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A New Biorefinery Model For Livestock Farming: Microalgae Cultivation For Animal Slurries Valorization

Ph.D. Thesis

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Summary

Abstract

Development of livestock farming sector poses serious concerns on its environmental impact due to the production of huge volume of slurries characterized by high concentrations of organic and mineral pollutants, mainly in the form of ammonia, phosphates and carbon compounds.

The aim of this work was to evaluate the possibility to use microalgae as a biological tool for the depuration of such wastewaters, assimilating the nutrients and producing an added value biomass to be used in different sectors such as the food and feed, nutraceutical and bioenergy.

Chapter 1 deals with the study of a digestate treatment plant characterized by a series of physical-chemical treatments which depurate the liquid fraction of the digestate through membrane technology (ultrafiltration and reverse osmosis) allowing to discharge 50% of the slurry as clean water and to produce ammonium sulfate and nutrients-rich solids/concentrates usable as fertilizers. The ultrafiltration step produces also a clear permeate still rich in soluble nutrients that could be further used as a growth medium for microalgae production. *Chapter 2* intended to demonstrate the possibility to integrate microalgae production with this system, helping to reduce the cost of slurry treatment and improving the energy balance of the process. The tolerance of the microalga *Scenedesmus* sp. to the permeate was evaluated,

results demonstrating that percentage upper than 10% inhibited the growth of this microalga, but below this value productivity up to 124 mg L^{-1} d^{-1} could be obtained. The composition of the culture medium also influenced the biomass composition, with protein, carbohydrate and lipid content being a direct function of ammonia concentrations. It was then demonstrated that integrating microalgae production with anaerobic digestion it is possible to produce 166-190 t y^{-1} of microalgal valuable biomass.

Chapter 3 focused on the possibility to exploit a wild microalga strain (*Chlorella* sp.), isolated in the farm, to improve the depuration of the digestate and the two digestate liquid fraction after centrifugation and ultrafiltration. The results demonstrated that digestate could not support a good growth, as the other two liquid stream, because of low light availability in the culture. Ultrafiltrate, on the other hand, resulted in the best biomass productivity (0.21 g $L^{-1}d^{-1}$) comparable to that obtained in a synthetic medium. All the streams were depurated with ammonia, phosphorus and COD reduction up to 98%, 99% and 70% respectively

Besides these encouraging data it has been found that only 30% of the nitrogen were successfully incorporated in the microalgal biomass due to stripping processes, posing serious environmental concerns on the process

In *Chapter 4* astaxanthin-producer *Haematococcus pluvialis* was cultivated in a treated swine slurry with low-cost cascade filters. Although this microalga is slow-growing and very susceptible to contamination, it showed a sustained growth (up to 60 mg L^{-1} d^{-1} of biomass) in the waste stream reducing all the pollutants present in the wastewater. Moreover it accumulated a good amount of astaxanthin, improving the overall feasibility and sustainability of the process.

Introduction

1. Premise

Human activities, in all their forms, have always been a source of consistent organic and mineral pollution with a significant impact on the environment. Furthermore, the problems of water scarcity and their supply for domestic, industrial or agricultural activities, have highlighted the need to develop and optimize processes that allow an efficient recycling of water resources, both in terms of overall microbiological and chemical quality, and from an economical point of view.

In particular, livestock manure is characterized by a high organic and mineral load that would be appropriate to reduce and/or remove, in order to reuse the effluent directly, as a part of a productive process. Different physical, chemical and biological systems allow the accumulation of organic matter and nutrients in products with more or less high quality features. However, often, the cost of construction, maintenance and operation of these treatment plants are not offset by the value of the produced material, representing a significant cost to companies and public sector.

The development of innovative techniques for the treatment and exploitation of livestock waste is therefore a paramount to successfully address a purification process and ensure high quality standards of treated wastewater.

2. Livestock manure

Livestock manure are defined as waste products excreted by livestock or a mixture of litter and wastes, even in a processed form (Nitrates Directive 91/676/EEC).

These products are exerting pressure on land due to the high amount of nutrients that characterizes them: the composition of the wastewater is highly dependent on the species, the possible dilution with washing water and the type and amount of bedding materials.

It is also influenced by factors that determine the characteristics of the manure, such as nutrition, physiological stage and race of livestock heads.

The proper use of animal manure is, therefore, an opportunity for the maintenance of a balance between the livestock sector and the environment. This kind of wastewater is indeed a useful product for fertilizing land but often treatment is necessary to reduce the nutrients load and/or its volume to export it easily from the farm (Piccinini and Bonazzi , 2005).

The practice of agricultural land fertilization, carried out through manure spreading is subjected to a specific regulation aimed at protecting the groundwater and surface waters from pollution caused, primarily, by the nitrogen (in the form of ammonia and nitrate) present in the wastewater.

The EU Directive 91/676/EEC has dictated the fundamental principles to which subsequent national legislation has equalized with the DL 152/1999 and the DM of April 7th 2006.

The Directive indicates:

• A designation of nitrate vulnerable zones NVZ), in which there is a ban on animal slurry spreading to a maximum annual limit of 170 kg of nitrogen per hectare;

• The regulation of agronomic use of manure with the definition of the "Program of Action".

The DM MIPAF of April 7th 2006 was born aiming at nationally harmonize the diverse regional regulations that have created disparities situations between more or less virtuous regions (Corradini , 2007).

