiniec, L, Physiological and developmental regulation of seed oil production. *Prog Lipid Res* **49**: 235-249 (2010).
142. Radakovits R, Jinkerson, RE, Darzins, A, Posewitz, MC, Genetic engineering of algae for enhanced biofuel production. *Eukaryot Cell* **9**: 486-501 (2010).
143. Greenwell H, Laurens, L, Shields, R, Lovitt, R, Flynn, K, Placing microalgae on the biofuels priority list: a review of the technological challenges. *J R Soc Interface* rsif20090322 (2009).
144. Agrawal GK, Rakwal, R, *Seed Development: OMICS Technologies toward Improvement of Seed Quality and Crop Yield*. Springer: 2012.
145. Plaxton WC, Podestá, FE, The functional organization and control of plant respiration. *Crit Rev Plant Sci* **25**: 159-198 (2006).
146. Liu B, Benning, C, Lipid metabolism in microalgae distinguishes itself. *Curr Opin Biotech* **24**: 300-309 (2013).
147. Moellering ER, Benning, C, RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. *Eukaryot Cell* **9**: 97-106 (2010).
148. Chapman KD, Dyer, JM, Mullen, RT, Biogenesis and functions of lipid droplets in plants Thematic Review Series: Lipid Droplet Synthesis and Metabolism: from Yeast to Man. *J Lipid Res* **53**: 215-226 (2012).
149. Msanne J, Xu, D, Konda, AR, Casas-Mollano, JA, Awada, T, Cahoon, EB, Cerutti, H, Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. *Phytochemistry* **75**: 50-59 (2012).
150. Courchesne N, Parisien, A, Wang, B, Lan, C, Enhancement of lipid production using biochemical, genetic and

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

transcription factor engineering approaches. *J Biotechnol* **141**: 31 - 41 (2009).

151. Wang ZT, Ullrich, N, Joo, S, Waffenschmidt, S, Goodenough, U, Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot Cell* **8**: 1856-1868 (2009).

152. Ungerer J, Tao, L, Davis, M, Ghirardi, M, Maness, P-C, Yu, J, Sustained photosynthetic conversion of CO₂ to ethylene in recombinant cyanobacterium *Synechocystis* 6803. *Energy Environ Sci* **5**: 8998-9006 (2012).

153. Rahman A, Putman, RJ, Inan, K, Sal, FA, Sathish, A, Smith, T, Nielsen, C, Sims, RC, Miller, CD, Polyhydroxybutyrate production using a wastewater microalgae based media. *Algal Res* **8**: 95-98 (2015).

154. Leite GB, Abdelaziz, AE, Hallenbeck, PC, Algal biofuels: challenges and opportunities. *Bioresour Technol* **145**: 134-141 (2013).

155. Singer SJ, Nicolson, GL, The fluid mosaic model of the structure of cell membranes. *Science* **175**: 720-731 (1972).

156. Hallenbeck PC, *Microbial technologies in advanced biofuels production*. Springer: 2012.

157. Qiao K, Abidi, SHI, Liu, H, Zhang, H, Chakraborty, S, Watson, N, Ajikumar, PK, Stephanopoulos, G, Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. *Metab Eng* **29**: 56-65 (2015).

158. Dunahay TG, Jarvis, EE, Roessler, PG, Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. *J Phycol* **31**: 1004-1012 (1995).

159. Trentacoste EM, Shrestha, RP, Smith, SR, Glé, C, Hartmann, AC, Hildebrand, M, Gerwick, WH, Metabolic engineering of lipid catabolism increases microalgal lipid

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

accumulation without compromising growth. *P Natl Acad Sci* **110**: 19748-19753 (2013).

160. Tam L-W, Lefebvre, PA, Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA insertional mutagenesis. *Genetics* **135**: 375-384 (1993).

161. Kilian O, Benemann, C, Vick, B, High-efficiency homologous recombination in the oil-producing alga, *Nannochloropsis*. Google Patents: 2011.

162. Gomma AE, Lee, S-K, Sun, SM, Yang, SH, Chung, G, Development of stable marker-free nuclear transformation strategy in the green microalga *Chlorella vulgaris*. *African Journal of Biotechnology* **14**: 2715-2723 (2015).

163. Run C, Fang, L, Fan, J, Fan, C, Luo, Y, Hu, Z, Li, Y, Stable nuclear transformation of the industrial alga *Chlorella pyrenoidosa*. *Algal Res* **17**: 196-201 (2016).

164. Roessler PG, Effects of silicon deficiency on lipid composition and metabolism in diatom *Cyclotella cryptica*. *J Phycol* **24**: 394-400 (1988).

165. Zhu S, Wang, Y, Xu, J, Shang, C, Wang, Z, Xu, J, Yuan, Z, Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella sp.* under nitrogen depletion. *Bioresour Technol* **198**: 165-171 (2015).

166. Liu Z-Y, Wang, G-C, Zhou, B-C, Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresour Technol* **99**: 4717-4722 (2008).

167. Takagi M, Yoshida, T, Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. *J Biosci Bioeng* **101**: 223-226 (2006).

168. Tornabene T, Holzer, G, Lien, S, Burris, N, Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. *Enzyme Microb Technol* **5**: 435 - 440 (1983).

2. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production

169. Eustance E, Wray, JT, Badvipour, S, Sommerfeld, MR, The effects of cultivation depth, areal density, and nutrient level on lipid accumulation of *Scenedesmus acutus* in outdoor raceway ponds. *J Appl Phycol* 1-11 (2015).
170. Leite GB, Paranjape, K, Hallenbeck, PC, Breakfast of champions: Fast lipid accumulation by cultures of *Chlorella* and *Scenedesmus* induced by xylose. *Algal Res* **16**: 338-348 (2016).

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

Abstract

The paper concerns the possibility of including the culturing of microalgae in the wastewater treatment sequence by growing them on the blackwater from biosolid dewatering to produce biomass to feed the anaerobic digesters. Two photobioreactors were used: a 12 L plexiglas column for indoor, lab-scale tests and a 85 L plexiglas column for outdoor culturing. Microalgae (*Chlorella sp* and *Scenedesmus sp*) could easily grow on the tested blackwater. The average specific growth rate in indoor and outdoor batch tests was satisfactory, ranging between 0.14-0.16 d⁻¹. During a continuous test performed under outdoor condition from May to November, and using the off-gas as the CO₂ source, the average biomass production was 50 mgTSS L⁻¹ d⁻¹. Microalgal growth was affected by environmental conditions.

Biochemical methane potential tests showed that the production of biomethane from algal biomass was slower but its final value was slightly higher than that from waste sludge (208 mLCH₄ gVS⁻¹ vs. 190 mLCH₄ gVS⁻¹).

Keywords

Microalgae; blackwater; outdoor cultivation; wastewater treatment plant

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3.1 Introduction

Microalgae are photosynthetic microorganisms with simple growing requirements that can produce lipids, proteins and carbohydrates in large amounts over short periods of time ¹. This is the chief reason why microalgal cultivation is gradually increasing worldwide. One further attracting aspect of microalgae culturing consists in the possibility of using them as a biofuel feedstock, as their biomass and oil productivity exceed those of vascular plants ². Moreover, their water quality and quantity requirements are lower than those of land crops traditionally used for producing biofuels, their culture does not involve the use of herbicides and pesticides and, of course, they don't need arable lands. Their potential for absorbing CO₂ can be exploited in the treatment of gaseous emissions, as demonstrated by Wang et al. (2008)³.

Microalgae can also be used in wastewater treatment since many species are able to utilize organic carbon (mixotrophic growth) ⁴ and inorganic nutrients ⁵ that are

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abundant in wastewaters. Moreover, the presence of microalgae may improve the oxidation of organic matter in wastewater thanks to the photosynthetic oxygenation⁶. While the idea of using microalgae in wastewater treatment dates back to 50 years ago, with large installations tested in California during decades (e.g. Green et al., 1995⁷), the circular economy concept which requires the wastewater treatment plant (WWTP) to serve as a resource recovery platform has recently rose interest in microalgal-based processes⁸. Several long-term experiences using pilot/demonstrative plants have been run in New Zealand⁹, Morocco, California¹⁰ and Spain¹¹. Results of these experiences confirm the applicability of high rate algal ponds for successful wastewater treatment¹². Although results are encouraging, microalgal-based treatments may be applied only if solar irradiance and temperature support algal growth on a year-round basis, which hampers the applicability of this process at high latitudes¹³. Moreover, those processes can be implemented only in new installations, being the treatment train quite different from the typical one.

Nonetheless, microalgal based processes can be also conceived as side-stream processes to be integrated in existing WWTPs with the aim of improving their energy or nutrient recovery capacity. In this contest, the blackwater (BW) from biosolid dewatering can serve as nutrient source, while the off-gas from the combined heat and power unit can serve as CO₂ source¹⁴.

Interesting applications are reported on the growth of microalgae on the effluents from anaerobic digesters fed on waste sludge¹⁵ and on agro-wastes¹⁶. Microalgae are able to grow on wastewaters of different origins: domestic¹⁷, industrial¹⁸ and agricultural^{19,20}. Interesting applications

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are reported for the treatment of effluents from anaerobic digesters fed on waste sludge^{21,22} and on agro-wastes²³.

The latter have been used after pretreatment including chemical precipitation²⁴, microfiltration²⁵, centrifugation^{26,27}, decanting,^{28,29} macro or micronutrient adjustment/supplementation³⁰, and dilution³¹. The reported data demonstrate that high nutrient removal rates could be achieved. The growth of microalgae on blackwater (BW) from biosolid dewatering from anaerobic digesters fed on waste sludge have been also tested at lab-scale^{14,32}. The highest net biomass yield was found in the genera of *Chlorella* and *Scenedesmus* (biomass productivity 183 and 247 mg L⁻¹ d⁻¹, respectively³³).

The harvested algal biomass is rich in nutrients and energy and can be used as for soil amendment or converted into biofuels. In the latter case, biogas production can be achieved by taking advantage of the anaerobic digesters already available in conventional WWTP. The biochemical methane production (BMP) of the algal biomass is strictly related to the anaerobic degradability of the selected algal species and, in particular, of their cell wall. Indeed, the results of BMP measurements reported in literature vary within a very wide range: 153 to 600 L CH₄ kgsv⁻¹, according to Ward et al. (2014)³. Blackwater is especially rich in ammonia nitrogen and this allows an intensive biomass growth but not a relevant lipid accumulation in the algal cells. In spite of this, some authors³⁵ found that methane production by anaerobic digestion of microalgae grown on such blackwater was comparable to biomethane production from waste activated sludge.

This paper discusses the possibility of including microalgal culturing in the wastewater treatment sequence by growing

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them on the BW from biosolid dewatering in order to produce biomass to feed the anaerobic digesters.

The microalgal growth tests have been performed indoors (lab-scale) and outdoors (pilot-scale), in order to compare microalgal growth velocity under environmental vs. controlled conditions. This allowed to evaluate the effect of environmental parameters (such as temperature and irradiance) on microalgal production and nutrient removal. In the pilot-scale test, the off-gas from the full-scale combined heat and power unit (CHP) was used as CO₂ source. Finally, we compare BMP from microalgal biomass with BMP from sewage sludge in order to assess the possibility of producing energy from the anaerobic digestion of algal biomass as such and mixed to waste sludge.

Therefore, the novelty of the present study consists in:

- (i) the use of raw BW from urban wastewater treatment plant as a substrate to grow microalgae, while the majority of literature data were obtained on pretreated (diluted and/or supplemented and/or furtherly clarified) BW samples;
- (ii) the comparison of PBR performances indoor conditions and outdoor;
- (iii) the use of a continuously fed PBR operated under natural and uncorrected environmental conditions;
- (iv) the use of the off-gasses from the CHP as the CO₂ source.

3.2 Methods

3.2.1 Wastewater treatment plant (WWTP)

The research was carried out in a municipal WWTP located in the outskirts of Milano (Bresso, Northern Italy), receiving wastewater from 220,000 inhabitant equivalents (I.E.). The water line is made of mechanical treatments, primary settling, secondary treatment by conventional activated sludge (nitrification/denitrification) followed by U.V. disinfection. The sludge line includes two anaerobic digesters operating under mesophilic conditions (35°C) followed by one post-digester. On average, the produced biogas is made of 67% CH₄, 31% CO₂ and minor or trace concentrations of other gasses. Biogas is sent to two Combined Heat and Power (CHP) units producing 220 and 320 kW_{el}, respectively. The digestate is centrifuged and a cationic polyelectrolyte is used to enhance sludge dewatering (EM516GK from SNF Italia S.p.A.).

3.2.2. *Microalgae inoculum*

A mixed microalgal community dominated by *Chlorella spp.*, acquired from EPSAG (Göttingen University, Germany), and *Scenedesmus spp.*, kindly supplied by INRA-LBE (Narbonne, France) previously adapted to grow on BW was used¹⁴.

3.2.3 *Photobioreactors for microalgal cultivation*

Two photobioreactors were used during the experimentation.

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In indoor tests, a 12 L plexiglas column ($V=12$ L, $\text{Ø}=10$ cm) served as the photobioreactor (IN_PBR). Light was provided by 6 fluorescent lamps (FLORA model from OSRAM, supplying a photosynthetic active radiation (PAR) of $230 \mu\text{mol m}^{-2} \text{s}^{-1}$), 18 W each one, with 12 h dark/light periods. At the bottom of the column, a fine bubble diffuser was available through which air, or a specific gas mixture, was flushed.

The outdoor experimentation was performed using a larger plexiglas column ($V= 85$ L, $\text{Ø}=29$ cm, OUT_PBR). The column was provided with an adjustable-flow peristaltic pump (max flow-rate of 0.1 L min^{-1}) for continuous feeding and with an overflow for continuous discharging of the microalgal suspension. The CHP off-gas was cooled and demisted and compressed at 0.12 vvm to the bottom of the plexiglas column through a fine bubbles diffuser. According to the WWTP operators, the CO_2 content in the off-gas was $10.8\pm 0.1\%$. Finally, 150-L tanks were available for feed and discharge storage.

3.2.4 Blackwater characterization

Blackwater (BW) from sludge dewatering was used as feed, as such or after dilution with the WWTP secondary effluent. The BW main characteristics are listed in **Table 3.1**. Because of the low P/N ratio, KH_2PO_4 was added to the BW to adjust the P/N ratio to 0.1 g/g. The nitrate content is likely to originate from the water used to prepare the polyelectrolyte suspension. The ammonia nitrogen content is quite low and this is due to the low percentage of total solids in the waste sludge fed to the anaerobic digester (23 gTS kg^{-1}). The low COD content suggests a well efficient

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anaerobic stabilization, while the low TSS content confirms an efficient solid/liquid step (centrifuging). Both the low ammonium and low TSS contents make this waste stream a good candidate for microalgae culturing.

Table 3.1: Chemical characterization of the BW used to grow microalgae.

Parameter	Average	Min	Max
N- NH_4^+ [mg L^{-1}]	264	41	437
N- NO_3^- [mg L^{-1}]	2.4	0.3	7.1
TSS [g L^{-1}]	0.1	0.002	1.1
COD [mg L^{-1}]	150	120	230
P-tot [mg L^{-1}]	3	0.9	10.9
Conductivity [$\mu\text{S cm}^{-1}$]	2230	1330	3480
pH	7.9	6.3	8.6

3.2.5 Experimental set-up

The experimentation included: 10 batch tests and a continuous test (160 days) HRT between 7 and 10 days.

Batch tests were carried out in parallel indoor, using the IN_PBR, and outdoor, using the OUT_PBR. Each test was started by mixing the microalgal inoculum (10% of the overall volume) with the BW as such or after dilution with the secondary effluent in order to adjust the initial ammonium concentration between 120 and 250 $\text{mg}_\text{N} \text{L}^{-1}$. Either air or the off-gas from the CHP unit were bubbled for mixing and as CO_2 supply. Tests lasted from 11 to 35 days depending on the algal growth rate. Ammonia nitrogen, pH, temperature (T), irradiance (I), conductivity, total suspended solids (TSS), and optical density (OD) at 665 nm were monitored regularly.

Relevant experimental conditions for the batch tests are summarized in **Table 3.2**.

Algal growth was assessed by following the OD data collected during the exponential growth phase. Specifically,

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the specific microalgal growth rate (μ_{\max} in d^{-1}) was calculated as the slope of the line fitting the OD data plotted in a log [OD(t)/OD(0)] versus time graph. Moreover, the specific ammonium removal rate (v_N in $\text{mgN g}_{\text{TSS}}^{-1} \text{d}^{-1}$) was assessed as the maximum ammonium removal rate referred to the TSS concentration. Finally, the length of the lag phase (t_{lag}) was also established observing the sub-maximum level of the specific growth rate, according to Shrestha et al. 2013³⁶.

Table 3.2: Relevant operational conditions for batch tests.

Test	PBR	Gas	N- NH ₄	T	pH	I
IN1	IN	Air	173	20	8.0	230
OUT1	OUT	Off	191	13	8.8	175
IN2	IN	Air	229	20	8.0	230
OUT2	OUT	Off	205	14	7.3	206
IN3	IN	Air	171	20	8.0	230
OUT3	OUT	Air	172	15	9.0	226
IN4	IN	Air	165	20	6.9	230
OUT4	OUT	Air	120	18	7.1	281
IN5	IN	Air	233	20	7.0	230
OUT5	OUT	Off	257	9	7.7	39

PBR: PhotoBioReactor (IN:indoor and OUT:outdoor); Gas: gas supplied (air or off-gas), N-NH₄: Influent N-NH₄ (mgN L^{-1}); T: Average temperature °C; pH: Average pH []; I: Average Irradiation (W m^{-2})

Finally, a *continuous test* was conducted outdoor, using the OUT_PBR. The BW was dosed continuously to maintain the HRT between 7 and 10 days. Total phosphorous, ammonium, nitrate, TSS, OD, conductivity and pH, were monitored from 1 to 3 times a week.

Daily average values for temperature and irradiance were obtained from the ARPA-Lombardia database for a meteorological station located few kilometers from the WWTP.

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Experimental data were processed as follows. Mass balances across each sampling point were set in order to assess nitrogen removal rate and microalgal production rate as both TSS and OD increase. The TSS production rate r_{TSS} ($\text{mgTSS L}^{-1} \text{d}^{-1}$) was calculated on the basis of the mass balance across the PBR:

$$\frac{[TSS]_{t_{i+1}} - [TSS]_{t_i}}{\Delta t} = \frac{[TSS]_{IN} - [TSS]_{t_i}}{HRT} + r_{TSS}$$

Were: $[TSS]_{t_i}$ and $[TSS]_{t_{i+1}}$ are the TSS concentrations (mg L^{-1}) measured at time t_i and t_{i+1} in the microalgal suspension; $[TSS]_{IN}$ is the TSS concentration in the BW fed to the microalgal column, Δt is the sampling interval (d). Similar mass balances were set to assess the rate of OD increase (r_{OD}), the ammonium removal rate (r_{NH_4}), the nitrate production rate, (r_{NO_3}).

Finally, the ammonium removal efficiency (% η_{NH_4}), total nitrogen removal efficiency (% η_{N}) and nitrification efficiency (% η_{nit}) were assessed by taking into account the ammonium loading rate (ALR) and the total nitrogen loading rate (NLR) as follows: $\eta_{\text{NH}_4} = r_{\text{NH}_4}/\text{ALR}$, $\eta_{\text{N}} = (r_{\text{NH}_4} - r_{\text{NO}_3})/\text{NLR}$ and $\eta_{\text{nit}} = r_{\text{NO}_3}/\text{ALR}$.

Free N-NH₃ (mg L^{-1}) was also computed from the total ammonia nitrogen (TAN) as:

$$\text{N-NH}_3 = \frac{1}{1 + 10^{-pH + 0.09018 + \frac{2729.92}{T(^{\circ}\text{C}) + 273.15}}} \times \text{TAN}$$

according to Anthonisen et al., (1976)³⁷.

3.2.6 Biochemical methane potential (BMP) tests

BMP tests were performed on microalgal biomass to assess their value as feedstock for anaerobic digestion. Algal biomass was produced in a semi-continuous system indoor, using the IN_PBR. Air was used for mixing and as a source of CO₂ and a mixture of BW (50%) and secondary effluent (50%) was used as nutrients source. Upon microalgal inoculation the OD was 0.483. The diluted BW was fed twice a week in order to maintain the HRT around 7-10 days. Microalgal biomass was harvested when the optical density at 665 nm of the suspension was around 2.2, after 22 days of semi-batch culturing. The microalgal biomass was harvested by centrifuging at 4000 rpm during 10 min.

The waste sludge was collected from the WWTP at the inlet to the anaerobic digesters and consisted in a mixture of primary and secondary waste sludge.