The introduction of this act has therefore changed the regulatory environment forcing farmers operating in NVZ to comply with the new regulation with the consequent sharp increase in manure management costs

that may occur by either increasing slurry spreading surface or through treatments that reduce the organic and mineral load of the wastewater.

3. Anaerobic digestion and digestate.

Anaerobic digestion (AD) is a useful technology to produce renewable energy from manure thus to encourage its proper treatment, recycling and disposal, instead of causing environmental pollution and uncontrolled release of greenhouse gases.

Digestate is the byproduct of the anaerobic digestion and, in relation to the quality of the material subjected to the process, is composed of a liquid and a solid phase, separable with a trivial centrifugation. The literature reports a partial amount of information on the digestate: we know that many organic molecules, such as carbohydrates, proteins, lipids and cellulose are totally or partially degraded to gaseous products (methane and CO_2) or transformed into structural components of microbial cells (Muller *et al*., 1998, Connaughton *et al*., 2006). The liquid phase contains dissolved solids and suspended solids. The anaerobic digestion, causing mineralization of most of the organic molecules, increases the nutrient content in mineral form, solubilizing them in the liquid phase.

The digestate contains high concentrations of nitrogen, phosphorus and all other macro and micro-nutrients. If the digested material contains potentially toxic substances, such as heavy metals or synthetic organic compounds (pesticides or polychlorinated biphenyls), digestion tends to solubilize such substances in the liquid phase (Massé, 2007).

Digestate has the features to represent an excellent fertilizer, as it contains the most important nutrients predominantly in the mineral form. Recent studies on the digestate, have focused the attention on many aspects such as organic matter content (OM) and composition, biological quality and stability, nutrient content and organisms and pathogens xenobiotics concentrations (Schievano *et al*., 2008). The results showed significant reductions of the load of OM during the anaerobic processes and a very high biological stability of the digested residue, similar to that obtainable by composting processes. It was also highlighted the presence of recalcitrant aliphatic and aromatic fractions, possible precursors of humus in agricultural soils. Therefore digestate could be considered an excellent soil conditioner (Schievano *et al*., 2008, Tambone *et al*., 2009).

In the perspective of agronomic reuse of these liquid masses, as is the case of most of the existing biogas plants in Italy and in Europe, the amount of N to be used for agriculture acitivities is subject to Directive 91/676/EEC. It is

then necessary to emphasize that in the event of nutrients excess or presence of heavy metals and xenobiotics, digestate should be subjected to purification treatments upstream agronomic use.

4. Microalgae and wastewater treatment

Microalgae are photosynthetic microorganisms, able to use solar light and carbon dioxide to produce the necessary compounds for their life cycle.

To do that they need to uptake organic and mineral nutrients from the environment incorporating them in the biomass.

Together with bacteria and yeasts belong to the class of microorganisms particularly interesting for their broad applications in both traditional and more innovative biotechnology (De la Noue and De Pauw, 1988).

Along with basic research, applied algology has developed strongly over the past 40 years, beginning in Germany and extending to the United States, Japan, Israel, Italy with the initial aim of getting high production of protein (SCP, Single Cell Protein) and fat (Burlew, 1953). In the 50s the production of *Chlorella* in Japan and Taiwan as a functional food (nutraceutical) had already started and established (Kawaguchi, 1980; Soong, 1980) while in the 60s and 70s took off the idea of using the microalgal growth for the treatment of waste water and for the production of fuels and fertilizers.

Currently are produced in the world, 5000 ton $year^{-1}$ of microalgae biomass with a market value of 1 billion € per year (Pulz and Gross, 2004) mainly within the sectors of the food, feed and nutraceuticals

Wastewater treatment through the use of microalgae is a well known process that exploits the ability of these microorganisms to remove high concentrations of nutrients, allowing their recycling in the biomass, in terms of protein (45-60% dry weight), nucleic acids and fatty acids (De la Noue *et al*., 1992). The removal of nutrients is further increased by the stripping of ammonia and by the precipitation of phosphorus due to the increase of the pH during the algal growth. This increase is also due to the death of many pathogens that do not survive the high pH values, thus allowing the sanitation of the wastewater (Aiba, 1982; Mallick, 2002; Mezrioui *et al*., 1994; Robinson, 1998; Schumacher *et al*., 2003).

Through photosynthesis provide oxygen (1.5-1.92 kg O_2 per kg of biomass) for heterotrophic bacteria that mineralizing organic pollutants, they release carbon dioxide directly usable by algae. This relationship between microalgae and bacteria enables high oxygenation of wastewater treatment tanks and therefore a substantial reduction of both the BOD, and costs due to mechanic oxygenation.

Some species of green algae, especially between *Chlorella* and *Scenedesmus* genera, have proved particular tolerance to high concentrations of nutrients in wastewater both urban and agricultural (E. Kessler, 1991), and for this have been widely studied with regard to their efficiency in removing mainly nitrogen and phosphorus (Bhatnagar *et al*., 2010; Ruiz-Marin *et al*., 2010). Their growth can in fact lead to high rates of removal, more than 80%, of ammonia, nitrates and total phosphorus (Marin *et al*., 2010; Martinez *et al*., 2000; Bhatnagar *et al*. , 2010), confirming that microalgae can be effectively used in their purification and enhancement. For example, it was seen as the green alga alga Botryococcus braunii, reach good levels of growth in a pig slurry containing a nitrate concentration of 788 mg L^{-1}, and with around 80% of removal (An *et al*., 2003).