BMP tests were started within 24 h from sample collections. By using an automated volumetric system (AMTPS, Bioprocess control, Sweden) using 500 mL glass bottles. The digestate from the full-scale anaerobic digester was used as inoculum after 7 days of endogenous incubation at 35 °C to reduce non-specific biogas generation. Test bottles were performed in duplicate and a blank bottle was included. The inoculum to substrate ratio was between 0.7 and 1 g_{vs} g_{vs}⁻¹. Oligo-elements were supplied by dosing a mineral medium, as suggested by OECD (2006). N₂ bubbling for few minutes was used to deoxygenate the suspension. Bottles were maintained at 35±0.5 °C, mechanically mixed. Tests were continued until the daily methane production was less than the 1% of the

accumulated methane during a minimum of 3 days. Data processing was performed by subtracting the methane production of the blank bottle and by referring this net methane production to the amount of volatile solids of the substrate (microalgal biomass or waste sludge).

3.2.7. Analytical methods

Analyses of total nitrogen, total phosphorus, COD and total and volatile suspended solids (TSS and VSS) as well as total and volatile solids (TS and VS) were carried out according to Standard Methods³⁹, while ammonia and nitrate nitrogen were measured using spectrophotometric test kits (Hach-Lange LCK303, LCK 339, respectively) on 0.45 μm filtered samples. Temperature, pH, conductivity and dissolved oxygen were measured directly on-site by portable probes. Optical density (OD) was measured at a wavelength of 665 nm.

The lipid content in the microalgal biomass was determined gravimetrically according to Bizzotto et al (2009)⁴⁰. Prior to measurements, the microalgal suspension was centrifuged at 4000 rpm during 10 min and lyophilized. Lipids were extracted in n-hexane by an ultrasonic device.

3.2.8. Statistical analyses

All the statistical analyses were conducted using the R Project software⁴¹ and the package Hmisc⁴².

Pearson's correlation coefficients and p-values were used to explore significant linear relationships between experimental parameters, microalgal growth and N-NH₄ removal (μ_{max} , v_{N}) in batch tests. Relationships having p-values < 0.05 were deemed to be statistically significant.

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The T test for paired data was performed to detect differences between indoor (IN) and outdoor (OUT) performances in terms of μ_{\max} , V_N and t_{lag} . Also in this case p-values < 0.05 were deemed to be statistically significant.

The experimental parameters tested for the batch tests were: lag phase length (days), mean values (calculated after the lag phase) of: total nitrogen (mg L^{-1}), total phosphorus (mg L^{-1}), N-NH_4 (mg L^{-1}), OD, conductivity ($\mu\text{S cm}^{-1}$), pH, temperature ($^{\circ}\text{C}$) and irradiance (W m^{-2}).

Generalized Linear Models (GLMs) were used to evaluate the results of the continuous trial since they allow to analyze categorical and continuous variables at the same time.

The experimental parameters included in the full GLM as independent variables were: irradiance, temperature, season (spring, summer and fall), pH, conductivity, OD, TSS concentration, N-NH_4 concentration in the BW (IN) and free ammonia and OD value (for η_{NH_4} , η_{nit} , η_{N}) in the column (OUT). The effect of such parameters was tested on the following dependent variables:

- rate of OD increase (r_{OD});
- ammonia removal efficiency (η_{NH_4});
- nitrification efficiency (η_{nit});
- total nitrogen removal efficiency (η_{N}).

Different authors^{43,44} used different integration times to compute average or cumulated values for temperature and irradiance. In our study various integration times (from 3 to 7 days) were tested. So, 20 variables were obtained (Cumulative Irradiance [CIn], Average Irradiance [AIn], Cumulative Temperature [CTn], Average Temperature [ATn], with n varying between 3 and 7).

First, the integration time that better explained the variation in OD was selected for the CI, AI, CT and AT separately.

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Then, cumulative and average values corresponding to the previously selected integration time were compared for both temperature and irradiance. In both cases, the Akaike Information Criterion (AIC) was used for the model selection. After, a one-way analysis of variance was used to test for differences in cumulated temperature and in cumulated irradiance among season (spring, summer and fall) to describe the general trend.

Restricted models were generated removing variables in a backward-stepwise procedure and compared with the full GLM model using the ANOVA test. The restricted models were maintained only if they did not change substantially the estimate ($p > 0.1$) when compared with more complex models.

3. Results and discussion

3.3.1. Microalgal growth during batch tests

Figure 3.1 reports an example of the typical trend of N-NH_4 concentration and OD during indoor and outdoor batch tests. First, a lag phase is observed, due to the need of acclimation to high nutrient concentrations and culture conditions. Then, N-NH_4 concentration started decreasing while the OD increased exponentially, indicating that unlimited growth was taking place. Eventually, algal growth slowed down as soon as ammonium became limiting. One can see that the increase of OD and the decrease of N-NH_4 concentration were faster in the indoor test while the lag-phase was shorter and this was probably due to the more constant and controlled light and temperature conditions under indoor conditions.

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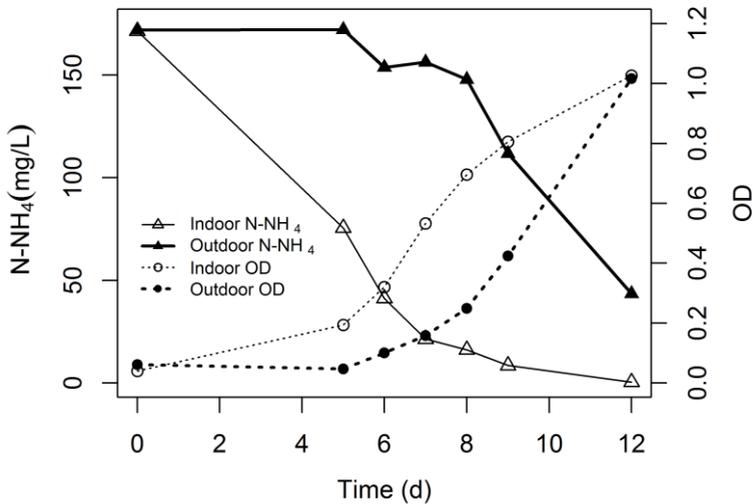


Figure 3.1: Microalgal density (expressed as absorbance at 665 nm =OD) and concentration of $N-NH_4$ during indoor and outdoor batch test 1.

The average length of the lag phase was 7 and 10 days for indoor and outdoor tests, respectively, as shown in **Figure 3.2**. However, according to the T test for paired data, t_{lag} was not significantly different between indoor and outdoor tests ($df = 4$, p -value = 0.30). For outdoor tests, the Pearson test showed that t_{lag} was negatively correlated with temperature and irradiance suggesting that high temperature and irradiance shortened the lag phase.

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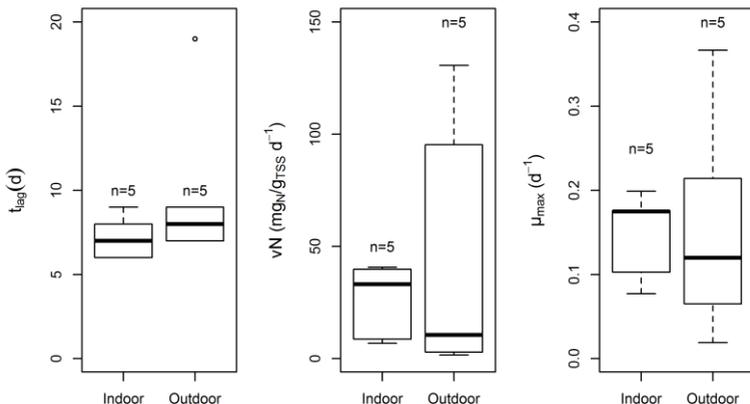


Figure 3.2: Box plot representation of the time lag (t_{lag}), the specific ammonium removal rate (vN) and the microalgal specific growth rates (μ_{max}), during batch tests indoor and outdoor. Boxes show the range between the 25th and 75th percentiles. The whiskers extend from the edge of the box to the minimum and maximum data values. The horizontal line indicates the median value.

As for the microalgal specific growth rates (μ_{max}), average μ_{max} for indoor and outdoor batch tests were $0.14 \pm 0.05 d^{-1}$ and $0.16 \pm 0.14 d^{-1}$ respectively. The T test for paired data did not suggest a significant difference in μ_{max} values ($df = 4$, p -value = 0.80) between indoor and outdoor tests. The Pearson test showed that OD, TSS concentration and pH are positively correlated with μ_{max} . Moreover, temperature was also found to be positively correlated with algal growth within the tested interval (9-20°C). On the contrary, irradiance was not significantly correlated with algal growth within the tested interval (32-352 $W m^{-2}$). It is true that the penetration of light decreases with increasing algal density: this would explain that a correlation was observed between irradiance and t_{lag} but not between irradiance and μ_{max} .

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The higher value of the standard deviation for outdoor μ_{\max} depends on the larger variability of environmental conditions in those tests.

The average values of v_N were 25 ± 16 and 48 ± 60 $\text{mg}_N \text{g}_{\text{TSS}}^{-1} \text{d}^{-1}$ indoor and outdoor, respectively. Once more, the T test for paired data showed no significant difference in v_N values ($df = 4$, $p\text{-value} = 0.49$) between indoor and outdoor tests. According to the Pearson test, OD, pH and concentrations of TSS, and N-NH_4 were all positively correlated with v_N . Moreover, a positive correlation was found between μ_{\max} and v_N being both values correlated to algal growth. Finally, the N-NH_4 removal was high and always above 90% in all the tests. Those results confirmed that microalgae were able to grow in batch on the tested BW and to remove ammonia nitrogen under the provided conditions.

No significant differences were found in algal growth rate, ammonium removal and lag phase length between outdoor and indoor tests, and this is likely due to the fact that the tested outdoor natural conditions varied around those adopted in indoor tests leading to larger standard deviations but similar average values. The obtained values compare well with literature data referred to batch tests on synthetic or real digestate from waste sludge digestion (**Table 3.3**). Generally speaking, all data on algal growth rate fall within 0 and 0.9 d^{-1} . The closer experiences are those reported by Uggetti et al. 2014³¹ and Fouilland et al., 2014²² that grew *Scenedesmus* spp. using digestate as feed; the reported interval for the specific growth rate are well in agreement with the values obtained in our experimentation. Differences may be attributed to the operational temperature (higher than in Uggetti et al., 2014³¹) or to the chemical composition of the digestate.

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Table 3.3: Summary of literature data on microalgal culturing on blackwater from biosolid dewatering.

Microalgae Strains	Nutrient Source	Culturing conditions	Growth rate μ (d^{-1})	Ref
<i>Nannochloris spp</i>	Synthetic digestate	Indoor batch T=19-35; pH=6.8-9.2 PAR = 11-360 $\mu E m^{-2} s^{-1}$	0.13-0.72	45
Mixed (<i>Scenedesmus</i> sp dominant)	Mixture of digestate and wastewater	Indoor batch T = 30 \pm 4 °C PAR = 80-90 $\mu E m^{-2} s^{-1}$ pH = 7-11	0.04-0.9	31
<i>Chlorella</i> sp. ADE5	Mixture of digestate and wastewater	Indoor batch T = 30 \pm 2 °C PAR = 200 $\mu E m^{-2} s^{-1}$ pH = 7	-	21
Mixed <i>Chlorella</i> spp. and <i>Scenedesmus obliquus</i>	Mixture of BW and secondary effluent	Indoor continuous (HRT = 10 d) I = 230 $\mu E m^{-2} s^{-1}$ T = 21 \pm 1 °C pH = 6.9-8.4	0.07	14
(a) <i>Scenedesmus</i> spp.; (b) <i>Nannochloris</i> spp.;	Digestate from secondary sludge	Indoor batch I = 261 \pm 20 $\mu E m^{-2} s^{-1}$ T = 21 °C	(a) 0-0.89 (b) 0-1.1	22
Mixed (a) <i>Chlorella</i> spp. (b) <i>Scenedesmus obliquus</i>	Synthetic medium	100 ml Erlenmeyer flask	(a) 0.75 (b) 0.76	46
<i>Scenedesmus</i> spp	Mixture swine digestate distilled water	Batch PBR Photoperiod 12:12 room temperature	0.25 \pm 0.1	47
Mixed <i>Chlorella</i> spp. and <i>Scenedesmus obliquus</i>	Mixture of BW from biosolid dewatering and secondary effluent	a) Indoor batch I = 230 $\mu E m^{-2} s^{-1}$ T = 20 \pm 1 °C pH = 8 \pm 0.5 b)Outdoor batch I=185 \pm 90 T=14 \pm 3 °C pH = 8 \pm 0.8	a) 0.07-0.2 b) 0.06-0.36	This study

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3.3.2. Microalgal growth during continuous tests

Table 3.4 summarizes the average test conditions and results of the continuous test and **Figure 3.3** shows the trend of N-NH_4 concentration and of OD during the course of the experiment.

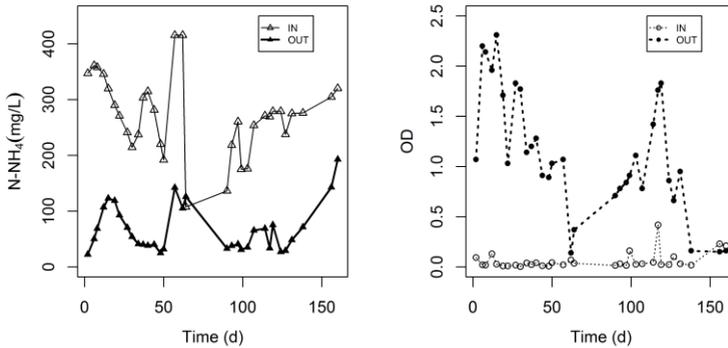


Figure 3.3: Trend of $\text{NH}_4\text{-N}$ concentration and optical density in the inlet (IN) and outlet (OUT) of the PBR during outdoor continuous test.

The difference between N-NH_4 concentration in the influent and in the effluent demonstrates a significant removal, and the increase of cell counts and OD values allows to quantify the algal growth during the trial. One only exception was observed around day 60 when a low-strength BW was collected from the WWTP whose ammonium content was even lower than that in the PBR.

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Table 3.4: Summary of different parameters studied during continuous outdoor test IN = in the fed BW and OUT= in the effluent stream.

Parameters	Average± Standard deviation
Cumulative Temperature °C	20.4±17.4
Cumulative Irradiance (W m ⁻²)	198±292
pH _{IN}	7.98±0.46
pH _{OUT}	7.22±0.99
Conductivity (µS cm ⁻¹) _{IN}	2,180±718
Conductivity (µS cm ⁻¹) _{OUT}	1,590±300
N-NH ₄ (mg L ⁻¹) _{IN}	272±70
N-NH ₄ (mg L ⁻¹) _{OUT}	68±43
N-NH ₃ (mg L ⁻¹) _{OUT}	4.2±9.2
N-NO ₃ (mg L ⁻¹) _{IN}	2.23±2.33
N-NO ₃ (mg L ⁻¹) _{OUT}	52±34
OD _{IN}	0.06±0.08
OD _{OUT}	1.12±0.61
<i>Chlorella sp</i> density (cells mL ⁻¹) _{OUT}	4.71E+05±1.97E+05
<i>Scenedesmus sp</i> density (cells mL ⁻¹) _{OUT}	6.20E+04±7.38E+04
TSS (mg L ⁻¹) _{IN}	67±133
TSS (mg L ⁻¹) _{OUT}	582±244
Biomass productivity (TSS mg L ⁻¹ d ⁻¹)	50±40

The biomass productivity, expressed as TSS mg L⁻¹ d⁻¹, is comparable with literature data, 23-40 mgTSS L⁻¹ d⁻¹ 48,49.

Generalized Linear Model results confirmed that CI₄ and CT₄ were able to better explain the variation in OD, suggesting that the 4-days cumulative temperature and Irradiance display the strongest correlation with algal growth if compared to average values and to cumulative values over shorter (3-days) or longer (5-7-days) integration intervals. Season had a significant main effect both on Cumulative Temperature (F_{2,30} = 5.99, p < 0.01) and on Cumulative irradiance (F_{2,30} = 7.91, p < 0.01). During fall, both Cumulative Temperature and Cumulative Irradiance were lower than in spring and in summer.

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Statistical analysis using GLMs were also used to understand the effect of environmental conditions and BW composition on microalgae growth and nitrogen removal in the continuous test.

The correlation between phosphorus removal and microalgal growth was weak, probably because phosphorus was added to adjust P/N ratio, so we did not include it in the statistical analyses.

According to the results summarized in **Table 3.5**, microalgal growth rate (r_{OD}) is negatively correlated to the optical density in the influent (IN) and to the concentration of free ammonia in the column, as determined in the effluent (OUT). The latter is inhibiting, while the former reduces light penetration within the columns. The microalgal growth rate increases with increasing temperatures. No effect on the microalgal growth was observed for pH, conductivity and influent ammonium concentration. Microalgal growth rate appears to be affected by the season, being fall the less favorable and spring the most favorable seasons, as also shown in **Figure 3.4**.

According to data in **Table 3.5**, nitrification efficiency (η_{nit}) was positively affected by temperature, confirming that nitrification is faster at higher temperatures. Moreover, nitrification appears to decrease with increasing optical density and microalgal growth rate (data not shown). Both evidences suggest that microalgae and nitrifying bacteria may compete for common substrates, such as P or CO₂, and/or that environmental conditions that favor microalgal growth are unfavorable to nitrifying bacteria. The effluent COD was not monitored, therefore the relevance of heterotrophic bacteria respiration could not be quantified. However, heterotrophic bacteria are expected to be present and to play a role in the complex relationships among the

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

various groups of organisms in the column as suggested in previous works^{17,50,51}.

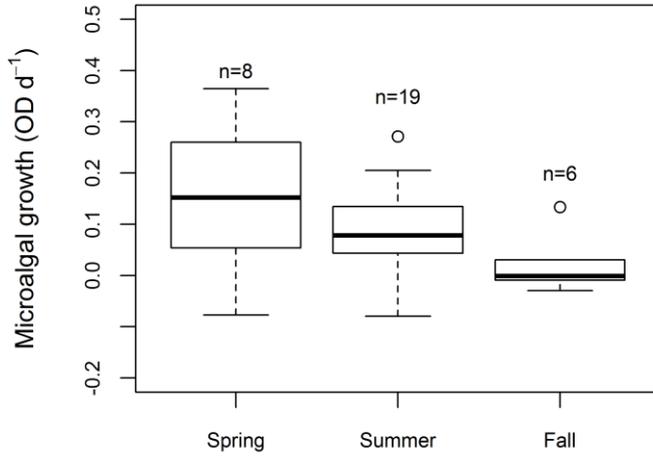


Figure 3.4: Boxplots representation the microalgae production in different seasons (spring, summer and fall). Boxes show the range between the 25th and 75th percentiles. The whiskers extend from the edge of the box to the minimum and maximum data values. The horizontal line indicates the median value.

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Table 3.5: Estimate of the statistical analysis on data from the continuous experimentation. Season is a three levels categorical variable (spring, summer and fall).

Dependent variables	Independent variables			
	r_{OD} (OD (d ⁻¹))	Γ_{nit} %	Γ_N %	Γ_{NH_4} %
OD _{IN}	-183.661***			
OD _{OUT}	N.D.	-0.149*	0.207***	
N- NH ₄ IN			0.006**	0.405***
N-NH ₃ OUT	-2.791***		-0.036***	-1.849**
Conductivity _{IN}			-0.001***	-0.080***
Irradiance			0.001*	
Temperature	1.410**	0.004'	-0.006*	0.506'
Season fall	-62.523*			92.868*
Season spring	38.400*			87.046*
Season summer	-13.684*			191.886*
pH			0.144*	

Significance levels (code) referred to ANOVA test: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), $p < 0.1$ ('). N.D. not considered

The nitrogen removal efficiency (η_N) is the result of the overall effect of algal growth and ammonia stripping, both processes contributing to the total nitrogen removal. Apparently, nitrogen removal is fostered by high optical density in the column (that corresponds to high levels of microalgal cells), by intense irradiance, by high level of pH, by high ammonium concentration and by low conductivity which, in its turn, does not play a significant role on microalgal growth. This would suggest that ammonium and optical density positively affect ammonia stripping while conductivity reduces the stripping efficiency since high salinity reduces the efficiency of liquid/gas transfer⁵². As for free ammonia, high concentrations were previously found to negatively affect algal growth, while they are expected to foster ammonia stripping, thus leading to an overall mild negative impact on the total nitrogen removal.

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Finally, in the last column of **Table 3.5**, the coefficient of the effect on the ammonium removal efficiency (η_{NH_4}) is summarized. Ammonium removal depends on all the above listed processes (microalgal growth, stripping and nitrification). As expected, the coefficient is negative for both free ammonia and conductivity, due to their negative effect on algal growth and conductivity. The effect of irradiance, that is strong on algal growth, is not relevant on the overall ammonium removal since it does not affect stripping nor nitrification, while the coefficient is positive for temperature, season and ammonium in the influent. This suggests that ammonium removal rate increases with increasing ammonium concentration in the influent. No effect is observed for optical density that has contrasting effects on the three above listed processes contributing to ammonium removal.