References

- Aiba S., 1982. Growth kinetics of photosynthetic microorganisms. Adv. Biochem. Eng. 23: 85-156.
- An J.Y., Sim S.J., Lee J.S., Kim B.W., 2003. Hydrocarbon production from secondarily treated piggery wastewater by the green alga Botryococcus braunii. J. Appl. Phycol. 15: 185-191.

- Bhatnagar A., Bhatnagar M., Chinnasamy S., Das K., 2010. Chlorella minutissima - a promising fuel alga for cultivation in municipal wastewaters. Appl. Biochem. Biotechnol. 161: 523-536.
- Burlew J.S., 1953. Algal culture, from laboratory to pilot plant. ISBN-13 978-0872796119.
- Connaughton S., Collins G., O'Flaherty V., 2006. Development of microbial community structure and activity in a high-rate anaerobic bioreactor at 18° C. Water Research 40: 1009-1017.
- Corradini E, 2007. Direttiva nitrati e reddito, un peso insostenibile?. Supplemento Suinicoltura 11.
- De la Noüe A., Laliberte G., Proulx D., 1992. Algae and wastewater. J. Appl. Phycol. 4: 247-254.
- De la Noue J., De Pauw N, 1988. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotech. Adv. 6: 725-770.
- Decreto Legislativo n°217/2006. Revisione della disciplina in materia di fertilizzanti. Gazzetta Ufficiale n° 141, Supplemento Ordinario n°152.
- Direttiva Nitrati 91/676/CEE

- Kawaguchi K., 1980. Microalgae production systems in Asia. In Algae Biomass, Shelef and Soeder (Eds), Elsevier/North-Holland Biomedical Press, Amsterdam, 25-33.
- Kessler, E., 1991. Scenedesmus: problems of a highly variable genus of green algae. Bot. Acta. 104, 169-171.
- Mallick N., 2002. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: A review. BioMetals 15: 377–390.
- Martin C., De la Noüe J., Picard G., 1985. Intensive cultivation of freshwater microalgae on aerated pig manure. Biomass, 7: 245-259.
- Martinez M.E., Sanchez S., Jimenez J.M., El Yousfi F., Munoz L., 2000. Nitrogen and phosphorous removal from urban wastewater by the microalga Scenedesmus obliquus. Bioresour. Technol. 73: 263-272.
- Massé, D.I. , Croteau F. Masse L., 2007 The fate of crop nutrients during digestion of swine manure in psychrophilic anaerobic sequecing batch reactors. Bioresour. Technol. 98: 2819-2823.
- Mezrioui N., Oudra B., Oufdou K., Hassani L., Loudiki M., Darley J., 1994. Effect of microalgae growing on waste-water batch culture

on Escherichia coli and Vibrio cholerae survival. Water SciDe la Noüe. Technol. 30: 295-302.

- Muller W., Fricke K., Vogtmann H., 1998. Biodegradation of organic matter during mechanical biological treatment of MSW. Compost Science & Utilisation 6 (3): 42-52.
- Piccinini S., Bonazzi G., 2005. Sistemi integrati aerobici e anaerobici: nuove strade per smaltire gli effluenti zootecnici. L'Informatore Agrario n° 7/2005.
- Pulz O., Gross W., 2004. Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol., 65: 635-648.
- Robinson P.K., 1998. Immobilized algal technology for wastewater treatment purposes. In: Wong, Y.S. and N.Y.F. Tam (eds.), Wastewater Treatment with Algae, Pp: 1–16. Berlin: Springer-Verlag and Lands Bioscience.
- Ruiz-Marin A., Mendoza-Espinosa L.G., Stephenson T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresour. Technol. 101, 58-64.

- Schievano A., Adani F., Tambone F., D'Imporzano G., Scaglia B., Genevini P.L., 2008. "What is the digestate?" In Anaerobic Digestion, opportunity for agriculture and environment. Pag. 7-18.
- Schumacher G., Blume T., Sekoulov I., 2003. Bacteria reduction and nutrient removal in small wastewater treatment plants by an algal biofilm. Water Sci. Technol. 47, 195-202.
- Soong P., 1980. Production and development of Chlorella and Spirulina in Taiwan. In Algae Biomass, Shelef and Soeder (Eds), Elsevier/North-Holland Biomedical Press, Amsterdam, 97-113.
- Tambone, F., Genevini, P., D'Imporzano, G., Adani, F., 2009. Assessing amendment properties of digestate by studying the organic matter composition and the degree of biological stability during the anaerobic digestion of the organic fraction of MSW. Bioresource Technol. 100(12), 3140–2.

Nitrogen and water recovery from animal slurries by a new integrated ultrafiltration, reverse osmosis and cold stripping process: a case study.

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Abstract

The correct management of livestock manure represents one of the major challenge for the agricultural sector development, as it may ensure environmental and economic sustainability of livestock farming. In this work, a new treatment process called N-Free®, was monitored on two plants treating digested cattle manure (DCM) and digested swine manure (DSM). The process is characterized by sequential integration of solid/liquid separations, ultrafiltration, reverse osmosis and cold ammonia stripping.

Solid and liquid streams were characterized regarding TS, TKN, N-NH_4^+, P and K content allowing to draw a complete mass balance. The main results were a substantial reduction of initial digestate volume (38 and 51% in DCM and DSM respectively) as clean water and a high N-NH_4^+ removal percentage (47 and 71% in DCM and DSM respectively), through cold ammonia stripping, allowing the production of up to 1.8 m^3 concentrated ammonium sulfate, every 100 m^3 of digestate treated. The concentrated streams, rich in either organic or mineral N, P and K, can be efficiently used for land application. The N-Free® technology demonstrated to be a valuable candidate for the path towards nutrient and water recycle, in a new sustainable agriculture and farming concept.

Keywords: livestock manure; agricultural sustainability; N management; solid/liquid separation; membrane technology.

1. Introduction

The development of livestock production sector has led to a great concern for its environmental impact, due to the production of huge volumes of wastewaters characterized by a high organic and mineral load, mainly nitrogen (N) and phosphorous (P) (Petersen *et al*., 2007).

It is well known that losses of N due to spread of manure on land may contribute to environmental pollution through several ways: ammonia and nitrogen oxides emission to atmosphere (ApSimon *et al*., 1991; Bouwman, 1990; Skiba *et al*., 1997) and nitrate leaching to ground and surface water bodies (Smith and Chambers, 1997).

NH_3 in atmosphere is known to accelerate the formation of particulate matter (PM) and can make up a large portion of its final mass (Clarisse *et al*., 2009). Ammonium sulfate and nitrate can make up to 60% of fine particulate $PM_{2.5}$ and 40% of the total mass respectively (Clarisse *et al*., 2009). NH_3 accounts for almost half of all reactive nitrogen released in the atmosphere, having an important role in the acidification and the eutrophication processes (Clarisse *et al*., 2009).

In aquatic ecosystems, excess N and P concentrations might cause diverse problems such as toxic algal blooms, oxygen depletion, fish kills, loss of biodiversity and aquatic plant beds. Nutrients enrichment degrades aquatic ecosystems and impairs the use of water for drinking, industry, agriculture and other purposes (Carpenter *et al.*, 1998).

Current environmental legislations do not deal with, hence do not restrict, potassium (K) application in agricultural soils. Nevertheless, K excess related to its growing concentration in soil, has been reported as a cause for

mineral deficiencies, imbalances, immune suppression and reproductive losses in herbivores (Masse *et al*., 2007).

During the last decades, the problem of manure management has dramatically grown forcing European Community to draw Nitrate Directive guidelines (91/676/CEE) followed by corresponding Italian Rules (Dlgs. 152/99, Dm. 7/4/2006), born to overcome these issues by the application of recommended management practices (RMPs), in particular regulating manure disposal and its agronomical use, limiting nitrogen load in agricultural soils (170 kg N ha^{-1}). In Northern Italy, over 70% of Utilized Agricultural Area (UAA) is defined as Nitrate Vulnerable Zone (National Strategic Nitrate Plan, 2009) while 81% of total ammonia emission in the atmosphere is directly correlated with manure management (ARPA, 2010). Moreover, manure incorrect land application leads to a substantial decrease in nutrients availability for plant growth (Chambers *et al*.,1999), implying the need to integrate cultures fertilizing with chemical products and the increase of agronomical practices economic and environmental costs.

Anaerobic digestion (AD) is a useful technology to produce renewable energy from manure thus to encourage its proper treatment, recycling and disposal, instead of causing environmental pollution and uncontrolled release of greenhouse gases (Kaparaju and Rintala, 2011). The AD

treatment of manure and wastewater results, also, in the production of a biologically stable and partially hygienized organic product, i.e. the digestate that could be efficiently used in agriculture as fertilizer and/or organic amendment (Tambone *et al*., 2009).

Nevertheless, farm land availability is often not sufficient to meet in force regulation on nutrients load limits hence it is necessary to reduce and/or export materials/nutrients to other land. To do this, digested wastewater or slurry must be concentrated or separated into different fractions to be exported from the farm in an economically feasible way.

Solid-liquid separation has been commonly used as a physical treatment process for animal slurries, mainly to improve slurry handling properties, by removing coarse solids and fiber (Burton, 2007). Relatively low cost and simple technologies such as settling basins, screen and press screw separators, have also been applied for the removal of solid material from dilute slurries (Preez *et al*., 2005). More advanced technologies such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) have long been employed in both municipal and industrial wastewater operations (Rautenbach *et al*., 1996) and are more recently exploited for nutrient concentration and liquid manure treatment (Masse *et al*., 2007).

In this broad context a new process called N-Free® based on solid/liquid separation and membrane technology was developed, allowing fractionation of digestates and recovery of concentrated streams. This technology has found, recently, in collaboration with the University of Milan (Italy), full scale application in North Italy to treat anaerobic digested animal slurries because anaerobic pre-treatment allows chemical and physical modification of slurries, i.e. organic matter degradation and organic-N ammonification (Schievano *et al*., 2009) allowing high process performances.

The objective of this research was to monitor this process at full scale level to evaluate its efficiency in terms of mass and nutrients balances, with particular reference to N removal from digestate.