Figure 3.5 shows η_{nit} , $\eta_{\text{N-NH}_4}$ and η_{N} during continuous test (22 samples for each independent variable were considered); nitrification gave the lowest efficiency, while the ammonium removal efficiency, which depends on microalgal growth, stripping and nitrification processes, gave the highest.

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

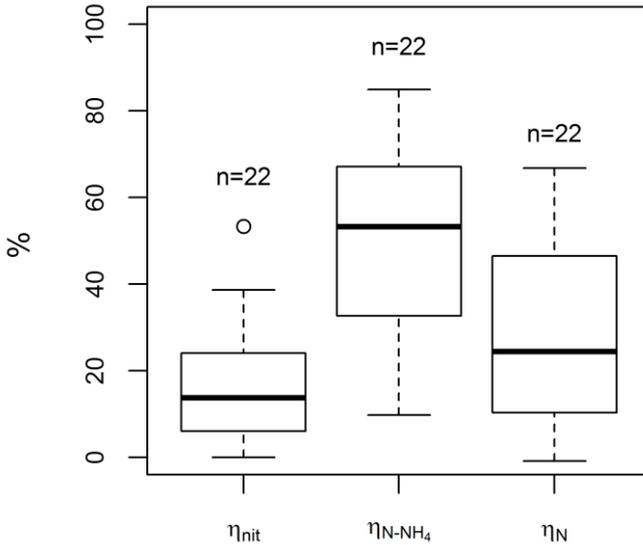


Figure 3.5: Boxplot representation of % nitrification efficiency (η_{nit}), % ammonium removal efficiency (η_{NH_4}), % nitrogen removal efficiency (η_N) during continuous phase. Boxes show the range between the 25th and 75th percentiles. The whiskers extend from the edge of the box to the minimum and maximum data values. The horizontal line indicates the median value.

3.3. Microalgal lipid content

The lipid content of the microalgal suspension was determined in samples collected from the semi continuous test. The microalgae lipid content was between 1 and 2.4 % of total microalgae dry weight, quite low if compared to the values reported in literature. Sialve et al., (2009)⁵⁴ indicated a range of 7–23% into microalgae grown on synthetic medium. On the other hand, many factors affect the accumulation of lipids in algal biomass and the pattern of the fatty acids inside the algal cells. As demonstrated by Makulla (2000)⁵⁵, the growth rate (which, in turns, depends on a number of factors) and the nutrient availability are both important and their respective roles have practically never been distinguished at experimental level.

High lipid cell content is usually found in cells grown under stress conditions, typically under nutrient limitation, which are often associated with low biomass productivity⁵. In the present experimentation, N-NH₄ concentration in the feed was always over 100 mg L⁻¹ and algal productivity was quite high, so none of the cited conditions for high lipid cell concentration occurred, possibly explaining the low lipid content in algal biomass. On the other hand, the total lipid content could have been underestimated by the hexane extraction. There is still uncertainty about the best extractant for algal lipids and Prommuak et al. (2013)⁵⁶ for instance, report that the best one is a mixture of chloroform and methanol at the ratio of 2:1 (v/v). In spite of the low lipid content, the results of BMP tests were satisfactory, as shown below.

3.3.4 BMP tests

BMP tests were conducted on three samples:

- (i) algae collected at the end of the semi continuous operation,
- (ii) waste sludge (mixture of primary and secondary sludge, WS) from the full scale WWTP
- (iii) a mixture of algal biomass and waste sludge (32% and 68% w/w based of the volatile solid content, respectively).

The trend of the average value of methane production on duplicates is reported in **Figure 3.6** (the average distance was always lower than 5%). The methane production is faster when waste sludge is degraded with 50 % and 90% of the ultimate methane production achieved after 4 and 11 days, respectively. The conversion of microalgae biomass into methane follows a slower kinetics with 50% and 90% of the final production achieved after 24 and 41 days, respectively. However, the ultimate specific methane production is slightly higher (+9.5%) for microalgae ($208 \text{ mL}_{\text{CH}_4} \text{ gVS}^{-1}$) than for waste sludge ($190 \text{ mL}_{\text{CH}_4} \text{ gVS}^{-1}$). By knowing the ultimate specific methane production of microalgae and waste sludge, the expected methane production from a mixture of them was computed by the linear composition of each contribution according to their percentage in the mixture. This expected methane production compares well with the measured one. Apparently, no significant synergistic nor antagonistic effects are to be expected by co-digesting microalgae in the existing anaerobic digester designed to treat waste sludge.

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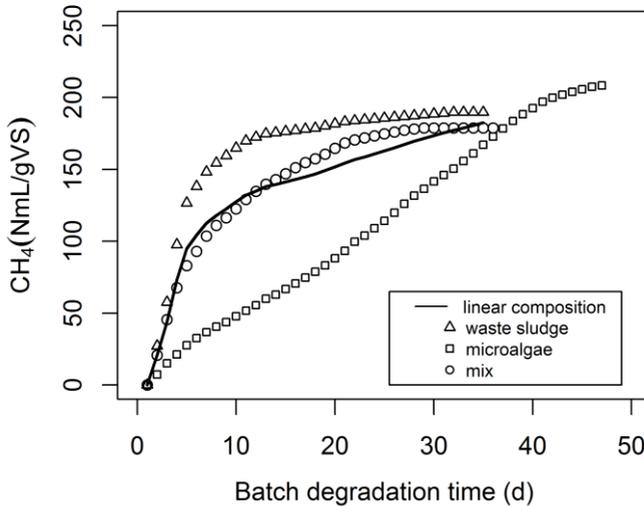


Figure 3.6: Results of BMP tests on microalgal biomass, waste sludge and a mixture of microalgal sludge and waste sludge (WS) (32% and 68% w/w respectively). The solid line corresponds to the expected methane production by assuming the additivity of WS and microalgae. Average distance between replicate was below 5% and is not reported on each data-set to improve readability.

The methane potential of the microalgal biomass remains within the broad range reported by Ward et al. (2014)³⁴ which, upon reviewing literature data indicated a range of 153-600 mL_{CH₄} g_{VS}⁻¹. Indeed, data from this experimentation are within the low range and this is in agreement with the low lipid content of the algal biomass. Indeed, the algal BMP is expected to be higher for those microalgae that are naturally richer in lipid or that are grown under environmental conditions that can induce lipid production (^{57,58} among others).

The slow degradation of the microalgal biomass was previously ascribed to the time required to hydrolyze the

microalgal cell wall which can resist anaerobic degradation even after prolonged exposure to the anaerobic microbial community, thus preventing their degradation⁵⁹. Indeed, both *Chlorella spp.* and *Scenedesmus spp.* have a cellulose or hemicellulose-based cell membrane which has to be first hydrolyzed by endoglucanase, esoglucanase and xylanase. Those enzymes are normally produced by the anaerobic community⁶⁰, however pretreatments can be used to speed-up the process³⁴. The combination of Anaerobic Membrane Bioreactor (AnMBR) and microalgae-based technologies can be considered an interesting approach for recovering nutrients and energy from sewage whilst reducing carbon foot- print, as demonstred by Viruela et al (2016)⁶¹.

3.4 Conclusions

The main outcome of this experimentation is the pilot-scale demonstration that microalgae can grow on BW from biosolid dewatering in natural, uncontrolled conditions, within the tested range of environmental parameters (Northern Italy from May to November). The tested blackwater had a quite high N-NH₄ concentrations (120 to 257 mg L⁻¹ in batch tests and 272 ± 70 mgL⁻¹ in the continuous test) and the average microalgae growth rate in indoor and outdoor batch tests ranged between 0.14-0.16 d⁻¹. The average algal biomass production during the outdoor continuous cultivation was 50 mgTSS L⁻¹ d⁻¹ and the harvested algal biomass had a biochemical methane potential slightly higher than that of the waste sludge typically fed to the anaerobic digester of the WWTP (208 mLCH₄ gVS⁻¹ vs 190 mLCH₄ gVS⁻¹). Statistical analyses confirmed to be affected by environmental conditions

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

(temperature and season) and to be negatively correlated with the occurrence of nitrification.

These results suggests that microalgal cultivation can be integrated into existing WWTP implementing the anaerobic digestion of waste sludge allowing to foster biogas production thus improving the energy balance of the WWTP.

3.5 Acknowledgments

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3.6 References

1. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**, 294–306 (2007).
2. Nielsen, S. L., Enríquez, S., Duarte, C. M. & Sand-Jensen, K. Scaling maximum growth rates across photosynthetic organisms. *Funct. Ecol.* **10**, 167–175 (1996).
3. Wang, B., Li, Y., Wu, N. & Lan, C. Q. CO₂ bio-mitigation using microalgae. *Appl. Microbiol. Biotechnol.* **79**, 707–718 (2008).
4. Skorupskaitė, V., Makarevičienė, V. & Levisauskas, D. Optimization of mixotrophic cultivation of microalgae *Chlorella sp.* for biofuel production using response surface methodology. *Algal Res.* **7**, 45–50 (2015).
5. Xin, L., Hu, H., Ke, G. & Sun, Y. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus sp.* *Bioresour. Technol.* **101**, 5494–5500 (2010).
6. Muñoz, R. & Guieysse, B. Algal-bacterial processes for the treatment of hazardous contaminants: a review. *Water Res.* **40**, 2799–815 (2006).
7. Green, F. B., Lundquist, J. T. & Oswald, W. J. Energetics of advanced integrated wastewater pond systems. *Water Sci. Technol.* **31**, 9–20 (1995).
8. Bahr, M., Díaz, I., Dominguez, A., González Sánchez, A. & Muñoz, R. Microalgal-biotechnology as a platform for an integral biogas upgrading and nutrient removal from anaerobic effluents. *Environ. Sci. Technol.* **48**, 573–581 (2014).
9. Craggs, R., Sutherland, D. & Campbell, H. Hectare-scale demonstration of high rate algal ponds for

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

- enhanced wastewater treatment and biofuel production. *J. Appl. Phycol.* **24**, 329–337 (2012).
10. Borowitzka, M. A. & Moheimani, N. R. *Algae for Biofuels and Energy*. (Springer Netherlands, 2013). doi:10.1007/978-94-007-5479-9
 11. Acién, F. G., Fernández, J. M. & Molina-Grima, E. Economics of Microalgae Biomass Production. *Biofuels from Algae* 313–325 (2013).
 12. Lourie, E., Patil, V. & Gjengedal, E. Efficient purification of heavy-metal-contaminated water by microalgae-activated pine bark. *Water, Air, Soil Pollut.* **210**, 493–500 (2010).
 13. Slegers, P. M., Lösing, M. B., Wijffels, R. H., van Straten, G. & van Boxtel, a. J. B. Scenario evaluation of open pond microalgae production. *Algal Res.* **2**, 358–368 (2013).
 14. Ficara, E., Uslenghi, A., Basilico, D. & Mezzanotte, V. Growth of microalgal biomass on supernatant from biosolid dewatering. *Water Sci. Technol.* **69**, 896–902 (2014).
 15. Ge, S., Champagne, P., Plaxton, W. C., Leite, G. B. & Marazzi, F. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production. *Biofuels, Bioprod. Biorefining* (2016). doi:10.1002/bbb
 16. Monlau, F., Sambusiti, C., Ficara, E., Aboulkas, a., Barakat, a. & Carrère, H. New opportunities for agricultural digestate valorization: current situation and perspectives. *Energy Environ. Sci.* 2600–2621 (2015). doi:10.1039/C5EE01633A
 17. Arcila, J. S. & Buitrón, G. Microalgae-bacteria aggregates: Effect of the hydraulic retention time on the municipal wastewater treatment, biomass

- settleability and methane potential. *J. Chem. Technol. Biotechnol.* **91**, 2862–2870 (2016).
18. De-Bashan, L. E. & Bashan, Y. Immobilized microalgae for removing pollutants: review of practical aspects. *Bioresour. Technol.* **101**, 1611–1627 (2010).
19. González, C., Marciniak, J., Villaverde, S., García-Encina, P. a & Muñoz, R. Microalgae-based processes for the biodegradation of pretreated piggery wastewaters. *Appl. Microbiol. Biotechnol.* **80**, 891–8 (2008).
20. Posadas, E., Bochon, S., Coca, M., García-González, M. C., García-Encina, P. a. & Muñoz, R. Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability. *J. Appl. Phycol.* (2014). doi:10.1007/s10811-014-0263-0
21. Cho, S., Lee, N., Park, S., Yu, J., Luong, T. T., Oh, Y.-K. & Lee, T. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. *Bioresour. Technol.* **131**, 515–520 (2013).
22. Fouilland, E., Vasseur, C., Leboulanger, C., Le Floc’h, E., Carré, C., Marty, B., Steyer, J.-P. & Sialve, B. Coupling algal biomass production and anaerobic digestion: Production assessment of some native temperate and tropical microalgae. *Biomass and Bioenergy* **70**, 564–569 (2014).
23. Gonzales, L. E., Canizares, R, Baena, S. Efficiency of ammonia and phosphorus removal from a colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. **60**, 259–262 (1997).
24. Chen, R., Li, R., Deitz, L., Liu, Y., Stevenson, R. J.

- & Liao, W. Freshwater algal cultivation with animal waste for nutrient removal and biomass production. *Biomass and Bioenergy* **39**, 128–138 (2012).
25. Bchir, F. S., Gannoun, H., Herry, S. El & Hamdi, M. Optimization of *Spongiochloris sp.* biomass production in the abattoir digestate. *Bioresour. Technol.* **102**, 3869–3876 (2011).
26. Franchino, M., Comino, E., Bona, F. & Riggio, V. a. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. *Chemosphere* **92**, 738–744 (2013).
27. Marcilhac, C., Sialve, B., Pourcher, A.-M., Ziebal, C., Bernet, N. & Béline, F. Control of nitrogen behaviour by phosphate concentration during microalgal-bacterial cultivation using digestate. *Bioresour. Technol.* **175**, 224–230 (2015).
28. Olguín, E. J., Hernández, B., Araus, a., Camacho, R., González, R., Ramírez, M. E., Galicia, S. & Mercado, G. Simultaneous high-biomass protein production and nutrient removal using *Spirulina maxima* in sea water supplemented with anaerobic effluents. *World J. Microbiol. Biotechnol.* **10**, 576–578 (1994).
29. Blier, R., Lalibert, G. & Notie, J. De. Tertiary treatment of cheese factory anaerobic effluent with *Phormidium bohneri* and *Micractinum pusillum*. **52**, 151–155 (1995).
30. Marcilhac, C., Sialve, B., Pourcher, A. M., Ziebal, C., Bernet, N. & Béline, F. Digestate color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem. *Water Res.* **64**, 278–287 (2014).
31. Uggetti, E., Sialve, B., Latrille, E. & Steyer, J. P.

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

- Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. *Bioresour. Technol.* **152**, 437–443 (2014).
32. Ge, S. & Champagne, P. Nutrient removal, microalgal biomass growth, harvesting and lipid yield in response to centrate wastewater loadings. *Water Res.* **88**, 604–612 (2016).
33. Quiroz, C. E., Peebles, C. & Bradley, T. H. Scalability of combining microalgae-based biofuels with wastewater facilities: A review. *Algal* **9**, 160–169 (2015).
34. Ward, a. J., Lewis, D. M. & Green, F. B. Anaerobic digestion of algae biomass: A review. *Algal Res.* **5**, 204–214 (2014).
35. Wang, M. & Park, C. Investigation of anaerobic digestion of *Chlorella sp.* and *Micractinium sp.* grown in high-nitrogen wastewater and their co-digestion with waste activated sludge. *Biomass and Bioenergy* **80**, 30–37 (2015).
36. Shrestha, R. P., Haerizadeh, F. & Hildebrand, M. *Handbook of Microalgal Culture. Handb. Microalgal Cult. Appl. Phycol. Biotechnol.* (2013). doi:10.1002/9781118567166
37. Anthonisen, A. C., Srinath, E. G., Loehr, R. C. & Prakasam, T. B. S. Inhibition of nitrification and nitrous acid compounds. *Water Environ. Fed.* **48**, 835–852 (1976).
38. OECD. Test n 311. Anaerobic Biodegradability of Organic Compounds in Digested Sludge by Measurement of Gas Production. (2006).
39. APHA. *Stand. Methods Exam. Water Wastewater – 21th Ed. Am. Public Heal. Assoc. Washingt. DC,*

- USA (2005).
40. Bizzotto, E. C., Villa, S. & Vighi, M. POP bioaccumulation in macroinvertebrates of alpine freshwater systems. *Environ. Pollut.* **157**, 3192–3198 (2009).
 41. R core Team. R: A Language and Environment for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org>. (2015).
 42. Harrel, F. & Dupont, C. Hmisc: Harrell Miscellaneous. R package version 3.17-1. <http://CRAN.R-project.org/package=Hmisc>. (2015).
 43. González-Fernández, C., Molinuevo-Salces, B. & García-González, M. C. Nitrogen transformations under different conditions in open ponds by means of microalgae-bacteria consortium treating pig slurry. *Bioresour. Technol.* **102**, 960–966 (2011).
 44. Fernández, I., Acién, F. G., Berenguel, M. & Guzmán, J. L. First principles model of a tubular photobioreactor for microalgal production. *Ind. Eng. Chem. Res.* **53**, 11121–11136 (2014).
 45. Vasseur, C., Bougaran, G., Garnier, M., Hamelin, J., Leboulanger, C., Chevanton, M. Le, Mostajir, B., Sialve, B., Steyer, J. P. & Fouilland, E. Carbon conversion efficiency and population dynamics of a marine algae-bacteria consortium growing on simplified synthetic digestate: First step in a bioprocess coupling algal production and anaerobic digestion. *Bioresour. Technol.* **119**, 79–87 (2012).
 46. Roberts, K. P., Heaven, S. & Banks, C. J. Comparative testing of energy yields from microalgal biomass cultures processed via anaerobic digestion. *Renew. Energy* **87**, 744–753 (2016).
 47. Prandini, J. M., da Silva, M. L. B., Mezzari, M. P.,

3. Factors Affecting the Growth of Microalgae on Blackwater from Biosolid Dewatering

- Pirolli, M., Michelon, W. & Soares, H. M. Enhancement of nutrient removal from swine wastewater digestate coupled to biogas purification by microalgae *Scenedesmus spp.* *Bioresour. Technol.* **202**, 67–75 (2016).
48. Pittman, J. K., Dean, A. P. & Osundeko, O. The potential of sustainable algal biofuel production using wastewater resources. *Bioresour. Technol.* **102**, 17–25 (2011).
49. Chinnasamy, S., Bhatnagar, A., Hunt, R. W. & Das, K. C. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresour. Technol.* **101**, 3097–3105 (2010).
50. Osundeko, O. & Pittman, J. K. Implications of sludge liquor addition for wastewater-based open pond cultivation of microalgae for biofuel generation and pollutant remediation. *Bioresour. Technol.* **152**, 355–363 (2014).
51. Park, J. B. K., Craggs, R. J. & Shilton, a N. Wastewater treatment high rate algal ponds for biofuel production. *Bioresour. Technol.* **102**, 35–42 (2011).
52. Weiland, R. & Hatcher, N. Stripping sour water: the effect of heat stable salts. *Pet. Technol. Q.* **17**, 105–109 (2012).
53. Sialve, B., Bernet, N. & Bernard, O. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. *Biotechnol. Adv.* **27**, 409–16 (2009).
54. Sialve, B., Bernet, N. & Bernard, O. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. *Biotechnol. Adv.* **27**,

- 409–416 (2009).
55. Makulla, A. Fatty acid composition of *Scenedesmus obliquus*: Correlation to dilution rates. *Limnol. - Ecol. Manag. Int. Waters* **30**, 162–168 (2000).
56. Prommuak, C., Pavasan, S., Quitain, A. T., Goto, M. & Shotipruk, A. Simultaneous Production of Biodiesel and Free Lutein from *Chlorella vulgaris*. *Chem. Eng. Technol.* **36**, 733–739 (2013).
57. Li, Y., Horsman, M., Wang, B., Wu, N. & Lan, C. Q. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Appl. Microbiol. Biotechnol.* **81**, 629–936 (2008).
58. Mooij, P. R., Stouten, G. R., Tamis, J., van Loosdrecht, M. C. M. & Kleerebezem, R. Survival of the fattest. *Energy Environ. Sci.* **6**, 3404 (2013).
59. Mussgnug, J. H., Klassen, V., Schlüter, A. & Kruse, O. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J. Biotechnol.* **150**, 51–56 (2010).
60. Sambusiti, C., Rollini, M., Ficara, E., Musatti, a., Manzoni, M. & Malpei, F. Enzymatic and metabolic activities of four anaerobic sludges and their impact on methane production from ensiled sorghum forage. *Bioresour. Technol.* **155**, 122–128 (2014).
61. Viruela, A., Murgui, M., Gomez-Gil, T., Duran, F., Robles, A., Ruano, M. V., Ferrer, J. & Seco, A. Water resource recovery by means of microalgae cultivation in outdoor photobioreactors using the effluent from an anaerobic membrane bioreactor fed with pre-treated sewage. *Bioresour. Technol.* **218**, 447–454 (2016).

4. Microalgae to remove nitrogen from agro-digestates: the MICROGATE project

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4.1 Introduction

The biogas technology in Lombardy has spread remarkably in the last years, so that the region accounts for approximately one third of the national biogas production. Anaerobic digestion has ensured an improved environmental sustainability of animal breeding but has not solved the issue of excessive nitrogen (N) discharge into this nitrate sensitive area. To reduce the N load into the fields, farms are forced to use expensive technical solutions, thus increasing production costs¹. This motivates the impellent need of searching environmentally and economically sound technologies to reduce the N load in agricultural wastewaters. Microalgae are

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fast growing photosynthetic organisms that require few simple elements such as light, carbon dioxide and nutrients (primarily N and P) that are largely present in the livestock wastes ². The oxygen produced during the photosynthetic process results in the oxidation of organic pollutants. Heavy metals may be also be uptaken or co-precipitated ³. Microalgal-based processes are technically simple and have a low energy request since they exploit solar energy. Moreover, they allows for the valorisation of waste heat streams and CO₂ that are available in biogas plants, resulting in a convenient process integration and in the reduction of the carbon footprint of the agricultural sector ⁴.

In this context, the Microgate (Use of **MICRO**algae to miti**GATE** nitrogen pollution from agricultural wastewaters) project intents to validate the feasibility to use microalgae to remove ammoniacal nitrogen from the liquid phase of digestates from animal manure anaerobic digestion. Currently, initial results are available on the characterization of selected waste stream and on the semicontinuous culturing of microalgae at lab scale on one of those streams.

4.2 Material and methods

4.2.1 Wastewater characterisation and analytical methods

The characterization of the following wastewaters was performed:

- W1: swine manure, from a farm breeding 20,000 pigs collected before and after the primary treatment by rotating sieve and dissolved air flotation;
- W2: digestate from a full scale digester fed on the solid fraction of swine manure, maize silage, poultry manure and cheese whey, more details in Scaglione et al (2015)

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⁵. Digestate was collected before and after solid/liquid separation by centrifugation;

- W3: digestate from a full scale digester fed on swine and dairy manure, maize silage, and minor contributions of poultry manure and cheese whey.

Digestate was collected before and after solid/ liquid separation by screw-press.

Three sampling were performed in May-July 2015. Samples were characterized for relevant chemical parameters: solid content (TS and VS), COD, N-NH₄, N-NO₃, P-PO₄, turbidity and pH according to Standard Methods ⁶.

4.2.2 *Microalgae culturing at lab-scale*

The liquid fraction of W2 was used. Semi-continuous culturing tests were performed in triplicate in 150 mL glass vials, initially prepared by mixing the microalgal inoculum, with 15 ml of W2 and tap water. A mixed microalgal community dominated by *Chlorella* spp. and *Scenedesmus* spp. previously adapted to grow on digestate was used. Light was provided by 6 fluorescent lamps (FLORA model from OSRAM), 18 W each one, with 12 h dark/light periods. Air was flushed at the bottom of each vial through a fine bubble diffuser. Every 1 or 2 weeks, a fraction (10 out of 150 mL) of the algal suspension was withdrawn and substituted by an equivalent volume of W2. The frequency of feeding was adjusted in order to achieve an almost complete depletion of the ammonium concentration to avoid too high ammonium concentration (below 160 mgN L⁻¹) after each feeding event. The concentration of N forms was constantly monitored and correlated to the microalgal concentration, which was measured spectrophotometrically (optical density at 680 nm).

4.2.3 Results and discussion

The main results of the wastewater characterization are reported in **Table 4.1**. In some cases, the values of total and soluble COD seem inconsistent (soluble slightly higher than total) but this depends on the non homogeneous presence of solids in the sample aliquots used for the analyses of total COD. Air flotation and centrifugation allowed for significant reductions in the solid content and total COD. Nutrient concentrations were less affected, with some P reduction likely due to precipitation. In view of using those wastewaters to grow microalgae, the liquid fraction of W1 has a suitable turbidity and solid content, acceptable ammonium concentration and N/P ratio; the COD/N ratio is quite high suggesting that heterotrophic bacteria are expected to grow as well. The W2 liquid fraction has the lowest turbidity value and low COD/N; however, the high ammonium content and high N/P value are potential issues. On the contrary, W3 has unacceptable levels of both solid content and turbidity which would seriously limit light penetration. Therefore, the liquid fraction (L) of W1 and W2 were selected as candidates for algal culturing tests.

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Table 4.1. Wastewater characteristics of raw samples (TQ) or liquid fractions after solid/liquid separation (L).

Parameter	Samples					
	W1 TQ	W1 L	W2 TQ	W2 L	W3 TQ	W3 L
TS (g L ⁻¹)	5±1	2±0.3	67±7	9±2	60±2	53±0.7
TS (g L ⁻¹)	5±1	2±0.3	67±7	9±2	60±2	53±0.7
VS (g L ⁻¹)	3±0.6	0.74±0.1	47±5	4±3	38±1.5	32±0.4
pH	7.4±0.2	7.3±0.08	7.7±0	7.4±1	7.8±0.06	8.2±0.08
N-NH ₄ (mg L ⁻¹)	200±110	200±80	220±400	960±400	2200±100	2200±200
P-PO ₄ (mg L ⁻¹)	28±15	17±7	100±47	34±11	178±109	234±70
Turbidity (FAU)	194±90	202±23	9,600±7,500	1,600±320	2,800±2,000	3,500±2,200
S-COD (g L ⁻¹)	1.3±0.2	1±0.1	4.7±0.7	7.2±7.5	22.4±31.0	5.0±0.18
T-COD (g L ⁻¹)	4.6±0.6	0.95±0.11	65±11	6.2±4	55.6±4.6	40±2.2
N/P	13±4	17±7	23±14	32±24	26±23	11±5

*S-COD soluble COD, T-COD total COD

Results of the first semi-continuous tests of algal growth on W2 L with a concentration of 1730 mgNH₄-N L⁻¹ are reported in **Figure 4.1-4.2**. Ammonium was efficiently (>97%) removed and the microalgal concentration increased over time up to 3-4 times the initial value, above which light penetration was probably insufficient to sustain further algal growth. However, long average hydraulic retention times (above 80 d) were requested due to the high ammonium concentration in the wastewater.

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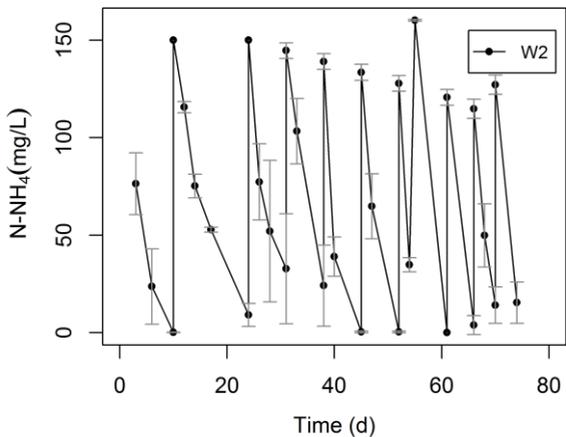


Fig. 4.1. Results of the semi-continuous growth test on W2: ammonium concentration. All data are mean \pm standard error ($n = 3$).

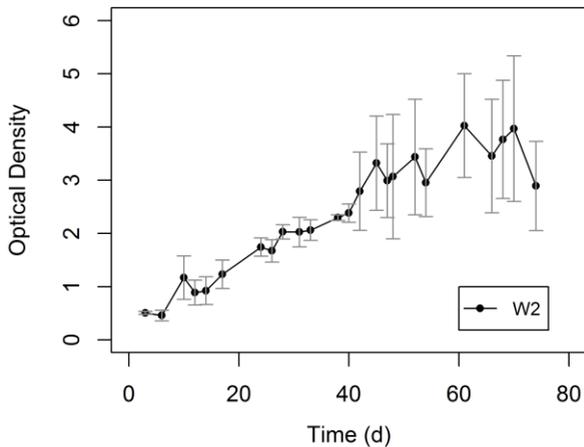


Fig. 4.2. Results of the semi-continuous growth test on W2: Optical Density. All data are mean \pm standard error ($n = 3$).

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4.3 Conclusions

The encouraging lab scale results obtained justify a pilot-scale continuous test with a 800L race-way pond treating W2 L has been just started up in a farm and will last for around 6 months (May 2016-October 2016).

4.4 Acnowledgments

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4.5 References

1. Posadas, E., García-Encina, P. A., Soltau, A., Domínguez, A., Díaz, I. & Muñoz, R. Carbon and nutrient removal from concentrates and domestic wastewater using algal-bacterial biofilm bioreactors. *Bioresour. Technol.* **139**, 50–58 (2013).
2. Wang, M. & Park, C. Investigation of anaerobic digestion of *Chlorella* sp. and *Micractinium* sp. grown in high-nitrogen wastewater and their co-digestion with waste activated sludge. *Biomass and Bioenergy* **80**, 30–37 (2015).
3. Lourie, E., Patil, V. & Gjengedal, E. Efficient purification of heavy-metal-contaminated water by microalgae-activated pine bark. *Water, Air, Soil Pollut.* **210**, 493–500 (2010).
4. Menger-Krug, E., Niederste-Hollenberg, J., Hillenbrand, T. & Hiessl, H. Integration of microalgae systems at municipal wastewater treatment plants: Implications for energy and emission balances. *Environ. Sci. Technol.* **46**, 11505–11514 (2012).
5. Scaglione, D., Ficara, E., Corbellini, V., Tornotti, G., Teli, A., Canziani, R. & Malpei, F. Autotrophic nitrogen removal by a two-step SBR process applied to mixed agro-digestate. *Bioresour. Technol.* **176**, 98–105 (2015).
6. APHA. *Stand. Methods Exam. Water Wastewater – 21th Ed. Am. Public Heal. Assoc. Washingt. DC, USA* (2005).

5 A novel option for reducing the optical density of liquid digestate to achieve a more productive microalgal culturing

Abstract

The liquid fraction of digestate produced by agricultural biogas plants is rich in macro- and micro-nutrients that are valuable for the culturing of microalgae. Nonetheless, the high ammonium concentration may cause toxicity and the high optical density may reduce light penetration, negatively affecting the biomass production rate. Dilution with fresh water has been frequently suggested as a mean for improving the digestate characteristics in view of microalgal culturing. In this paper, the feasibility of culturing microalgae on undiluted raw digestate or on digestate after pretreatment by stripping and adsorption was investigated.

First, adsorption tests were performed using commercial activated carbon from wood in order to identify appropriate conditions for optical density (OD) reduction. Up to 88% reduction was obtained by dosing 40 g L⁻¹ after 24 h of contact time. Then, semi-continuous culturing tests were performed on a microalgal inoculum including mainly *Chlorella* spp. and *Scenedesmus* spp. under controlled temperature and light conditions during 6-14 weeks. Raw, stripped, and stripped and adsorbed digestate samples were tested. The biomass production rate increased from 27±13 mgTSS L⁻¹ d⁻¹ on raw digestate, to 82±18 mgTSS L⁻¹ d⁻¹ mgTSS L⁻¹ d⁻¹ by using stripped digestate, and to 220±78 mgTSS L⁻¹ d⁻¹ by using the stripped and adsorbed digestate. Moreover, nitrification was

constantly suppressed when using the stripped and adsorbed digestate, while relevant nitrite built-up was observed when using raw and stripped digestate. These results suggest that microalgae are able to grow on the raw digestate, provided that long hydraulic retention times are applied. A much faster grow (up to 10 times) can be obtained by pretreating the liquid fraction of digestate by stripping and adsorption, which may be an effective means of improving the areal productivity of microalgal culturing on digestates.

Keywords: Microalgae; digestate; optical density; stripping; adsorption.

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5.1 Introduction

In agricultural areas, biogas technology is extensively used for reducing organic matter load from livestock wastewater effluents, through the transformation of organic carbon into biogas as a sustainable and renewable source of fuel ¹. Anaerobic digestion (AD) has ensured an improved environmental sustainability of animal breeding and full-scale installations (biogas plants) have spread rapidly in Europe. As a result of this rapid development, huge amounts of digestate, are produced and generally separated into liquid/solid fractions at farm scale. Liquid digestate, rich in nitrogen and potassium, is normally spread into fields as fertilizer, whereas the solid phase, rich in phosphorous and stabilized carbon, is generally used as soil amendment. Nonetheless, due to European Nitrate Directive (91/676/CEE), but also to economic and environmental issues, its conventional valorization route as soil amendment and/or fertilizer does not always fit the local context of intensive livestock areas ².

Nutrients discharged from biogas plants and liquid digestate spreading cause significant environmental problems such as water contamination and eutrophication ³. This aspect has prompted attention toward technical solutions to deal with this nitrogen load either by chemical-physical processes such as stripping, evaporation, membrane filtration ⁴ or with advanced biological processes, such as the anammox process ⁵. Recent studies have focused on the possibility to use liquid digestate as a nutrient source for microalgae growth ² and ⁶. According to Xia and Murphy³ the land requirement for microalgal cultivation is estimated as 3% of traditional direct land application of digestate. Furthermore, in a concept of circular

economy, microalgae growth on digestate effluents can be further send it back to the anaerobic digester as co-digesting feedstock or be used as a slow-release fertilizer in agronomy^{2,7,8}.

However, two major drawbacks of using liquid digestate as a substrate for microalgae growth are related to its high nitrogen content and turbidity level^{3,9}. Indeed, liquid digestate is often characterized by: a high concentration of total suspended solids, causing an inherently high turbidity and reducing light transmission, and a high ammonia concentration ($>100 \text{ mg}\cdot\text{L}^{-1}$), which could potentially be toxic to microalgae¹⁰. Thus, pre-treatments have been often adopted to remove or dilute the undesirable compounds from supernatants. As concerns turbidity, the most common pre-treatments include solid/liquid separation¹¹, filtration¹² and dilution¹³ which also helps in lowering the ammonium concentration. Another novel and promising option for turbidity reduction is the use of activated carbon (AC) for reducing the turbidity level of liquid digestate, and thus facilitating microalgae growth. The use of activated carbon has attracted attention due to its high sorption capacity of various kinds of components^{14,15,16}. Furthermore, this activated carbon could be produced from renewable biomasses like lignocellulosic biomasses (i.e. wood) through a process in two steps: char production through pyrolysis followed by a steam or chemical/thermal activation^{17,18}. The use of AC for turbidity reduction has been highly investigated for wastewater or seawater treatment with good results^{19,20,21}. Nonetheless, until now, no studies reported the use of AC to reduce the turbidity of liquid digestate in view of microalgae culturing.

The main objective of this study was to evaluate the feasibility of growing microalgae on digestate without addition of fresh water for dilution. To this aim, undiluted (raw) digestate was

first tested as microalgal growth medium. Then, pretreatments (ammonia stripping and a combination of stripping and adsorption on AC) were applied to digestate to reduce ammonium concentration, turbidity, and optical density, thus facilitating microalgae growth. First, the effects of the AC concentration (in the range 1 to 40 g·L⁻¹) and time contact (in the range 0 to 1440 min) were investigated on optical density reduction. Then semi-continuous microalgae culturing tests were conducted on raw and pretreated liquid digestate.

5.2 Materials and methods

5.2.1 *Liquid digestate characterization*

Liquid digestate (DIG_L) came from a piggery farm in Northern Italy, breeding up to 20.000 pigs. At the hosting farm, a full-scale wastewater treatment plant (WWTP) and a full-scale anaerobic digester followed by a solid/liquid separation were in operation (more details in Scaglione et al. 2015)⁵. Digestate was collected after solid/liquid separation performed by centrifugation and is here after referred to as DIG_L.

5.2.2 *Adsorption tests*

Adsorption tests were carried out by using a commercial activated carbon (AC) from wood (Norit® CA1, Sigma Aldrich, Saint Louis, USA). Tests were carried out at room temperature (20°C), under static conditions, by varying the solid loading of AC from 1 to 40 g·L⁻¹, and the adsorption time from 0 to 1440 min. After adsorption, samples were

centrifuged at 12200 rpm for 2 min (MiniSpin®, Eppendorf, Hamburg, Germany) and the supernatant was separated and kept for optical density (OD) analysis.

Optical density (OD) at 680 nm was used to quantify the clarification effect of the adsorption process. Indeed, the primary pigment involved in photosynthesis is chlorophyll a, which has strong absorption bands in the regions 400–450 and 650–700 nm²². According to preliminary tests (data not shown), the mixed microalgal community used in the algal growth tests showed two maxima in the adsorption spectrum corresponding to 420 and 680 nm. A strong correlation was observed between OD at 680 and at 420 nm measured on samples of adsorbed DIG_L, suggesting that just one OD measure at these two wavelengths could be measured and used to assess the clarification effect of the AC adsorption process. Therefore, the clarification effect of the adsorption process was assessed from the OD level at 680 nm of the original DIG_L (ODdigestate@680nm) and of the adsorbed digestate (Odadsorbed digestate@680 nm), as follows:

$$\text{OD reduction (\%)} = (\text{OD digestate@680 nm} - \text{OD adsorbed digestate@680 nm}) / (\text{OD digestate@680 nm}) \quad \text{Eq. 1}$$

5.2.3 *Semi-continuous culturing tests*

Semi-continuous culturing tests were performed in 150 mL glass test tubes (4.5 cm diameter, 20 cm height) . Light was provided by 6 fluorescent lamps (FLORA model, OSRAM, Munich, Germany), 18 W each one, with 12 h dark/light periods. Air was flushed from the bottom of each flask through a fine bubble diffuser to maintain well mixed conditions. Temperature remained around 20±2°C. As inoculum, a mixed

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microalgal community dominated by *Chlorella* spp. and *Scenedesmus* spp., and previously adapted to grow on the liquid digestate from a digester fed on waste sludge, was used²³.

5.2.3.1 Tests on raw liquid digestate

Each test tube was prepared by mixing the concentrated microalgal inoculum (10 mL) with 15 mL of the DIG_L and tap water (125 mL). The initial condition was selected in order to start from a non-inhibiting ammonium concentration and to ensure sufficient algal inoculum to ensure a rapid colonization. Specifically, the following conditions were set in each test tube: 160 mgN·L⁻¹ of ammoniacal nitrogen concentration and 0.5 of OD.

Every 1 or 2 weeks, a sample (10 out of 150 mL) of the algal suspension was withdrawn and substituted by an equivalent volume of DIG_L. The frequency of feeding was adjusted in order to achieve an almost complete depletion of the ammonium concentration before each feeding, thus avoiding too high ammonium concentration after each feeding event, which remained below 160 mgN·L⁻¹. Semi-continuous culturing tests were performed in triplicate. The concentration of N forms, phosphorus, total and volatile suspended solids, pH, temperature, OD at 680 nm and algal counts were assessed on samples of the algal suspension withdrawn from the test tubes.

5.2.3.2 Tests on stripped and adsorbed liquid digestate

During these experiments, microalgae were cultured on two types of pretreated liquid digestates. The first one was pretreated by a stripping phase and will be referred to as

DIG_S; the second one was prepared by further treating the stripped digestate with AC in order to reduce the OD (DIG_S&A). The stripped digestate was prepared using an air compressor providing 0.6-1.2 vvm through a fine bubble diffuser. No alkaline chemical was used since alkaline pH (around 9-9.5) was naturally achieved during air bubbling. The stripping process lasted from 5 to 7 days at $20\pm 2^\circ\text{C}$.

The DIG_S&A was prepared by treating the stripped digestate with activated carbon. Following the results of the adsorption tests, a solid load of 40 gAC L^{-1} was used (section 2.2). The adsorption process was carried out under static conditions for 10 minutes at ambient temperature (20°C). Then, the activated carbon was separated by centrifuging (3000 rpm for 5 minutes).

Three batches of DIG_S and DIG_S&A were prepared to be used to feed the microalgal cultures. Each one was prepared from a freshly collected DIG_L which was first stripped and then adsorbed on AC. Each batch of DIG_S and DIG_S&A was then stored at 4°C and used for microalgal feeding for no more than 2 weeks. Optical density at 680 nm, turbidity, ammonium, nitrate, nitrite, soluble COD and phosphate concentrations were assessed for each batch of DIG_S and DIG_S&A.