2. Methods

2.1. N-Free® process

The N-Free® process (Fiolini e Savani s.r.l., Brescia, Italy) is characterized by a series of physical/chemical treatments which allow separation of digestate liquid fraction from digestate solid fraction by means of solids and concentrates and the production of ammonium sulfate. The N-Free® unit operates in batch mode, treating about 14 m^3 of digestate for each cycle. The number of cycles per day, depending on total daily treated volume (50 or

100 m^3 day^{-1}), normally ranges from 4 to 7. In this work, two N-Free® plants located in Northern Italy Nitrate Vulnerable Zones were monitored: the first treats 50 m^3 day^{-1} of digested cattle manure (further called DCM) and the second treats 100 m^3 day^{-1} of digested swine manure (further called DSM).

The process scheme is summarized in Figure 1. The first separation is achieved with a screw press (SP) separator (Doda, Mantova-Italy). The separation resulted in a solid and liquid stream. As second step, the liquid fraction of the SP is added of a polyamide flocculant and send to a decanter centrifuge (DC) (MAMMOTH 570/3, Pieralisi, Jesi-Italy), allowing the separation of another solid fraction vs. liquid stream. The liquid enters in the UF-unit, that is equipped with a 40 kDa grafted Polyacrylonitrile membrane (PAN) (Orelis, France) with a maximum exercise pressure ranging from 3.5 to 4.5 bar. The permeate liquid proceeds to the last separation unit, i.e. the RO–step, which includes two consecutive passages on RO membranes (Dow, USA). The permeate from the RO is finally refined in zeolites bed and then discharged to surface-water bodies. On the other hand the concentrate from the RO enters into the cold stripping unit; sludge is added with 15-18 kg m^{-3} sludge of lime which allows pH raising up to 12-12.5. At these conditions, the equilibrium NH_4^+/NH_3 is completely shifted to NH_3 which is easily stripped as gaseous ammonia by using a controlled air flow.

Air plus ammonia is then scrubbed with sulfuric acid producing liquid ammonium sulfate (8% on a wet weight basis as N). Any further missing technical characteristics and data regarding the N-Free® system design and operation were not available from manufacturer as protected by patent.

2.2. Sampling and chemical characterization of N-Free® liquid and solid streams

Liquid and solid streams were sampled from the observed N-Free® plants 3 times during 3 months of plant normal functioning, after the end of one batch cycle. Each sample was took in triplicates, for a total of 9 samples for each step of the process. Samples were stored in 1 l bottles at 4°C overnight and then analyzed.

Total Solids (TS) were measured according to standard procedures (APHA, 1998). Total Kjeldhal Nitrogen (TKN), ammonia nitrogen ($N\text{-}NH_4^+$) were determined using fresh material according to the analytical methods for wastewater sludges (IRSA-CNR, 1994).

P and K contents were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian. Fort Collins, USA) according to 3051A and 6020A EPA methods (EPA, 2007): briefly a representative sample of up to 0.5 g for the solids and 3 g for the liquids were digested in 10 ml of

concentrated nitric acid using microwave heating with a suitable laboratory microwave unit. The sample and the acid were placed in a fluorocarbon (PFA or TFM) microwave vessel which is capped and heated in the microwave unit. After cooling, the vessel content was diluted to 50 ml, filtered with 0.45 μm cellulose acetate filters and then analyzed. A certified standard reference material (GBW 07405, soil) from the National Centre for Standard Materials (Beijing, China) was used in the digestion and analysis. Average recovery was 92 ± 4% for all the metals determined. To ensure the accuracy and precision in the analyses, reagent blanks were run with samples. All analyses were performed in triplicate.

2.3. Calculations of solid-liquid separation efficiencies

Solid-liquid separation efficiency is the total mass recovery of solids or nutrients in the solid fraction as a proportion of the total input of solids or nutrients (Svarovsky, 1985):

$$E_t = \frac{U \times M_c}{Q \times S_c} \quad (1)$$

where E_t is the simple separation efficiency; U (kg) the quantity of the solid fraction; M_c (g kg^{-1} w.w.) the concentration of the component (TS, TKN, N-NH_4^+, P, K) in the solid fraction; Q (kg) the amount of slurry

treated and S_c (g kg^{-1} w.w.) is the concentration of the considered component in the slurry. Nevertheless, it is useful to consider the separation effect taking into account the increase in concentration of the components in the solid fraction. Most widely used definition of separation efficiency is the reduced efficiency index E'_t (Svarovsky, 1985):

$$E'_t = \frac{E_t - R_f}{1 - R_f} \quad (2)$$

where R_f=U/Q (solid fraction to total slurry ratio) and E_t=simple separation efficiency.

Eq. (2) satisfies the basic requirement for an efficiency definition whereas it becomes 0 when no separation takes place (E_t=R_f) and 1 when separation is complete (Svarovsky, 1985).

3. Results and discussion

3.1 Chemical characterization of solid and liquid streams

Chemical characterization of liquid and solid streams of both DCM and DSM is shown in Table 1 and 2. All concentrations were reported on wet weight (w.w.) basis.

The TS content was almost double in DCM, as compared to the DSM (71.5 ± 1.7 and 39.2 ± 2.6 g kg^{-1} w.w. respectively). Regarding different N forms concentrations, DCM digestate was characterized by higher concentration of TKN, as compared to DSM digestate (3678 ± 146 and 3351 ± 380 mg kg^{-1} w.w. respectively). In contrast, N-NH_4^+ concentration was slightly higher in DSM (2091 ± 244 mg kg^{-1} w.w.) than in DCM (1786 ± 86 mg kg^{-1} w.w.). As consequence, strong differences in the two initial slurries were found for both organic N content and N-NH_4^+/TKN ratio; DCM showed higher N-org concentration (1892 ± 61 mg kg^{-1} w.w.) than that found in DSM (1259 ± 159 mg kg^{-1} w.w.). Conversely, as expected, N-NH_4^+/TKN ratio was higher in DSM (62.4 ± 3.9 %) compared to that found for DCM (48.5 ± 0.5 %).