Semi-continuous culturing tests were performed in duplicate on both DIG_S and DIG_S&A. Initially, each test tube was prepared by mixing the microalgal inoculum with 150 ml of DIG_S or DIG_S&A, respectively. Every 2 – 5 days, a fraction (70 mL) of the algal suspension was withdrawn and substituted by DIG_S or DIG_S&A, respectively. The feeding frequency was the same for all the test tubes and defined in order to allow for the almost complete depletion of ammonium in those test tubes fed on DIG_S&A. The same feeding frequency was also adopted for test tubes fed on DIG_S.

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The monitoring plan was similar to the one described previously in section 5.2.3.

5.2.4 Analytical determinations

Total and volatile suspended solids (TSS and VSS), COD, BOD₅, BOD₂₀ were analyzed according to Standard Methods (APHA, 2005). Ammoniacal, nitrite and nitrate nitrogen (N-NH₄, N-NO₃ and N-NO₂) and phosphate (P-PO₄) were measured using spectrophotometric test kits (Hach-Lange, Germany, LCK 303, LCK 340, LCK 342 and LCK 348, respectively), on 0.45 µm filtered samples. pH and conductivity were measured by a portable probes (XS PC 510 Eutech Instruments, USA). Optical density (OD) was measured using a DR 3900 Hach Lange, Germany, spectrophotometer at a wavelength of 680 nm and by using a 1 cm path length cuvette.

Microalgae were counted using a Hemocytometer, (Marienfeld, Germany) and a microscope (B 350, Optika, Italy). A sample 1 mL on microalgal suspension from each vial was withdrawn and conveniently diluted (dil. 1:10); then, 0.1 ml of the sample was injected into the hemocytometer chamber. The number of *Scenedesmus* and *Chlorella* algal cells were distinguished according to their morphological characteristics and counted, and the final estimated cell number was obtained from the mean of 9 square (1 mm²) readings. In case rotifers and nematodes were spotted during the microscopic observation, their presence and qualitative abundance was recorded.

5.2.5 Statistical analysis

All of the experiments were performed in duplicates, all data are mean \pm standard error ($n = 2$). The statistical analyses were conducted using the R Project software (R core Team 2015). The T test for paired data was performed to detect differences between daily biomass production ($\text{mgSST L}^{-1} \text{d}^{-1}$) and biomass concentration (in term of optical density), when using stripped digestate (DIG_S) and stripped digestate with activated carbon (DIG_S&A) as feed. p-values < 0.05 were deemed to be statistically significant.

5.3 Results and discussion

5.3.1 Chemical composition of liquid digestate

Three samples of the liquid fraction of digestate were collected from the farm within a 6 months period. The average chemical composition is reported in **Table 5.1**. One can see that liquid digestate has highly variable characteristics, which can be chiefly explained by seasonal variations in the piggery waste production, and in the digested co-substrates, as already shown in Scaglione et al. (2015)⁵.

Specifically, liquid digestate had a high OD due to the high concentration of suspended and dissolved solids which limit light penetration and therefore photosynthesis and microalgae growth, as already pointed out in previous^{6,24,25}. As regard nutrients and COD their values fall within typical literature intervals³. It is worth noting that the liquid digestate had an ammoniacal nitrogen concentration between 0.6 and 1.7 $\text{gN}\cdot\text{L}^{-1}$; high level of ammoniacal nitrogen have previously been suggested be potentially toxic to microalgae¹⁰, while low

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P concentration could have limiting effect on microalgal growth²⁶. As for the N:P ratio it varied within a large interval (48 ± 32 gN gP⁻¹). Such values are well above the theoretically optimum value of 10-16 molN molP⁻¹ (corresponding to 4.5-7.2 gN gP⁻¹) suggested by Geider & La Roche²⁷. However, the N:P ratio is within the range of other digestates previously tested for their capacity to sustain microalgal growth. Indeed, Marcilhac et al.²⁸ observed that the N:P ratio of digestate samples ranged from 38 to 135 gN gP⁻¹ varying depending on the biogas plant and on time at a given plant. In order to use digestate as substrate for microalgae culture either dilution or pretreatment may be therefore useful to improve light penetration and to reduce ammonia concentration.

Table 5.1. Chemical composition of liquid digestate (DIG_L) measured on three samples at different sampling time, and average value (mean±std).

Parameter	mean± std
TSS (mg L ⁻¹)	1,400±1,600
VSS (mg L ⁻¹)	1,800±1,600
pH	7.7±0.8
N-NH ₄ (mg L ⁻¹)	1,200±500
P-PO ₄ (mg L ⁻¹)	30±10
Optical Density (-)	1.3±0.5
COD _{sol} (mg L ⁻¹)	4,000±6,000
Total COD (mg L ⁻¹)	6,300±4,000
BOD ₅ sol (mg L ⁻¹)	600±100
BOD ₂₀ sol (mg L ⁻¹)	1,000±200
N:P	48±32
COD:N	5.0±6.6

5.3.2 Effect of AC adsorption on the optical density of liquid digestate

Figure 5.1 shows the OD reduction of liquid digestate at different AC dosages (1 to 40 g·L⁻¹) and adsorption times (0 to

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1440 min). As observed, the efficiency of the optical density reduction increased as the AC concentration increased. The best OD reduction (88%) was obtained at a AC dosage of 40 g·L⁻¹ and at an adsorption time of 10 min. Indeed, as showed in **Figure 5.1**, after 30 min, the reduction of OD remained constant. A picture comparing the raw and adsorbed digestate obtained by applying the previously identified adsorption conditions can be found in **Figure 5.2**.

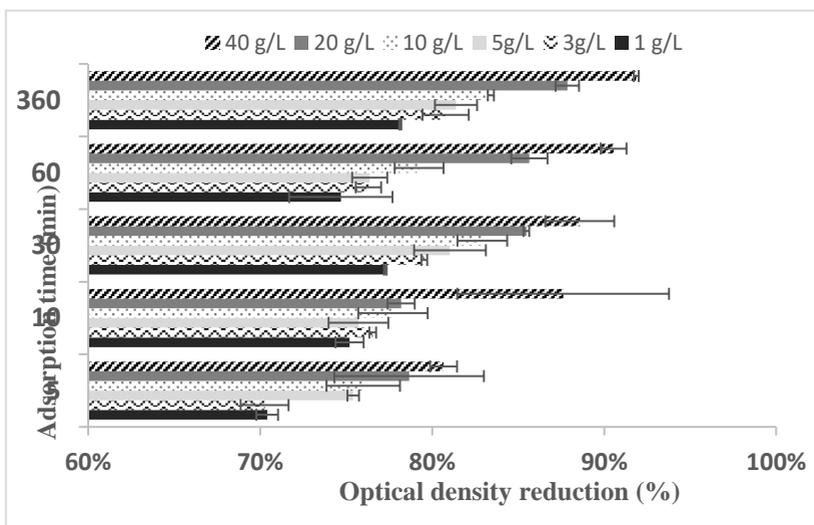


Figure 5.1. Optical density reduction (%) during the first 360 min, all data are mean \pm standard error ($n = 3$).



Figure 5.2. Visual evidence of the reduction of the optical density upon AC adsorption. DIG_L before (right vial) and after (left vial) the adsorption step.

5.3.3 Microalgal culturing tests

5.3.3.1 Growth on diluted liquid digestate

Results of the first semi-continuous tests of algal growth on DIG_L with a $1730 \text{ mgNH}_4\text{-N L}^{-1}$ concentration are reported in **Figure 5.3**. Microalgal density increased over time up to 3-4 times the initial value. Above such value, light penetration was probably insufficient to sustain further algal growth. Between day 41 and 80, a poor repeatability was observed associated to a significantly higher biomass density in one of the triplicate test tubes during that same period. From TSS data, the cumulated biomass production (CBP, mgTSS) was computed and compared to the cumulated volume of DIG_L fed to each test tube (**Figure 5.3**). Results show that the amount of algal biomass produced is proportional to the volume of DIG_L fed during each fed-batch cycle. The highest values of specific biomass production rate were observed between day 52 and 74 during which the average HRT was 55 d and corresponded to $27 \pm 13 \text{ mgTSS L}^{-1} \text{ d}^{-1}$.

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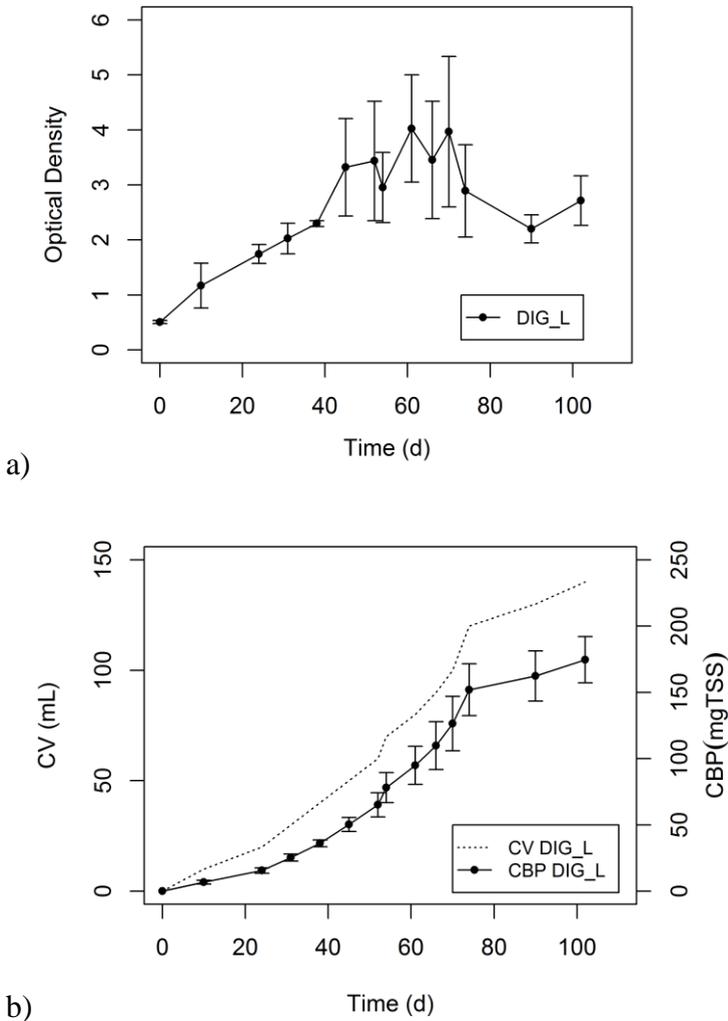


Figure 5.3. Results of the semi batch growth test on DIG_L: time trend of (a) optical density, (b) cumulated biomass production (CBP line) and cumulated treated DIG_L volume (CV, dashed line). All data are mean \pm standard error ($n = 2$)

Ammonium was efficiently (>97%) removed (Appendix A.1) with effluent concentrations (i.e. concentration measured at the end of each fed-batch cycle) ranging from $< 1 \text{ mg L}^{-1}$ to 35 mgN L^{-1} . Maximum ammonium removal rate, assessed as the slope of the time trend of ammonium concentration under non-limiting ammonium concentration ($> 10 \text{ mgN L}^{-1}$, data not shown) was found to be: $24 \pm 1.5 \text{ mgN L}^{-1} \text{ d}^{-1}$ for the three flasks. However, ammonium removal was also affected by other concomitant processes. Stripping is likely to have played a significant role since pH was averagely 9.6 ± 0.6 leading to relevant free ammonia concentrations. Moreover, nitrification was also observed although its appearance and relevance differed remarkably among each test tube and in time. As for nitrate, the average concentration slightly increased from day 24 onward from 2.1 ± 0.2 to $5 \pm 0.4 \text{ mgN L}^{-1}$ suggesting a slow nitrification process which was similar in all replicates (Appendix A.1). On the contrary, nitrite (Appendix A.2) remained negligible in replicate 1, while it increased remarkably in replicate 2 and 3 after day 60, reaching a maximum level of 210 and 110 mgN L^{-1} , respectively, at day 96; later, its concentration decreased. Denitrification was unlike to take place since dissolved oxygen was continuously entering via aeration maintaining aerobic conditions during both light and dark hours.

Extra phosphorus was dosed at time 33, 61 and 74 to increase the P concentration (Appendix A.1) in the test tubes from few mgP L^{-1} to 15-20 mgP L^{-1} . Average P removal rate was $0.5 \text{ mgP L}^{-1} \text{ d}^{-1}$.

Even for phosphorus biological (assimilation/release) and chemical-physical (precipitation/dissolution) processes are expected to take place simultaneously.

Soluble COD in the liquid digestate increased during the first part of the test and eventually stabilized (Appendix A.1). The

COD removal rate remained quite constant during the whole test, with average removal efficiencies of $83\pm 4\%$ which could be supported by heterotrophic bacteria or by mixotrophic algal growth (among others ^{29,30,31,32}).

As for the algal counts (**Figure 5.5**), *Chlorella* spp. was found to be the prevailing algal species (by up to 2 order of magnitude) in all vials up to day 68, then *Scenedesmus* spp. prevailed, probably due to a decrease in the ambient temperature under 20°C. The total number of algal cells was well correlated with the OD (R^2 of 0.77). The maximum total algal counts recorded were $13 \text{ E}+06 \text{ cell mL}^{-1}$ for replicate 1, $4.6 \text{ E}+6 \text{ cell mL}^{-1}$ for replicate 2, and $9.4 \text{ E}+06 \text{ cell mL}^{-1}$ for replicate 3; those values were obtained between day 52 and 68 when absorbance was at its maximum as well. The mean number of *Chlorella* spp. and *Scenedesmus* spp. reached in the vials was $2.5 \text{ E}+6 \text{ cell mL}^{-1}$ and $0.9 \text{ E}+6 \text{ cell mL}^{-1}$. Replicate 1 was the vial with the larger algal growth, with a mean count of $4.1 \text{ E}+6 \text{ cell mL}^{-1}$ for *Chlorella* spp. and $1.1 \text{ E}+0.6 \text{ cell mL}^{-1}$ for *Scenedesmus* spp. Few rotifers, which feed on microalgae, were found in all the vials. Nematodes were found from day 33 in replicate 2, while in the other vials they were found sporadically later on (from day 61 for replicate 1 and from day 90 for replicate 3).

5.3.3.2 Growth on adsorbed/stripped liquid digestate

The second set of semi-continuous algal growth tests was conducted on the DIG_L after stripping (DIG_S) and stripping and adsorption (DIG_S&A), according to the procedure described in section 5.3.3.1. Three batches of DIG_S and DIG_S&A (B1, B2, B3) were prepared during the course of the experimentation and their characteristics are summarized in

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Table 5.2. The adsorption step caused a remarkable reduction in both optical density ($81\pm 7\%$, similar to the removal efficiency obtained during the adsorption tests and reported in section 3.2). Similarly, high reductions were achieved for soluble COD ($87\pm 3\%$) and for nitrate ($90\pm 3\%$). On the contrary, no significant variation was observed in the ammonium concentration. As shown in **Table 5.2**, phosphorus was released by the activated carbon, leading to a remarkable increase in the phosphorus concentration in the DIG_S&A.

Of the three batches described in **Table 5.2**, B1 was used from the beginning of the culturing test until day 14, B2 from day 15 until 28, and B3 from day 29 until 40, respectively. The feeding procedure was similar to that applied to grow microalgae on the raw digestate. Since the ammonium concentration in the feed was lower, the average HRT, set according to the time needed for ammonium depletion, was much shorter than in the first set of trials, being 11 days up to day 29 and 8.8 days from day 30 to day 40.

Table 5.2: Characterization of the stripped (DIG_S) and stripped/adsorbed (DIG_S&A) samples.

Parameter	DIG_S			DIG_S&A		
	B1	B2	B3	B1	B2	B3
Conductivity (mS cm ⁻¹)	3.5	4.7	4.2	3.6	4.2	4.7
OD (-)	0.27	1.04	0.98	0.04	0.28	0.14
N-NH ₄ (mg L ⁻¹)	220	530	560	250	520	560
N-NO ₃ (mg L ⁻¹)	9.3	10	10.5	1.0	0.68	1.3
Soluble COD (mg L ⁻¹)	1,410	1,370	1,410	156	229	170

According to the T test for paired data, daily biomass production (mgSST L⁻¹d⁻¹) and biomass concentration (in term of optical density), was significantly different between stripped digestate (DIG_S) and stripped and adsorbed digestate (DIG_S&A) as feed, (df = 10, p-value = 0.0001, and df=10, p-

value= 0.0009, respectively for biomass production and biomass concentration). As shown in **Figure 5.4a**, microalgal concentration remained always higher in the test tubes fed on the DIG_S&A than in those fed on DIG_S, differences becoming more and more significant with time. From day 15 to day 29, the optical density increased in the test tubes fed on DIG_S&A while it tended to decrease in those fed on DIG_S. This behavior suggests that a higher growth rate was achieved when feeding the stripped and adsorbed liquid digestate. Later, biomass concentration reduced in all the test tubes because of the reduction in the average HRT. The cumulated biomass production was computed from TSS data, and in **Figure 5.4b** it is compared to the cumulated volume of liquid digestate fed to the test tubes. The CBP appeared significantly higher in the test tubes fed on DIG S&A than in the other ones. Coherently, a significantly higher biomass production rate was observed in the test tubes fed on DIG_S&A ($220\pm 78 \text{ mgTSS L}^{-1} \text{ d}^{-1}$) than on those fed on DIG_S ($82\pm 18 \text{ mgTSS L}^{-1} \text{ d}^{-1}$). According to the T test for paired data, daily biomass production ($\text{mgSST L}^{-1} \text{ d}^{-1}$) and biomass concentration was significantly different between stripped digestate (DIG_S) and stripped digestate with activated carbon (DIG_S&A) as feed, ($df = 10$, $p\text{-value} = p\text{-value} = 0.0001$, and $df=10$, $p\text{-value}= 0.0009$, respectively for biomass production and biomass concentration).

A slightly higher removal efficiency of the ammoniacal nitrogen (Appendix B.1 and B.2) was observed, on average, in the test tubes fed on DIG_S&A ($96\pm 4\%$) than on those fed on DIG_S ($88\pm 9\%$).

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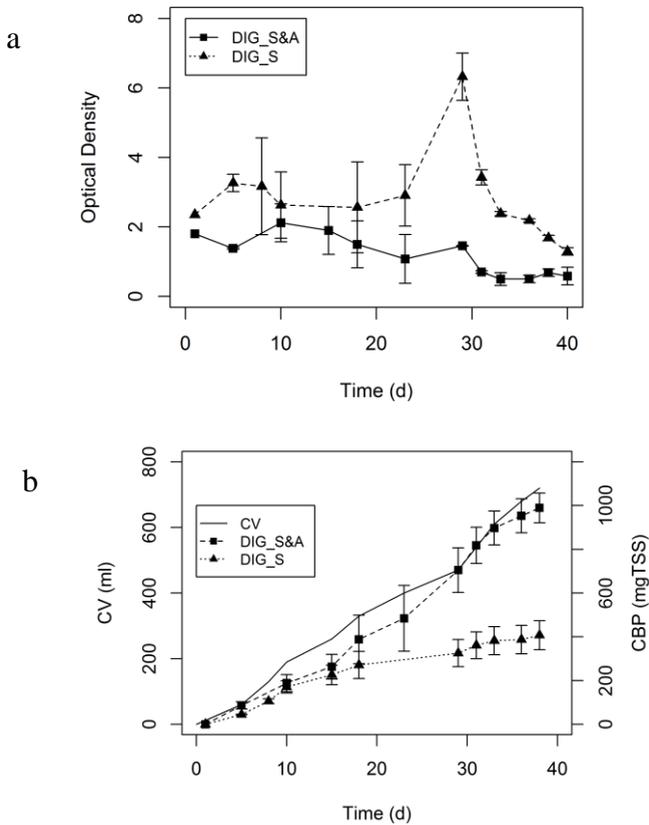


Figure 5.4. Results of the semi batch growth test on DIG_S and DIG_S&A : time trend of (a) optical density, (b) CV, solid line, cumulated biomass production and cumulated treated volume of pretreated digestate on the secondary y-axis(dashed line). All data are mean \pm standard error ($n = 2$)

Nitrification was almost negligible in the test tubes fed on DIG_S&A while an increase in nitrite (up to 30 mgN L^{-1}) with no significant production on nitrate was observed in test tubes fed on DIG_S from day 30 onward (Appendix B.1 and B.2).

As for phosphorus (Appendix B.1 and B.2), very different concentrations are found in the DIG_S and DIG_S&A, as commented before. In the test tubes fed on DIG_S, effluent concentrations decreased from 3 to 1 mgP L⁻¹ during the first two weeks; at day 15, extra P was dosed in order to increase the P level in each test tubes to 20 mgP L⁻¹ thus avoiding P-limiting conditions. Later P decreased slowly and, from day 20 onward, remained stable around 5 mgP L⁻¹. In the test tubes fed on DIG_S&A, the P level remained between 200 and 300 mgP L⁻¹.

A limited COD (Appendix B.1 and B.2) removal was observed in the test tubes fed on DIG_S (15±3% on average), while a 30% increase in the soluble COD was observed in the test tubes fed on DIG_S&A. This is due to the release of COD from the algal biomass that is more relevant than COD oxidation. Indeed, the residual COD after adsorption is poorly degradable thus making COD release more relevant than COD oxidation.

As for algal counts (**Figure 5.5**), *Chlorella* spp. was found to be the prevailing algal species in the test tubes fed on DIG_S&A, while in the DIG_S ones the prevalent species was *Scenedesmus* spp. However, in all vials the number of both microalgae species was of the same order of magnitude. The total number of algal cells was well correlated with the optical density (R² between 0.8 and 0.9). The maximum values of total number of algal cells were obtained on day 10 and on day 29 in DIG_S and in DIG_S&A, respectively, when the maximum absorbance values were also observed. In those days, the total algal count was 2.8 E+06 cell mL⁻¹ and 1.7 E+6 cell mL⁻¹ for the two replicates of the DIG_S set, and 4.9 E+06 cell mL⁻¹ and 5.3 E+06 cell mL⁻¹ for the two replicates of the DIG_S&A set. The mean number of *Chlorella* spp. and of *Scenedesmus* spp. in the DIG_S vials was 0.3E+6 cell mL⁻¹ and 0.4E+6

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cell mL⁻¹, respectively, while in the DIG_S&A vials it was 1.18 E+6 cell mL⁻¹ and 0.9 E+6 cell mL⁻¹, respectively. Rotifers were found in both the sets, but in DIG_S their number was significantly larger than in the DIG_S&A one.

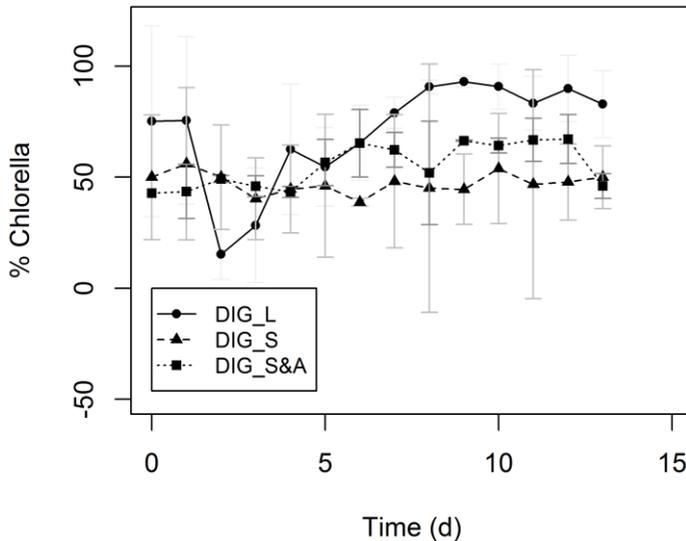


Figure 5.5. Average percent of *Chlorella* cells in the DIG_L (rounds), DIG_S (triangles) and DIG_S&A (squares) culturing tests with time, all data are mean \pm standard error ($n = 2$).

5.4. Discussion

Digestate is rich in nutrients that are needed in microalgal culturing. For this reason, the use of digestate for microalgal culturing has been proposed either as an opportunity to reduce microalgal-culturing costs or as a process to reduce the nutrient level in digestate thus reducing the land request for digestate spreading especially in livestock intensive breeding area. The dual-system “AD/microalgae” has recently attracted attention

for an alternative used of the rich nutrients fraction of liquid digestate ^{6,8,9}. Indeed, liquid digestate is the major fraction of digestate and may contain high levels of ammonia nitrogen ³. Nonetheless, some major limitations exist before an industrial scale-up of microalgae ponds using liquid digestate as the high ammonia and turbidity content of liquid digestate ² and ³³. Although ammonium is a preferable form for microalgal utilization ³⁴, high total ammonia nitrogen (TAN) (both ammonium and free ammonia) levels may lead to inhibition ³. Turbidity is another important parameters that could limit the availability of photosynthetically active radiation and consequently microalgae growth. Thus, dilution with fresh water is commonly applied to lower both the ammonium level and turbidity, thus improving the microalgae efficiency growth ¹³ but further affecting the overall economic and environmental balances of the dual system. In the present study, a stripping step was applied to reduce the ammonium concentration, which may allow for ammonium recovery and further AC were used for liquid digestate turbidity reduction in order to avoid the need for dilution.

Indeed, stripping is a well known chemical/physical process which allows for the recovery of nitrogen in the form of ammonium salt. Its applicability to the liquid fraction of digestate is still limited mainly due to the fact that the ammonium salts marketability is yet uncertain. The used of AC for turbidity reduction has been highly investigated for wastewater or seawater treatment with good results ^{19,20}. The results reported in this study are in accordance with Hatt et al.²⁰, who noticed a minimum of 80% turbidity removal on secondary wastewater by performing batch tests at 20°C, for 2h, under stirring conditions and by using different types of granular AC with a mean dosage of 400 mg L⁻¹. Interestingly, Hatt et al. ²⁰ highlighted a good correlation between the

efficiency of turbidity reduction ($Abs_{UV\ 400nm}$) and the available meso and macropores of AC. Furthermore, the lower concentration of AC used in wastewater treatment compared to our investigation on liquid digestate for similar turbidity reduction could be mainly explained by the lower turbidity of secondary wastewater (around 7 NTU³⁵). Nonetheless, the high production cost of AC remains a limitation for its full-scale industrial implementation and used in remediation process^{36,37}. Thus, the use of AC produced from low cost lignocellulosic biomasses (i.e wastes residues, agricultural wastes) may be considered as a good alternative in this specific application.

As shown in **Table 5.2**, the proposed pretreatments were successful in improving the treatability of digestate. Indeed, the ammonium concentration is lowered by the stripping process to acceptable levels (200-500 mgN L⁻¹). A digestate from the anaerobic digestion of waste sludge with similar ammonium level as been yet proven to be adequate for microalgal culturing²³. However, stripping does not significantly affect the optical density, which is reduced by the adsorption process. Moreover, the applied pretreatments (stripping and adsorption) allows to modify the N:P ratio, thus allowing for its potential optimization. Finally, as commented before, the optical properties are improved both in terms of optical density and of turbidity if compared to the original DIG_L.

Microalgal culturing tests were then performed to assess the impact of the applied pretreatments on the biomass growth rate. The raw digestate could be successfully used to grow microalgae and a microalgal production rate of 27 ± 13 mgTSS L⁻¹ d⁻¹ was obtained. In the literature, a large interval of microalgal growth rate can be found, spanning from 30 to 670 mgTSS L⁻¹ d⁻¹, and this is partly due to differences in the culturing mode, algal strains, and digestate pretreatments^{2,3}. The low value obtained in this experimentation can be partially

explained by the high free ammonia levels and by the high digestate optical density which limits light availability. In order to limit ammonia inhibition, a quite high hydraulic retention time had to be applied (around 100 days, on average, with lower values of 55 d during the stationary growth phase). Such a long HRT would result in huge pond volumes and the land request for the treatment could become prohibitive.

By applying pretreatments, the biomass production rate was indeed improved since it increased to $82 \pm 18 \text{ mgTSS L}^{-1} \text{ d}^{-1}$ when the stripped digestate was used and to $220 \pm 78 \text{ mgTSS L}^{-1} \text{ d}^{-1}$ when a further adsorption step on activated carbon was applied, which corresponds to an almost 10 fold increase if compared to results obtained on the raw digestate. This would result in proportional reductions in the land request. Nonetheless, the high production costs of AC remains a limiting factor in view of a full-scale application^{36,37}. Thus, as a perspective work, low cost adsorbent such as biochar and/or AC from renewable lignocellulosic biomasses should be investigated.

Moreover, the use of pretreatments may also affect the composition of the community of microalgae and bacteria that develops. Simultaneous activity of microalgae and nitrifying bacteria took place both in microalgae cultivation test with raw liquid digestate and stripped digestate while nitrification was not observed in test tube fed on stripped and absorbed digestate. Nitrite accumulation up to 210 mgN L^{-1} were registered indicating the presence of ammonia oxidizing bacteria activity, but this phenomenon was not stable and the triggering factors are not yet clear. Nitrate accumulation was not registered indicating a low activity of nitrite oxidizing bacteria (NOB). Relevant nitrite accumulation was observed when free ammonia levels approached and overcame $50 \text{ mgN-NH}_3 \text{ L}^{-1}$. These high free ammonia levels probably led to an inhibition

of NOB, which are known to be sensitive to free ammonia already at concentration of $1\text{--}5 \text{ mgN-NH}_3 \text{ L}^{-1}$, with an IC50 (50% inhibition concentration) of $10 \text{ mgN-NH}_3 \text{ L}^{-1}$ ³⁸. On the contrary, ammonia oxidizing bacteria (AOB) are more tolerant to high free ammonia levels, as suggested by the higher IC50 values reported in the literature varying within the range of $40\text{--}600 \text{ mgN-NH}_3 \text{ L}^{-1}$ ³⁹, depending on acclimation. Nitrification is indeed a common process taking place in algal ponds/photobioreactors treating digestate. Gonzalez-Fernandez et al. ⁴⁰ verified in an open pond treating digested piggyery slurry that nitrification (with mainly nitrite accumulation) resulted in the major nitrogen transformation of ammonium nitrogen: the percentage ranged 36–58% of the total nitrogen fed to the ponds. Marcilhac et al ²⁸ reported that microalgae outcompeted nitrifying bacteria since the nitrification rate decreased with the increase in microalgal N assimilation. Moreover, their experiments showed how microalgae become better competitors for nutrients under P-limiting ($<0.1 \text{ mgP L}^{-1}$) conditions. Uggetti et al. ⁹ reported the presence of nitrification and in their microalgal growth tests and observed that nitrite and nitrate production was enhanced at high microalgae concentration (1.3 or 1.8 gTSS L^{-1}). This evidence was explained by the fact that at higher algal concentration larger quantity of oxygen are produced stimulating ammonium oxidation by nitrifiers, while microalgal growth rate is negatively affected by a self-shading phenomenon. In the present experimentation, the only test tubes in which negligible nitrification activity was observed were those fed on DIG_S&A, in which the highest microalgal production rate and biomass concentration was observed. This observation is in disagreement with data from Uggetti et al. ⁹. This may be due to the fact that in our experiment air was supplied continuously leading to constantly non-limiting dissolved oxygen levels.

Moreover, in test tubes fed on the adsorbed digestate, the lower OD of the DIG_S&A allowed the culture to achieve higher algal concentration before it possible became light-limited. Despite being a commonly observed process, the true mechanisms and the driving forces of the competition between microalgae and nitrifiers still need to be fully elucidated. Nonetheless, the use of adsorbed digestate suppressed nitrifiers growth suggesting that the lower OD and higher P level in DIG_S&A compared to the other tested conditions contributed in favoring microalgae over nitrifiers.

As previously mentioned, the removal of COD was also very different from one case to another, reaching 83% in DIG_L, just 15% in DIG_S&A and being negative, with a 30% increase, in DIG_S. Of course, the starting COD was much higher in DIG_L, lower in DIG_S and minimum in DIG_S&A and the treatments are likely to have removed the more degradable fraction of the total. Actually, the final value of COD in algae/bacteria consortia, as it was the case in the described experiments, is the result of the combined effect of bacterial oxidation, algal degradation and release from death cells. Moreover, many authors reported effective mixotrophic growth of algae, such as various species of *Chlorella*, accounting for strong COD removal also in poorly degradable wastewaters, pointing out the double role of organic compounds as both carbon source and growth factors (among others ^{29,30,31,32}). On the contrary, in very few cases mixotrophic metabolism is reported for *Scenedesmus* species ⁴¹. In the three substrate culturing tests, the total number of algal cells is comparable but some differences exist in the percent of *Chlorella* cells, as shown in **Figure 5.5** reporting the time trend in the three cases. Actually, the greater density of *Chlorella* in L_DIG_L could depend on the larger availability of organic matter and could contribute to the greater COD removal, which

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could thus be due not only to bacterial activity. The percent of *Chlorella* decreases from DIG_L to DIG_S and then to DIG_S&A, as the efficiency of COD removal in the three cases.

5.5. Conclusions and perspectives

Results of this experimentation suggest the feasibility of culturing microalgae on the liquid fraction of digestate without the need for dilution. However, long hydraulic retention times had to be used when using the raw DIG_L and low biomass productivity were obtained. In view of reducing the land request, pretreatments (stripping/adsorption) were tested as a mean to sustain faster microalgal grow. Commercial activated carbon proved to be effective in optical density reduction. By feeding the pretreated digestate, much faster microalgal grow was obtained. Indeed, these pretreatments allowed to increase the microalgal production rate by almost 10 times. Nonetheless, the high production cost of AC remains a limitation for its full-scale industrial use. AC produced from renewable biomasses like lignocellulosic substrates may be considered as a perspective alternative in this specific application. A crucialpoint is due to ammonia removing by stripping, which is not environmentally due to the high greenhouse potential of $\text{NH}_4\text{-N}$.

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5.6 Acknowledgment

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5.7 References

1. Fuchsz, M., & Kohlheb, N. (2015). Comparison of the environmental effects of manure-and crop-based agricultural biogas plants using life cycle analysis. *Journal of Cleaner Production*, **86**, 60-66.
2. Monlau, F. Sambusiti, C. Ficara, E. Aboulkas, A. Barakat, A. Carrère, H. (2015) New opportunities for agricultural digestate valorization: current situation and perspectives. *Energy & Environmental Science* 2600-2621.
3. Xia, A., Murphy, J.D., 2016. Microalgal Cultivation in Treating Liquid Digestate from Biogas Systems. *Trends in Biotechnology* **34**, 264-275.
4. Fuchs W., Drosch B. (2013) Assessment of the state of the art of technologies for the processing of digestate residue from anaerobic digesters. *Water Sci Technol.* 2013;**67**(9):1984-93.
5. Scaglione, D., Ficara, E., Corbellini, V., Tornotti, G., Teli, G., Canziani, R., Malpei, F. (2015). Autotrophic nitrogen removal by a two-step SBR process applied to mixed agro-digestate. *Bioresource Technology*, Volume **176**, 98-105.
6. Uggetti, E., Sialve, B., Trably, E., & Steyer, J. P. (2014). Integrating microalgae production with anaerobic digestion: a biorefinery approach. *Biofuels, Bioproducts and Biorefining*, **8**(4), 516-529.
7. Mulbry, W., Westhead, E.K., Pizarro, C., Sikora, L., 2005. Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer. *Bioresource Technology* **96**, 451-458.
8. Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to make

- microalgal biodiesel sustainable. *Biotechnology Advances* **27**, 409-416.
9. Uggetti, E., Sialve, B., Latrille, E., & Steyer, J. P. (2014). Anaerobic digestate as substrate for microalgae culture: the role of ammonium concentration on the microalgae productivity. *Bioresource technology*, **152**, 437-443.
 10. Källqvist, T., & Svenson, A. (2003). Assessment of ammonia toxicity in tests with the microalga, *Nephroselmis pyriformis*, Chlorophyta. *Water research*, **37**(3), 477-484.
 11. González-Fernández, C., M-Salces, B., García-González, M. C. (2011). Nitrogen transformations under different conditions in open ponds by means of microalgae–bacteria consortium treating pig slurry. *Bioresource Technology*, **102**, 960-966.
 12. Halfhide T, Dalrymple, OK, Wilkie, AC, Trimmer, J, Gillie, B, Udom, I, Zhang, Q, Ergas, SJ, Growth of an Indigenous Algal Consortium on Anaerobically Digested Municipal Sludge Centrate: Photobioreactor Performance and Modeling. *BioEnergy Res* **8**: 249-258 (2015).
 13. Bahr, M., Díaz, I., Dominguez, A., González Sánchez, A., Muñoz, R., 2014. Microalgal-biotechnology as a platform for an integral biogas upgrading and nutrient removal from anaerobic effluents. *Environ. Sci. Technol.* **48**, 573–581. doi:10.1021/es403596m.
 14. Zhang, S., Shao, T., Karanfil, T., 2011. The effects of dissolved natural organic matter on the adsorption of synthetic organic chemicals by activated carbons and carbon nanotubes. *Water Research* **45**, 1378-1386.
 15. Ioannidou, O., Zabaniotou, A., 2007. Agricultural residues as precursors for activated carbon

- production—A review. *Renewable and Sustainable Energy Reviews* **11**, 1966-2005.
16. Monlau, F., Sambusiti, C., Antoniou, N., Zabaniotou, A., Solhy, A., & Barakat, A. (2015). Pyrochars from bioenergy residue as novel bio-adsorbents for lignocellulosic hydrolysate detoxification. *Bioresource technology*, **187**, 379-386.
 17. Zabaniotou, A.A., Antoniou, N., Stavropoulos, G., 2014. Novel sorbent materials for environmental remediation via depolymerization of used tyres. *Desalination and Water Treatment* **56**, 1264-1273.
 18. Ioannidou, O.A., Zabaniotou, A.A., Stavropoulos, G.G., Islam, M.A., Albanis, T.A., 2010. Preparation of activated carbons from agricultural residues for pesticide adsorption. *Chemosphere* **80**, 1328-1336.
 19. Singh, K.P., Mohan, D., Sinha, S., Tondon, G.S., Gosh, D., 2003. Color Removal from Wastewater Using Low-Cost Activated Carbon Derived from Agricultural Waste Material. *Industrial & Engineering Chemistry Research* **42**, 1965-1976.
 20. Hatt, J. W., Germain, E., & Judd, S. J. (2013). Granular activated carbon for removal of organic matter and turbidity from secondary wastewater. *Water Science and Technology*, **67**(4), 846-853.
 21. Ayotamuno, M.J., Olatunji O M, Akor, A.J., 2013. Waste Water Reclamation and Reuse in Yenegoa Using Activated Carbon and Zero B. *International Journal of Advancements in Research & Technology* **2**, 159-166.
 22. Williams, P. J. L. B., & Laurens, L. M. (2010). Microalgae as biodiesel & biomass feedstocks: review & analysis of the biochemistry, energetics & economics. *Energy & Environmental Science*, **3**(5), 554-590.

23. Ficara, E., Uslenghi, A., Basilico, D., Mezzanotte, V., 2014. Growth of microalgal biomass on supernatant from biosolid dewatering. *Water Sci. Technol.* **69**, 896–902.
24. Cheng, J., Xu, J., Huang, Y., Li, Y., Zhou, J., and Cen, K. (2015). Growth optimisation of microalga mutant at high CO₂ concentration to purify undiluted anaerobic digestion effluent of swine manure. *Bioresource Technology*, Elsevier Ltd, **177**, 240–246.
25. Hena, S., Fatima, S., and Tabassum, S. (2015). Cultivation of algae consortium in a dairy farm wastewater for biodiesel production. *Water Resources and Industry*, Elsevier, **10**, 1–14.
26. Molinuevo-Salces, B., Garcia-Gonzalez, M. C., and Gonzalez-Fernandez, C. (2010). Performance comparison of two photobioreactors configurations (open and closed to the atmosphere) treating anaerobically degraded swine slurry. *Bioresource Technology*, Elsevier Ltd, **101**(14), 5144–5149.
27. Geider, R. J. & La Roche, J., 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *European Journal of Phycology*, Volume **37**, pp. 1-17.
28. Marcilhac, C., Sialve, B., Pourcher, A.-M., Ziebal, C., Bernet, N., Béline, F., 2014. Digestate color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem. *Water Research* **64**, 278-287.
29. Hemalatha, M., & Mohan, S. V. (2016). Microalgae cultivation as tertiary unit operation for treatment of pharmaceutical wastewater associated with lipid production. *Bioresource technology*, **215**, 117-122.