Although these differences, both digestates showed weak differences in P and K content (768 ± 15 and 620 ± 2 mg kg^{-1} w.w. for P; 2899 ± 128 and 2524 ± 35 mg kg^{-1} w.w. for K in DCM and DSM respectively).

Solid/liquid separation processes were strictly dependent on the initial slurry characteristics (Table 1 and 2). As result of the first solid/liquid separation step (i.e. SP separator) TS content in the liquid stream was found double in DCM (56.7 ± 3.1 g kg^{-1} w.w.) than in DSM (28 ± 6 g kg^{-1} w.w.). This was probably due to dry matter quality of digestates, i.e. DCM showed an higher amount of floating undigested fiber than DSM, retained in the liquid

fraction after SP separation, such as previously reported, also, for cattle manure (Nielsen and Voorburg, 2004; Burton, 2007). Conversely, DSM showed slightly higher TS content in the solid stream than DCM (262 ± 8 and 227 ± 34 g kg^{-1} w.w. for DSM and DCM respectively).

As consequence of the different behavior at the SP, which is correlated to initial slurries physical-chemical differences, strong variation was found in N content in both liquids and solids. Looking at TKN concentration, while both liquid streams showed similar TKN content (3455 ± 113 and 3255 ± 247 g kg^{-1} w.w. for DCM and DSM respectively), strong divergence was underlined in SP solid streams (3266 ± 70 and 5692 ± 165 mg kg^{-1} w.w. for DCM and DSM respectively).

Organic N content reflected TKN fate: in fact, while comparable N-org concentrations were found in DCM liquid and solid fractions (1818 ± 3 and 1900 ± 272 mg kg^{-1} w.w. for SP liquid and solid respectively), for DSM N-org was more concentrated in the solid fraction (1169 ± 157 and 4036 ± 373 mg kg^{-1} w.w. for SP liquid and solid respectively).

In contrast, $N\text{-}NH_4^+$ was similarly concentrated in both DSM and DCM solid (1656 ± 360 and 1326 ± 293 mg kg^{-1} w.w., respectively) and liquid (2086 ± 90 and 1702 ± 26 mg kg^{-1} w.w., respectively) fractions. Regarding P content, similar concentrations were found in the SP liquid fraction of DCM

and DSM (693 ± 18 and 480 ± 91 mg kg^{-1} w.w. for DCM and DSM respectively). On the other hand, solid fractions out of SP of DCM and DSM showed a strong difference in P concentration (812 ± 17 and 5100 ± 556 mg kg^{-1} w.w. for DCM and DSM respectively).

Probably this difference may be correlated with the diverse degradation potential of the two biogas plants feeding mixtures upstream the N-Free® units. Swine manure feeding was characterized by higher degradability than cattle manure such as stated by higher anaerobic biogasification potential (ABP) and oxygen demand after 20 h of incubation (OD_{20}) compared to those found for cattle manure feeding (609 ± 5 and 324 ± 18 L biogas kg^{-1} dry weight, and 291 ± 18 and 78 ± 2 mg O_2 g^{-1} dry weight, for swine and cattle manure feeding, respectively).

Several authors reported anaerobic processes as a way for P removal by means of organisms growth and consequent P assimilation (Chiou *et al*., 2001; Meyer *et al*., 2005). In this way, high degradability of swine manure promoted microorganism biomass production and lead to high P accumulation in microbial bodies that concentrated in the solid fraction after SP separation. This fact can explain, also, TKN accumulation in the solid fraction of DSM that was, above all, under organic fraction.

K content was found to be similar in both DCM and DSM in the liquid and in the solid fractions (2935 ± 64 and 2496 ± 19 mg kg^{-1} w.w. respectively, for the liquids; 2482 ± 242 and 2993 ± 82, respectively, for the solids).

Proceeding to the DC step, similar behavior was found in both DCM and DSM for TS fractionation, as both liquids and solids are characterized by comparable TS content (17 ± 2 and 12.1 ± 0.5 g kg^{-1} w.w. in the liquids; 192 ± 5 and 196 ± 8 g kg^{-1} w.w. in the solids, for DCM and DSM respectively). Despite this, while both DC liquid streams were characterized by similar TKN content (2037 ± 24 and 2194 ± 59 mg kg^{-1} w.w. for DCM and DSM respectively), strong differences could be observed in the solid fractions (7307 ± 98 and 10179 ± 764 mg kg^{-1} w.w. for DCM and DSM respectively). These differences can be explained, again, taking into consideration different biomass degradability leading to microorganism biomass production and subsequent N and P accumulation in the solid fraction, such as before reported.

DSM liquid stream showed a slightly higher N-NH_4^+ concentration (1895 ± 135 mg kg^{-1} w.w.) compared to that found in DCM (1661 ± 12 mg kg^{-1} w.w.). In the solid fractions this difference was more evident, with the DSM characterized by much higher N-NH_4^+ concentrations (2585 ± 401 mg kg^{-1} w.w.) than that measured in DCM (1340 ± 113 mg kg^{-1} w.w.). Similar N-org

content in both DC liquids and solids fractions were found (390 ± 5 and 374 ± 103 mg kg^{-1} w.w. in the liquids; 6023 ± 116 and 7594 ± 1083 mg kg^{-1} w.w. in the solids, for DCM and DSM respectively).

Looking at P content, DSM, compared to DCM, showed different behavior, as lower concentrations were found in the liquid fraction (53.1 ± 9.8 and 118 ± 22 mg kg^{-1} w.w. respectively) and, conversely much higher P concentrations were observed in the solid fraction (4702 ± 148 and 2731 ± 160 mg kg^{-1} w.w. for DSM and DCM respectively). These data reflected P behavior in solid-liquid separation processes as, in manures and slurries, it is mainly present in the solid-phase fractions (Smith *et al*., 1998).

Besides, in both DCM and DSM, K concentrations remained similar in the liquid and solid streams (2634 ± 134 and 2237 ± 12 mg kg^{-1} w.w. in the liquids; 2751 ± 128 and 2546 ± 116 mg kg^{-1} w.w. in the solids for DCM and DSM respectively).

UF process in both N-Free® plants showed comparable behavior. No substantial differences in ultrafiltration permeates and concentrates were found regarding TS concentrations (8.9 ± 0.4 and 9.5 ± 0.5 g kg^{-1} w.w. in the liquids; 30.1 ± 0.2 and 33 ± 1 g kg^{-1} w.w. in the solids for DCM and DSM respectively) despite starting liquid streams showed more marked differences (see Centrifuge liquid stream in Table 1 and 2). Therefore UF

was more efficient when digested cattle slurry was used because UF cut off particles larger than 40 kD independently of their concentration so that ultrafiltration permeate tended to assume similar TS concentration. This fact indicated that the amount of TS having size lower than 40 kD was similar for the two liquid streams.

TKN content in the liquid after UF was found slightly higher in the case of DSM (1882 ± 35 mg kg^{-1} w.w.), compared to DCM (1460 ± 14 mg kg^{-1} w.w.) and most of the permeated TKN was in the form of $N\text{-}NH_4^+$ as this form is water soluble and so permeated the membrane (97.5 ± 0.7 and 97.3 ± 0.5 % for $N\text{-}NH_4^+$/TKN ratio, in DSM and DCM respectively).

In parallel, $N\text{-}NH_4^+$ concentration was slightly higher in the DSM permeate (1815 ± 25 mg kg^{-1} w.w.) compared to the DCM permeate (1460 ± 14 mg kg^{-1} w.w.). In contrast, both concentrates showed similar TKN content (2936 ± 175 and 2996 ± 204 mg kg^{-1} w.w. for DSM and DCM respectively) and similar $N\text{-}NH_4^+$ concentrations (1852 ± 444 and 1522 ± 48 mg kg^{-1} w.w. for DSM and DCM respectively).

In the case of P, higher concentrations were found in the DSM permeate (39.6 ± 3.1 mg kg^{-1} w.w.) than that in the DCM permeate (8.70 ± 1.04 mg kg^{-1} w.w.). On the other hand, DCM concentrate showed higher P content (320 ± 20 mg kg^{-1} w.w.) as compared to DSM concentrate (255 ± 7 mg kg^{-1}

w.w.). These results indicated that above all P was present under unsoluble form (or organic form) preferably in DCM.

K was found in comparable concentrations in permeates (2419 ± 153 and 2340 ± 59 mg kg^{-1} w.w. for DCM and DSM respectively) and in the concentrates (2523 ± 159 and 2230 ± 416 mg kg^{-1} w.w. for DCM and DSM respectively).

RO step accounted for the complete removal of TS, as negligible TS was left in both permeates (u.d.l. and 0.34 ± 0.04 g kg^{-1} w.w. for DCM and DSM respectively).

A different behavior was observed regarding TKN and N-NH_4^+ since the RO permeate of DSM was characterized by higher nitrogen content (88.5 ± 5.2 and 72.5 ± 9.1 mg kg^{-1} w.w. for TKN and N-NH_4^+ respectively) compared to that of DCM (8 ± 1 and 7.1 ± 1.5 mg kg^{-1} w.w. for TKN and N-NH_4^+ respectively). These results indicated that RO was effective in the ammonia removing in both liquid streams, giving comparable final results.

Almost complete P removal from the liquid fraction was observed in the case of DSM (0.85 ± 0.04 mg kg^{-1} w.w.), while in the case of DCM permeate relatively higher concentration was found (3 mg kg^{-1} w.w.). Conversely, DSM concentrate was characterized by 5-fold higher P concentration than DCM concentrate (149 ± 11 and 25.3 ± 1.3 mg kg^{-1} w.w.

for DSM and DCM respectively), because of the higher concentration characterizing ultrafiltration liquid fraction of DSM than DCM (Table 1 and 2). K was also efficiently removed from permeates, in both case studies, and found in concentrates, DCM showing a much higher content (9936 ± 85 mg kg^{-1} w.w.) than that found in DSM (7685 ± 38 mg kg^{-1} w.w.).

Cold ammonia stripping led to the production of ammonium sulfate, which in the case of DSM was characterized by slightly higher N content than that found in DCM (61.1± 0.5 and 51.2 g kg^{-1} w.w. for DSM and DCM respectively).