30. Kim, S., Park, J., Cho, Y.B., Hwang, S.J. (2013). Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. *Bioresource Technology* **144**, 8–13.
31. Kandimalla, P., Sreekanth, D., Vurimindi, H. (2016): Mixotrophic cultivation of microalgae using industrial flue gases for biodiesel production. *Environ Sci Pollut Res*, **23**:9345–9354.
32. Ren, X., Chen, J., Deschênes, J.S., Tremblay, R., Jolicoeur, M. (2016). Glucose feeding recalibrates carbon flux distribution and favours lipid accumulation in *Chlorella protothecoides* through cell energetic management *Algal Research* **14** (2016) 83–91
33. Marcilhac, C., Sialve, B., Pourcher, A., Ziebal, C., Bernet, N., Béline, F., (2015) Control of nitrogen behaviour by phosphate concentration during microalgal-bacterial cultivation using digestate. *Biores. Technol.* **175**, 224–230.
34. Pérez-Garcia, O., Escalante, F., De-Bashan, L. & Bashan, Y., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. *Water Research*, Volume **45**, pp. 11-36.
35. Safari, G. H., Yetilmezsoy, K., Mahvi, A. H., & Zarrabi, M. (2013). Post-treatment of secondary wastewater treatment plant effluent using a two-stage fluidized bed bioreactor system. *Journal of Environmental Health Science and Engineering*, **11**(1), 1.
36. Luo, C., Lü, F., Shao, L., He, P., 2015. Application of eco-compatible biochar in anaerobic digestion to relieve acid stress and promote the selective

- colonization of functional microbes. *Water Research* **68**, 710-718.
37. Li, Y., Shao, J., Wang, X., Yang, H., Chen, Y., Deng, Y., Zhang, S., Chen, H., 2013. Upgrading of bio-oil: removal of the fermentation inhibitor (furfural) from the model compounds of bio-oil using pyrolytic char. *Energy. Fuels* **27** (10), 5975-5981.
38. Jubany I. G., Lafuente J., Baeza J.A., Carrera J. (2009) Total and stable washout of nitrite oxidizing bacteria from a nitrifying continuous activated sludge system using automatic control based on oxygen uptake rate measurements. *Water Research* **43**, 2761–2772.
39. Scaglione D., Tornotti G., Teli A., Lorenzoni L., Ficara E., Canziani R. and Malpei F. (2013) Nitrification denitrification via nitrite in a pilot-scale SBR treating the liquid fraction of co-digested piggery/poultry manure and agro-wastes. *Chemical Engineering Journal*, **228**, 935-943.
40. Gonzalez-Fernandez C., Molinuevo-Salces B. and García-González M. (2011) Nitrogen transformations under different conditions in open ponds by means of microalgae–bacteria consortium treating pig slurry. *Biores. Technol.* **102**, 960-966.
41. Leite, G. B., Paranjape, K., & Hallenbeck, P. C. (2016). Breakfast of champions: Fast lipid accumulation by cultures of *Chlorella* and *Scenedesmus* induced by xylose. *Algal Research*, **16**, 338-348.

6 Microalgal based treatment of digested agro wastes - lab and pilot-scale experiments

Abstract

Microalgae can be used to remove nutrients from waste streams while their biomass can be further used to produce bio-fuels. In this contest, an experimental research was carried out to investigate the use of microalgae to remove N from the digestate produced by the co-digestion of agro-wastes and to produce bioenergy from the microalgal biomass by its anaerobic digestion. In an outdoor bubble column, inoculated with a mixed consortium of *Chlorella vulgaris* and *Scenedesmus sp.*, and fed on the diluted (1:4, to obtain an ammonia nitrogen concentration of 200-250 mgN L⁻¹) liquid fraction of digestate, a 80% nitrogen removal was observed with the production of 50 mgTSS L⁻¹ d⁻¹. In a following indoor test, microalgal flocculation and nitrate and nitrite accumulation were observed, which are critical issues to be further investigated. The methane yield of the microalgal biomass in a 60 days batch test was higher than that of manure (207 versus 160 NL kgVS⁻¹).

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Keywords: Agrowaste digestate supernatant, anaerobic digestion, microalgal growth, nitrogen removal

6.1 Introduction

Microalgae are well known for their capacity of growing fast on waste streams and can thus be useful for nitrogen removal from concentrated wastewater flows ¹, while their biomass can be exploited for energy production ².

Literature reports a very wide range of nitrogen removal efficiencies (20-100%), but, in most cases, the experimental results show that microalgae are able to remove over 60% of nitrogen, especially of ammonia nitrogen³.

The biomass productivity of microalgae exceeds those of vascular plants ⁴, moreover, the average lipid content varies between 1 and 70% of dry weight (for example *Chlorella* sp. 10–48%) and under certain conditions (N deficiency) some species can reach 90%, with a maximum lipid productivity of 0.35 g L⁻¹ d⁻¹ ⁵. For those characteristics, microalgae have been recognized as the feedstock for the so-called "third-generation" biofuel². However, to improve the economics and the overall environmental impacts of biofuel production from microalgae, the use of reclaimed nutrients and CO₂ sources has been suggested to be a key step⁶. Moreover, growing microalgae in waste streams may also be seen as a mean to remove nutrients to meet discharge limits enforced by the Nitrate Directive (91/676/EEC).

Within this context, an experimental research has been carried out to investigate the possibility of using microalgae to remove nitrogen from the digestate produced by the codigestion of piggery manure and agrowastes and to produce energy from the microalgal biomass by anaerobic digestion.

6.2 Material and Methods

6.2.1 Photobioreactors for microalgal cultivation

The first experimentation was used to assess microalgal growth, the second to produce algal biomass to be tested for biogas production by anaerobic digestion tests. In both cases a mixed culture of *Chlorella vulgaris* and *Scenedesmus sp* grown on a municipal digestate was used as inoculum.

The pilot-scale experimentation was located at a piggery farm in Northern Italy (Cremona), breeding up to 20,000 pigs and hosting a full scale wastewater treatment plant (WWTP) and an anaerobic digester (more details in: Scaglione et al., 2015⁷). A pilot-scale photobioreactor (PS-PB) was installed outdoor and run in natural light and temperature conditions. The PS-PB was a 67 L plexiglas column ($\text{Ø} = 29$ cm) fed on a mixture of digestate supernatant after centrifuge separation and WWTP final effluent (1:4 v/v). CO_2 and mixing were provided by bubble aeration at a flow rate of 0.2-0.3 vvm. Phosphorus was added to the feed to adjust the N/P ratio to 1:10. The PS-PB was run in batch during the first 9 days, later it was fed continuously for 25 d, with 10 d hydraulic retention time (HRT).

The lab-scale photobioreactor (LS-PB) was a 12 L plexiglas column (net volume =10 L, $\text{Ø}=10$ cm). Light was provided by 6 fluorescent OSRAM, FLORA, 18 W lamps with dark/light period about 12 hours. CO_2 and mixing were provided by bubble aeration. The LS-PB was fed on a mixture of digestate supernatant and tap water. The LS test was started in semi-batch during the first 17 days, at an average HRT of 10 d, and

decreasing gradually the fraction of tap water in the feed, so that $\text{NH}_4\text{-N}$ concentration in the feed rose from 104 to 640 mg L^{-1} (corresponding to a volumetric loading rate of 10.4 to 64 $\text{mgN L}^{-1}\text{d}^{-1}$ photobioreactor). During the last 10 days, the column was run in batch.

6.2.2 Analytical determinations

Analyses of total nitrogen (N_{tot}), total phosphorus (P_{tot}), COD and Total Suspended Solids (TSS) were carried out according to Standard Methods 5220-D (APHA-AWWA-WPCF, 1992), while ammoniacal and nitrate nitrogen were measured using Hach Lange kits (LCK 302 and 340, respectively). Prior to chemical analyses, samples were filtered on 0.45 μm pore size nitrocellulose membranes. Optical density was measured at 665 nm. Temperature, pH, conductivity and dissolved oxygen were measured directly on site by suitable probes.

6.2.3 Biochemical methane production (BMP) tests

The algal biomass grown in the LS-PB was harvested by settling and centrifuging (4000 rpm, 10 minutes), and tested for biomethane potential. BMP tests were performed in duplicate using a commercial laboratory instrument (AMTPS, Bioprocess control, Sweden). Relevant testing conditions were: temperature 35 ± 0.5 °C, digestate from the farm as inoculum (12 gVS kg^{-1}), inoculum to substrate ratio between 1.1 and 2.5 (gVS gVS^{-1}) and digestion time 60 days. The BMP of algal biomass was compared to that of cow manure.

6.3 Results

During pilot scale test, algae grew rapidly, as demonstrated by the immediate raise of pH over 9 and by the doubling of the optical density during the first 9 d of operation in batch mode. During the continuous mode, algal density remained quite constant, around the average value of 0.55 gTSS L^{-1} , and the efficiency of N_{tot} and of N-NH_4 removal were both very high (on average 80 and 87%, respectively). **Figure 6.1** reports the concentrations of TSS and N-NH_4 at the inlet and at the outlet of the photobioreactor during the continuous run.

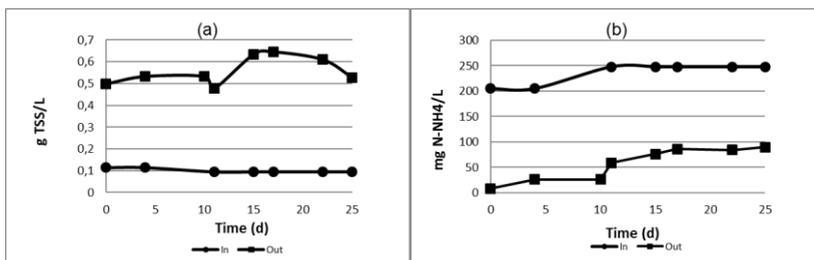


Figure 6.1 TSS (a) and $\text{NH}_4\text{-N}$ (b) concentrations at the inlet and at the outlet of the PS photobioreactor.

As for the LS-PB, the $\text{NH}_4\text{-N}$ removal was good (87.5%); however, nitrification occurred and $\text{NO}_3\text{-N}$ concentration reached 100 mg L^{-1} after 16 days. Algal cells formed aggregates, probably due to the high residual polyelectrolyte concentration in the digestate supernatant, and did not grow as expected. TSS concentration slightly decreased to 1.3 g L^{-1} , from the initial value of 1.6 g L^{-1} . Therefore, the feeding was stopped and the column was run in batch. In the meantime the polyelectrolyte had probably been degraded and algae had the

time for acclimation. After 10 d of batch mode, algal biomass had increased to 1.8 gTSS L⁻¹ and NH₄-N concentration was 12.9 mg L⁻¹. However, nitrification was still observed with significant production of nitrate and nitrite (see **Table 6.1**). Apparently, a mixed consortium of microalgae, N-NH₄ and N-NO₂ oxidising bacteria developed which can compete for CO₂ and for N itself, as also observed by Risgaard-Petersen et al. (2014)⁸. The causes and effects of NO₂ accumulation should still be studied: in principle algae should be able to use N-NO₂ but high concentrations could be inhibitory⁹.

Table 6.1 Concentrations (mg L⁻¹) of the different nitrogen forms in the LS-PB at the starting (T17) and at the end (T27) of the batch period.

Parameters (mg L ⁻¹)	Time	
	T17	T27
Total Nitrogen	400	389
N-NH ₄	80	12.9
N-NO ₂	n.d.	95
N-NO ₃	110	268

In **Figure 6.2**, results of the BMP test are reported. Experimental data were fitted to a first order kinetic model ($BMP(t) = BMP_{\infty} \times (1 - e^{-k_1 \times t})$). As summarised in **Table 6.2**, microalgae showed a higher ultimate anaerobic degradability although the degradation kinetics was slower than that of manure. However, co-degradation of microalgae and manure appears feasible when digesters with long HRT are used, as those used for co-digestion of manure and energy crops.

Table 6.2 Results of BMP tests on microalgae and manure samples

Parameters	Samples	
	Microalgae	Manure
BMP ₆₀ [L@273K,1atm/kgVS]	207	160
BMP _∞ [L@273K,1atm/kgVS]	243	163
k ₁ [d ⁻¹]	0.032	0.080
R ² [-]	0.995	0.998

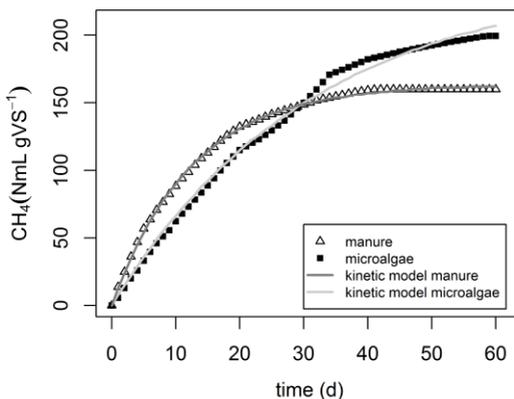


Figure 6.2 Specific methane production (CH_4 NmL gVS^{-1}) in BMP tests. Dots: experimental data; lines: first order kinetics model fitted on data.

6.4 Conclusions

The experimental results confirmed that algal based processes are promising for treating digestates from piggery farms and the produced biomass can be fed to anaerobic digesters to increase biogas and energy recovery. However, some aspects should be further investigated such as the occurrence of undesired flocculation, and the regulation of the nitrifiers/microalgal consortium.

6.5 Acknowledgment

This work has been supported by Fondazione Cariplo, grant n° 2014-1296.

6.6 References

1. Marazzi, F., Mezzanotte, V. & Ficara, E. Using microalgae for removing nitrogen from supernatant from biosolids dewatering. *IWA Spec. Conf. Nutr. Remov. Recover. Mov. Innov. into Pract. 18 – 21 May 2015, Gdańsk, Pol.* (2015).
2. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**, 294–306 (2007).
3. Sturm, B. S. M. & Lamer, S. L. An energy evaluation of coupling nutrient removal from wastewater with algal biomass production. *Appl. Energy* **88**, 3499–3506 (2011).
4. Nielsen, S. L., Enríquez, S., Duarte, C. M. & Sand-Jensen, K. Scaling maximum growth rates across photosynthetic organisms. *Funct. Ecol.* **10**, 167–175 (1996).
5. Li, Y., Horsman, M., Wang, B., Wu, N. & Lan, C. Q. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Appl. Microbiol. Biotechnol.* **81**, 629–936 (2008).
6. Clarens, A. F., Resurreccion, E. P., White, M. A. & Colosi, L. M. Environmental life cycle comparison of algae to other bioenergy feedstocks. *Environ. Sci. Technol.* **44**, 1813–1819 (2010).
7. Scaglione, D. *et al.* Autotrophic nitrogen removal by a two-step SBR process applied to mixed agro-digestate. *Bioresour. Technol.* **176**, 98–105 (2015).
8. Risgaard-Petersen, N., Nicolaisen, M. H., Revsbech, N. P. & Lomstein, B. A. Competition between ammonia-oxidizing bacteria and benthic microalgae. *Appl. Environ. Microbiol.* **70**, 5528–5537 (2004).

9. González-Fernández, C., Molinuevo-Salces, B. & García-González, M. C. Nitrogen transformations under different conditions in open ponds by means of microalgae-bacteria consortium treating pig slurry. *Bioresour. Technol.* **102**, 960–966 (2011).

7. Biogas production from microalgae grown on supernatant from biosolids dewatering

Abstract

Microalgae have been grown in a 85 L, outdoor bubble column located in a large wastewater treatment plant (WWTP) in Northern Italy. The supernatant from biosolid dewatering was used as N and P source while the off-gas from the CHP engine was used as CO₂ source. The microalgae suspension was used to perform solid/liquid separation tests and served as co-substrate with the mixed primary/secondary waste sludge from the same WWTP in anaerobic digestion semi-continuous tests.

Abstract book: World Conference on Anaerobic Digestion
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Keywords

Microalgae, cultivation, supernatant, harvesting, co-digestion

7.1 Introduction

Microalgae are nowadays considered as an important source of high value compounds as well as a potential renewable source of energy. In suitable culture conditions, microalgae are more efficient than vascular plants and their biomass and oil productivity greatly exceed that of vascular plants ¹. For example, the median value of the maximum specific growth rate of microalgal species is approximately 1 day^{-1} whereas for higher plants it is 0.1 day^{-1} or less ². Microalgae are able to sequester atmospheric or waste CO_2 ³, to remove nutrients from wastewaters ⁴ and to remove metals. A potential field of application of algal cultures is that of wastewater treatment plants (WWTPs). In fact, WWTPs are often operated with long HRT in order to maximize nitrification and achieve nitrogen removal by the subsequent denitrification and this causes the production of relatively low amounts of waste sludge with a high mineralization degree, so with a low methanogenic potential. Moreover, the sludge treatment line includes sludge dewatering producing a supernatant with high nutrient content which must be recycled to the water line for nutrient removal and which can be suitable for microalgal culturing. Methane yield values of various microalgal strains can be found in the literature and vary within large intervals ⁵ suggesting their potential as co-substrate in anaerobic digesters. The present paper focuses on the possibility of including algal cultures in the wastewater treatment sequence by growing them on the supernatants from sludge dewatering with the double aim of removing nitrogen and producing biomass to be fed to anaerobic digesters. Microalgal biomass needs to be harvested

before being sent to anaerobic digester and harvesting costs may account for up to 20–30% of the total cost of algal biomass production, due to small size of algal cells. Here the results of testing different harvesting processes are presented.

7.2 Materials and Methods

7.2.1 *Microalgae culturing*

Microalgae were grown in a 85 L, outdoor plexiglas column (150 cm height, 28.9 cm internal diameter), located in a large WWTP in Milano (220.000 I.E.), Italy. The column was connected to a feeding tank (150 L approx.) through a variable-flow peristaltic pump (Q_{\max} 120 mL min⁻¹). The produced suspension was sent to a storage tank (150 L approx.) by a gravity driven overflow. Additionally, flue gases coming from the CHP unit were collected, sent to a demister, and pumped to the column. The main monitoring parameters were the total and dissolved solid concentrations, nitrogen and phosphorus contents and optical density, temperature, pH, conductivity and dissolved oxygen. Sampling and analyses were performed 1-3 times a week within the period from 26 May to 28 November. The hydraulic retention time varied between 7 and 11 days, and the nitrogen content in the feed varied between 40 and 340 mg N L⁻¹. *Chlorella sp.* and *Scenedesmus sp.* were the main algal strain in the pilot plant.

7.2.2 *Biomass harvesting and solid/liquid separation tests*

Microalgal biomass was processed by three solid/liquid separation mechanisms. Gravity settling was performed during

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2 h in 2 L Imhoff cone (3 repetitions). Centrifugation tests were performed by using a lab centrifuge at 4000 rpm during 11 min (4 repetitions). The separated phases were characterized in terms of the volume, total solids (TS) and total volatile solids (TVS) content. Finally, flocculation tests were performed by using different dosages of the EM 516 GK polyelectrolyte (which is normally used at the WWTP in digestate centrifuging), and by characterizing the remaining supernatant through TSS measurements. The tested doses ranged between 1 and 4 mg g_{TSS}⁻¹. Tests were repeated 5 times.

7.2.3 Anaerobic digestion

A lab-scale anaerobic digester (Lab-AD) was used for sludge-algal co-digestion tests. The Lab-AD was a 2.4 L glass reactor, equipped with a thermostatic water bath. Stirring was obtained by a vertical mechanical stirrer operated at 80 rpm. One of the outlets of the digester was connected to a complementary system for measuring the produced methane comprising a plastic graduated cylinder (1000 mL) turned down inside a 1 N NaOH bath where the produced CO₂ is absorbed and the remaining gases, mainly CH₄, displaced the liquid volume according to its production. The Lab-AD was operated in a semi-continuous mode by manually feeding and discharging from 1 to 3 times a week during 6 months. Four feeding conditions were applied, as summarized in **Table 7.1**. As a reference, operational conditions of the full-scale anaerobic digester (*FS-AD*) are also listed.

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Table 7.1: Anaerobic digesters operational parameters.

Reactor	Phase	Time (d)	Algae %*	OLR g _{vs} L ⁻¹	HRT (d)	T (°C)
	1	63	0	0.79	21	32
	2	32	23	0.42	47	32
	3	33	24	0.58	34	30
Lab-AD	4	15	0	0.45	39	27
FS-AD	■	■	0	0.52	30	35

FS-AD ■Average annual conditions; *% of microalgal VS in the feed; OLR: Organic loading rate;HRT: Hydraulic Retention Time

7.2.4 Analytical methods

Total nitrogen and total phosphorus were measured using the persulfate method 4500-P J of (APHA, AWWA, 2005) while N-NH₄⁺ was analyzed according to the spectrophotometric-indophenol methodology of (IRSA-CNR, 2004). Temperature, pH, conductivity and dissolved oxygen were measured directly on field by suitable probes, while solid contents were determined according to methods. Optical density was measured at 665nm. The anaerobic digester monitoring consisted in the measurement of TS, TVS, pH, T, TKN and TN, following the recommended procedures of (APHA, AWWA, 2005)⁸, and FOS/TAC ratio using the method described by Lossie e Putz (2008)⁹.

7.3 Results and discussion

7.3.1 Microalgae biomass production

Microalgal production varied in the course of the experimentation according to the seasonal variations in temperature and irradiance and in the feeding strategy as

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reported in **Table 7.2**. The obtained microalgal suspension was in the range of 0.35-0.82 g_{TS} L⁻¹.

Table 7.2: Average values for the bubble column operational parameters

Month	HRT (d)	NLR (g _{vs} L ⁻¹ d ⁻¹)	I (W m ²)	T (°C)	Microalgae* (g _{TS} L ⁻¹)
June	10.4	29.8	257	24.0	0.82
July	9.9	20.4	232	23.5	0.42
August	10.9	18.0	205	24.7	0.35
September	7.4	34.3	166	20.9	0.53
October	7.4	39.5	91	15.1	0.52

*Concentration of microalgal suspension, I:irradiance (W m⁻²).

7.3.2 Microalgae harvesting efficiency

As seen before, the microalgal suspension produced in the bubble column is substantially diluted, making harvesting a necessary step before the AD process. In settling and centrifugation tests, the solid capturing efficiency (η_m defined as the fraction of microalgae mass retrieved in the separated phase) was computed (**Figure 7.1**).

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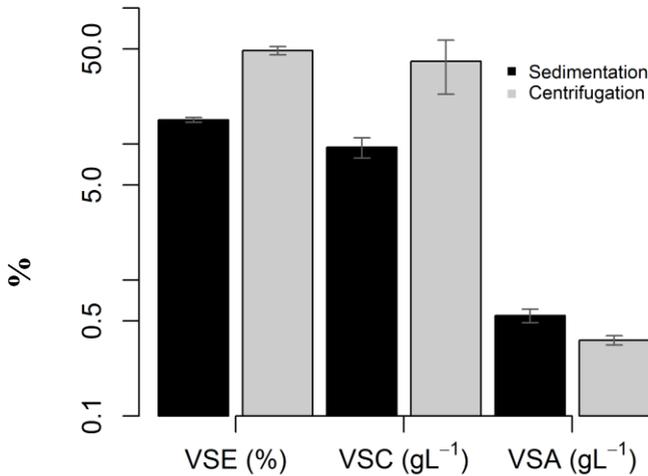


Figure 7.1. Results of settling and centrifugation tests. VSE is VS separation efficiency (%), VSC is VS present in solid cake (g L^{-1}) and VSA is VS present in algal suspension. All data are mean \pm standard error ($n = 3$).

Centrifugation was much more efficient than sedimentation in terms of both the η_m values, and the concentration of solids in the separated solid cake. While sedimentation achieved a maximum of $15.1\text{g}_{\text{TS}} \text{L}^{-1}$ ($11.3\text{g}_{\text{TVS}} \text{L}^{-1}$), centrifugation was able to achieve up to $71\text{g}_{\text{TS}} \text{L}^{-1}$ ($56\text{g}_{\text{TVS}} \text{L}^{-1}$). Additionally, η_m lower than 16% in sedimentation tests and up to 52% for centrifugation, which means there is still a significant amount of remaining biomass in the supernatant, which will be lost. To improve the separation efficiency flocculation tests were performed in order to assess the optimal flocculant dosage. The best results were obtained with $1.5 \text{ mg g}_{\text{TSS}}^{-1}$ which

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corresponded to the lower solid concentration in the clarified supernatant (**Figure 7.2**).

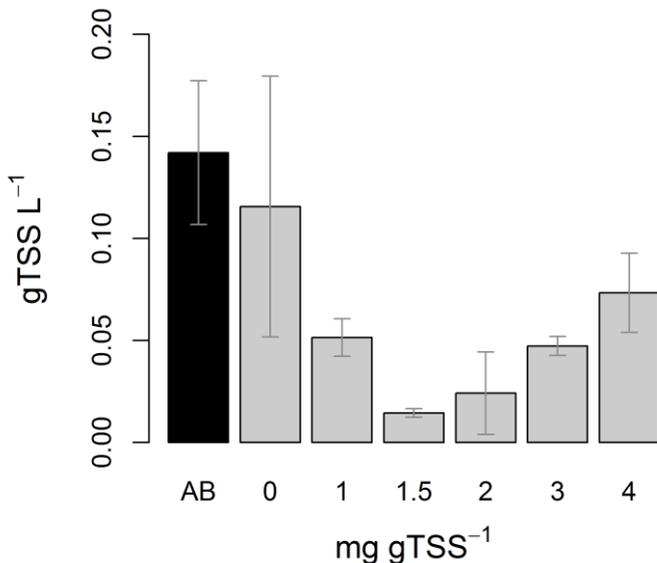


Figure 7.2. Flocculation tests: in x axis is the flocculant dosage, in y axis the TSS concentration in the clarified phase. AB is Algal Biomass. All data are mean \pm standard error ($n = 3$).

Those results are in agreement with other works^{11, 12}, that confirm centrifugation as the most efficient method (over 95% algal biomass could be obtained). However, high capital costs, energy input and operational costs prevent its at a larger scale, as shown in **Table 7.3**.

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Table 7.3: Summary of the performance and cost of different dewatering techniques, according with Uduman et al. (2010)¹².

Dewatering process	Highest possible yield	Energy consumption	Reliability	Limitation
Flocculation	>95% removal microalgae	Low for slow mixing; varies	Very good	Flocculants can be expensive, and their residues may cause problems
Centrifugation	12% TSS	Very high (8 kWh/m ³)	Very good	High energy input
Gravity sedimentation	0.5-1.5% TSS	0.1 kWh/m ³	Poor	Process is slow

Currently, centrifugation is most commonly used to harvest microalgae containing high-value bioproducts such as highly unsaturated fatty acids, pharmaceuticals and other.

7.3.3 Biogas production and organic matter degradation from microalgae-waste sludge codigestion

Once the microalgal biomass was concentrated, it was mixed with waste sludge in order to carry out the co-digestion tests in the lab-scale anaerobic digester. To compare the performance of the original AD process with waste sludge as the sole substrate with the proposed co-digestion alternative, a 7:1 ratio sludge-biomass was fed, using the specific methane yield (Y_{CH_4} , NL_{CH_4} g_{VS}^{-1}) and VS removal efficiency as main performance indicators. As detailed in **Table 7.1**, two reference phases were conducted, one at the beginning and one at the end of the experimentation which were different in the feeding conditions. Phase 1 was operated at a higher OLR than the FS-AD, while in Phase 4 a lower OLR and longer HRT were applied. However, higher Y_{CH_4} and VS removal were observed

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in phase 1 than in phase 4, suggesting a decreased degradability of the waste sludge fed to the lab-scale AD, depending of the operation of the water line of the full scale WWTP (**Table 7.4**). During the first phases of microalgae/sludge co-digestion, the OLR was reduced to $0.44\text{g}_{\text{vs}} \text{L}^{-1} \text{d}^{-1}$ in order to allow for the adaptation of the anaerobic consortium to the new feed. As a result, higher Y_{CH_4} and VS removal were observed and no detrimental effects of microalgae feeding were observed in the lab-scale AD performance. During phase 3 and 4 the same amount of waste sludge was fed to the lab-scale AD while an extra 24% of the OLR was obtained in phase 3 by adding the microalgal suspension. However, the overall methane production remained almost constant suggesting a poor conversion of the microalgal biomass into methane, possibly requiring longer degradation time. As suggested by Mussgnung et al., 2010¹⁰, the accessibility of the microalgal cell content to the anaerobic microbial consortium may depend on the presence and nature of the cell wall, leading to higher methane yields for microalgal species without or with a protein-based cell. *Scenedesmus sp* and *Chlorella sp* may express a low methane yield having a cellulose-based cell wall.

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Table 7.4: Performances of the lab-scale anaerobic digester.

Phase	Y_{CH_4} (NmL_{CH_4} g_{vs}^{-1})	VS removal efficiency (%)	pH	TKN ($mgN L^{-1}$)	NH_4^+ ($mgN L^{-1}$)
1	196	34	7.21	258	245
2	211	38	7.22	289	272
3	107	29	7.14	n.a.	n.a.
4	140	20	7.11	173	n.a.
FS-AD■	220±22	30±12.5			

■ Annual average values

7.4 Conclusions

Experimental results suggests that: microalgae can be cultured on the supernatant from biosolid dewatering in outdoor conditions. Microalgae can be co-digested with waste sludge without detrimental effects on the anaerobic consortium. However, longer degradation times than those available in the FS-AD could be required to guarantee an efficient methane production from the microalgal suspension.

Microalgae can be harvested by centrifugation and the flocculant used for biosolid dewatering appeared effective and could improve the microalgae harvesting efficiency.

7.5 Acknowledgment

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7.6 References

1. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**, 294–306 (2007).
2. Nielsen, S. L., Enríquez, S., Duarte, C. M. & Sand-Jensen, K. Scaling maximum growth rates across photosynthetic organisms. *Funct. Ecol.* **10**, 167–175 (1996).
3. Wang, B., Li, Y., Wu, N. & Lan, C. Q. CO₂ biomitigation using microalgae. *Appl. Microbiol. Biotechnol.* **79**, 707–718 (2008).
4. Sturm, B. S. M. & Lamer, S. L. An energy evaluation of coupling nutrient removal from wastewater with algal biomass production. *Appl. Energy* **88**, 3499–3506 (2011).
5. Ward, A. J., Lewis, D. M. & Green, F. B. Anaerobic digestion of algae biomass: A review. *Algal Res.* **5**, 204–214 (2014).
6. Standard-Methods. *Standard Methods for the examination of water and wastewater*. (American Public Health Association/American Water Works Association/Water Environment Federation, 2005).
7. IRSA-CNR. in *Metod. Anal. per le acqua* 16 (2004).
8. APHA, 2005. *Standard Methods for The Examination of Water Wastewater and Wastewaters*,.
9. Lossie, U. & Putz, P. *Targeted control of biogas plants with the help of FOS/TAC*. (2008).
10. Mussgnug, J. H., Klassen, V., Schlüter, A. & Kruse, O. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J. Biotechnol.* **150**, 51–56 (2010).
11. Zhou, Wenguang Min, Min Hu, Bing Ma, Xiaochen Liu, Yuhuan Wang, Qin Shi, Jian Chen, Paul Ruan,

- Roger Filamentous fungi assisted bio-flocculation: A novel alternative technique for harvesting heterotrophic and autotrophic microalgal cells. *Separation and Purification Technology*. **107**, 158–165 (2013).
12. Ge, S., Champagne, P., Plaxton, W. C., Leite, G. B. & Marazzi, F. Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production. *Biofuels, Bioprod. Biorefining* (2016). doi:10.1002/bbb

8. Conclusions

8.1 General conclusions

This thesis focuses on the potential of microalgae in the wastewater treatment field, with particular reference to nitrogen removal and biomass use for biogas production by anaerobic digestion. The chief requirements for algal growth are macronutrients (especially N), light and CO₂ as carbon source, all factors which are easily available in different kinds of wastewater and, especially, centrate, with seasonal limitations for light, according to site and climate.

Consequently, a great potential exists for placing an algal-based process in the sludge line, using the supernatant from sludge dewatering as a substrate and the off-gas from the cogeneration unit as an additional CO₂ source as well as to buffer pH, and exploiting natural sun radiation. The advantages of this option are related to the decrease of the N load recycled to the biological process and, thus, to the decreased energy needs for N oxidation. As presented in Chapter 1, the removal of nitrogen from supernatants is usually 10-20% of the total influent nitrogen load and involves significant energy costs due to the additional aeration. Moreover, the biogas production would increase using algal biomass as additional organic substrate for anaerobic digestion and to the CO₂ emissions from the co-generation unit would decrease.

The majority of literature data were obtained on pretreated centrate samples and controlled conditions, while the experimental work presented here was conducted also under outdoor pilot-scale conditions, with fluctuations in temperature and solar radiation intensity and showed that:

- Microalgae species as *Chlorella* and *Scenedesmus* can be cultured outdoor using centrate from sludge dewatering as the only nutrient source, in spite of its low phosphorus content.
- Microalgal culturing is also feasible using the liquid fraction of agro-digestate as substrate without any dilution. Commercial activated carbon (AC) proved to be effective to reduce optical density and thus improving light penetration. However, its high cost remains a limitation for a full-scale industrial application. AC produced from renewable biomasses like lignocellulosic substrates may be considered as a perspective alternative in this specific application.
- Average growth rates in indoor and outdoor batch tests were satisfactory and ranged between 0.14 and 0.16 d⁻¹, average biomass production in continuous tests was 50 mgTSS L⁻¹ d⁻¹ on centrate from municipal WWTP. In the case of agro-digestate the biomass production rate increased from 27±13 mgTSS L⁻¹ d⁻¹ on raw digestate, to 82±18 mgTSS L⁻¹ d⁻¹ on digestate undergone to ammonia stripping, and to 220±78 mgTSS L⁻¹ d⁻¹ on stripped digestate furtherly treated with activated carbon. For all the tests the N-NH₄ removal efficiency was up to 80%. The obtained results are in agreement to literature data (e.i. Uggetti et al., 2014¹ and Fouilland et al., 2014²)
- The ultimate specific methane production is slightly higher (+9.5%) for microalgae grown on municipal centrate (208 mL CH₄ gVS⁻¹) than for waste sludge (190 mL CH₄ gVS⁻¹). Similarly, microalgae grown on agriculture centrate: gave a slightly higher specific methane production than manure (207 mL CH₄ gVS⁻¹)

vs 160 mL CH₄ gVS⁻¹). However, the time needed to obtain the ultimate anaerobic degradation of algal biomass was quite long (around 40 days) due to the time required to hydrolyze the microalgal cell wall.

- Microalgae can be harvested by centrifugation and the coagulant used for biosolid dewatering in the WWTP where the experiments were carried out appeared effective and could improve the microalgae harvesting efficiency.

However, some aspects need further investigation.

- The seasonality of the outdoor process is a critical point, which is strongly dependent on light and temperature and on the stability of the culture, whose understanding still needs some sound investigations on the interrelations and ecological processes occurring within the algal/bacteria consortium including also other kinds of organisms (i.e. rotifers, nematodes, etc.).
- The processes for harvesting and dewatering of microalgae need to be compared in terms of efficiency and economic feasibility.^{3,4}
- The microalgal species and the operational parameters should be identified and optimized case by case, particularly for large scale application.

8.2 Future perspectives

Those results created the conditions to continue the experimental work: in the frame of two different projects two raceway pilot plants (1,000 and 800 L volume, respectively) have been installed at the municipal- and agro- WWTPs.

The first project called **IMAP** (**I**ntegration of **M**icro**A**lgae in conventional **W**W**T**P**s** for energy recovery) focuses on the integration of microalgal culturing units within a conventional wastewater treatment scheme with minor modifications of the existing treatment scheme. The second one, called **MICROGATE** (**U**se of **M**icro**A**lgae to **m**iti**G**A**T**E nitrogen pollution from agricultural wastewaters), aims at evaluating the use of microalgae to remove ammonia nitrogen from the liquid fraction of digestates from the anaerobic digestion of animal manure.

An important field which will have to be investigated concerns the recovery of materials after (or instead of) anaerobic digestion. Microalgae are well known for the important substances which can be extracted from their cells and be used for different purposes, including the preparation of nutraceutical, pharmaceutical and cosmetic products, etc. However, the laws in force are nor clear about the fate of algae grown on wastewaters. Many Authors have stressed the stimulant properties of algal biomass for plant growth. In this case also, however, no standard method to evaluate the potential use of algal biomass in the various kinds of agricultural production has been defined.

In the end, the production of new generation plastics could also be an important outcome and, apparently, could be feasible in spite of the absence of specific rules.

8.3 References

1. Uggetti, E., Sialve, B., Latrille, E. & Steyer, J. P. Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. *Bioresour. Technol.* **152**, 437–443 (2014).
2. Fouilland, E., Vasseur, C., Leboulanger, C., Le Floc'h, E., Carrè, C., Marty, B., Steyer, J. P. & Sialve, B. Coupling algal biomass production and anaerobic digestion: Production assessment of some native temperate and tropical microalgae. *Biomass and Bioenergy* **70**, 564–569 (2014).
3. Ahmad, A. L., Mat Yasin, N. H., Derek, C. J. C. & Lim, J. K. Comparison of harvesting methods for microalgae *Chlorella* sp. and its potential use as a biodiesel feedstock. *Environ. Technol.* **35**, 2244–2253 (2014).
4. Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J. & Chang, J.-S. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. *Bioresour. Technol.* **102**, 71–81 (2011).

Appendix

Appendix A.1: Effluent concentrations in microalgal growth tests on raw digestate (DIG_L). Mean and standard deviation refer to triplicate data.

Time	Average \pm standard deviation (mg L⁻¹)				
days	COD	N-NO ₃	N-NO ₂	N-NH ₄	P-PO ₄
10	2 \pm 0.2	2.6 \pm 1.21	038 \pm 0.35	0.13 \pm 0.11	2.4 \pm 3.6
24	4 \pm 0.1	0.6 \pm 1.08	0.09 \pm 0.16	6.7 \pm 5.3	0.11 \pm 0.01
31	7 \pm 0.2	2.2 \pm 0.20	0.08 \pm 0.01	17 \pm 28	0.28 \pm 0.05
38	11 \pm 0.4	2.7 \pm 0.10	0.26 \pm 0.30	12 \pm 21	9 \pm 4.3
45	16 \pm 0.5	2.3 \pm 0.09	0.08 \pm 0.01	0.32 \pm 0.42	4.2 \pm 2.6
52	19 \pm 1.1	3.1 \pm 0.29	0.93 \pm 1.4	0.33 \pm 0.49	2.1 \pm 1.2
54	24 \pm 1.4	3.4 \pm 0.1	2.4 \pm 2.8	38 \pm 5	0.32 \pm 0.11
61	28 \pm 1.9	3.5 \pm 0.15	0.24 \pm 0.41	0.06 \pm 0.1	1.4 \pm 1.5
66	34 \pm 2.2	4.1 \pm 0.29	4.9 \pm 4.23	16 \pm 20	6.4 \pm 7.5
70	40 \pm 2.5	4.7 \pm 0.28	18 \pm 22	22 \pm 19	2.6 \pm 2.3
74	51 \pm 2.7	4.5 \pm 0.25	47 \pm 49	12 \pm 14	6.4 \pm 6.6
90	58 \pm 2.7	5 \pm 0.4	60 \pm 56	31 \pm 53	22 \pm 1.4
102	65 \pm 2.5	4.4 \pm 0.39	57 \pm 57	5.6 \pm 10	10 \pm 6.3

Appendix A.2: Effluent nitrate concentrations from each replicate test vial (T1, T2, T3), in microalgal growth tests on raw digestate (DIG_L).

Time	N-NO₃ (mg L⁻¹)		
days	T1	T2	T3
10	0.03	0.72	0.38
24	0.00	0.00	0.27
31	0.09	0.07	0.08
38	0.10	0.61	0.07
45	0.08	0.08	0.06
52	0.25	0.00	2.5
54	0.01	5.4	1.7
61	0.00	0.00	0.72
66	0.02	7.3	7.4
70	0.20	10	43
74	0.09	43	98
90	0.07	67	112
102	0.08	56	113

Appendix B.1: Effluent concentrations from each duplicate test vial (T1, T2), in microalgal growth tests on stripped digestate (DIG_S).

Time days	Parameters (mg L ⁻¹)									
	COD		N-NO ₃		N-NO ₂		N-NH ₄		P-PO ₄	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
5	1,100	1,201	8.5	15.72	1.7	37	4.1	26	2.6	2.4
10	1,136	1,065	9.31	8.25	0.05	10	0.54	10	1.0	1.0
15	1,232	1,151	10.2	10.2	0.42	0.48	0.31	29	0.9	1.0
18	1,380	1,400	10.7	10.4	8.6	0.18	17	45	15	11
23	1,136	1,217	9.56	10.3	8.2	3.0	8.3	59	9.4	16
29	1,140	1,183	9.78	9.08	6.2	4.1	96	33	3.4	4.9
31	1,240	1,080	9.81	14.13	19	0.9	63	143	5.4	5.3
33	1,260	1,230	6.52	5.54	23	11	124	210	4.6	4.1
36	1,110	1,310	12.64	11.86	30	27	40	255	18	4.7
38	1,170	1,090	15.14	9.92	24	32	42	82	9.7	5.1
40	1,100	1,070	10.54	9.08	21	34	27	68	3.7	5.0

Appendix B.2: Effluent concentrations from each duplicate test vial (T1, T2), in microalgal growth tests on stripped and adsorbed digestate (DIG_S&A).

Time days	Parameters (mg L ⁻¹)										
	COD		N-NO ₃		N-NO ₂		N-NH ₄		P-PO ₄		
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	
5	n.a.	n.a.	0.60	0.54	0.04	0.00	n.a.	18	334	379	
8	14	15	0.7	0.8	0.0	0.0	n.a.	71	287	310	
10	26	28	0.8	0.9	0.0	0.0	n.a.	20	267	258	
15	44	49	1.0	1.1	0.0	0.0	2	7	218	241	
18	66	73	0.9	1.1	0.0	0.0	0.2	0.1	290	315	
23	84	90	0.8	0.8	0.1	0.0	0.0	0.0	299	390	
29	101	109	0.9	0.8	0.0	0.0	16	6	207	209	
31	119	127	0.8	0.7	0.1	0.0	24	39	216	228	
33	135	142	0.8	0.6	0.5	0.1	0.3	17	268	277	
36	143	157	1.0	0.6	0.4	0.0	14	77	208	262	
38	153	164	1.5	0.6	1.1	0.1	8.3	37	241	193	

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