After the zeolites refining, almost complete removal of all the considered components was observed with the only exception for K which in both cases was found to be at a similar concentration of that observed after RO (11.9 ± 1.2 and 40.4 ± 1.2 mg kg^{-1} w.w. for DCM and DSM respectively).

3.2 Mass balances and reduced separation efficiency indexes (E'_t).

The mass-balances of both N-Free® plants are summarized in Figure 2 and 3. Regarding wet weight balance, solid fractions after SP and DC separations represented the 32% and 12% of the initial mass while concentrated fractions (i.e. UF and RO concentrates) accounted for the 31.3% and 38.6% of the initial mass for DCM and DSM respectively. These data reflected TS concentration in the digested material (see Table 1 and 2)

and indicated that different digestate characteristics affected, above all, SP and DC steps as later performances resulted to be similar in terms of weight balance.

As a consequence of the process, clean water (dischargeable in the land) after zeolites treatment represented the 37% and the 49% of the total initial mass for DCM and DSM respectively. Differences were due to higher TS contents in cattle digestate than pig digestate that allowed high solid fraction production after SP and DC treatments.

Ammonium sulfate which can be readily used as a soil fertilizer accounted for the 1.2% and 1.8% of the initial slurry mass for DCM and DSM respectively. Difference in ammonia sulfate production depended by N-NH_4^+ content in starting digestate that, because it was higher for DSM (2091 mg kg^{-1} w.w.) than DCM (1786 mg kg^{-1} w.w.), allowed to get an higher ammonia concentration in the reverse osmosis concentrate of DSM (7263 mg kg^{-1} w.w.) than DCM (5690 mg kg^{-1} w.w.) before stripping step. Therefore it can be concluded that higher ammonia concentration in digestates lead to higher ammonia sulfate production.

For what concerns TS fractionation, the first step, i.e. SP separation, resulted more effective in the case of the DCM compared to the DSM, i.e. 31.7% and 20% of TS were removed from digestates (Figure 2 and 3) and

E'_t values was determined as 0.32 and 0.18 for DCM and DSM, respectively (Table 4). Higher performance obtained for DCM with respect to DSM was due to both high total solid content and high TS concentration (see Table 1 and 2).

Similarly, after DC, more effective fractionation was observed in the case of the DCM with 82.9% of TS removed from the liquid fraction coming from the SP with respect to a total TS removal of 64% calculated for the DSM (Figure 2 and 3). In this case, E'_t values for TS were calculated as 0.77 and 0.46 for DCM and DSM, respectively. The total TS removal provided by both solid/liquid separation steps (SP and DC) was of 65% of digestate TS (w/w) for DSM and of 91% of digestate TS (w/w) for DCM (Figures 2 and 3). These different performances can be explained taking into consideration the chemical-physical characteristics of the digestates. The double initial TS contents in DCM (Tables 1 and 2) certainly affected these results. Besides, probably the type of solids may have influenced the first two separations: DCM was characterized by a large portion of undigested ligno-cellulosic material (straw-like), while DSM showed a more homogeneous and fine grain structure. Higher E'_t for DSM than DCM was observed for TS at the UF step (Table 4), even if the liquid inputs (DC liquid fractions) had similar TS concentrations for both DCM and DSM (Table 1). Again, probably

different kind of molecules and suspended agglomerates were contained in the two liquids. The final step of the process, i.e. RO, accounted for almost 100% TS removal for both DCM and DSM, with E'_t ranging from 0.81 to 0.92 respectively (Table 4).

Regarding the fate of N, after the SP and DC steps, 52.5% retention of TKN in the solids fraction for the DCM was observed. In the case of DSM, these steps accounted only for 31.3% of TKN removed from the slurry. This is easily explained by the higher $N\text{-}NH_4^+$/TKN ratio found in DSM (Table 1), as soon as NH_4^+ is soluble and is not retained as solid fraction. After UF, the TKN removed from the liquid fraction was of 66.3% and 46.7% of the initial digestate TKN content for DCM and DSM, respectively (Figure 2 and 3). After RO, TKN was removed up to 89.6% and 93.1% of the initial amount, for DCM and DSM, respectively (Figure 2 and 3).

$N\text{-}NH_4^+$ fractionation was strictly influenced by the chemical-physical characteristics of the digestates, i.e. the TS content and $N\text{-}NH_4^+$/TKN ratio. As soon as $N\text{-}NH_4^+$ concentrations were found in similar concentrations in both liquid and solids fractions (Table 1), $N\text{-}NH_4^+$ mass balance was similar to the wet weight mass balance. While for the DCM the 38% of the initial $N\text{-}NH_4^+$ was retained in the solid streams and in the UF concentrate, only the 28.8% of initial $N\text{-}NH_4^+$ followed the same fate for DSM. In both DCM

and DSM, the rest of $N\text{-}NH_4^+$ was concentrated by the RO: 45.2% and 72.8% of the initial amount for DCM and DSM respectively (Figure 2 and 3). In the case of DCM, 16% of the initial $N\text{-}NH_4^+$ was missing in the balance, probably due to sampling uncertainties and variability. E'_t calculations indicated RO as the most effective separation process (1.00 and 0.96 for DCM and DSM respectively) leading to the complete $N\text{-}NH_4^+$ segregation in the RO concentrate.

In both DCM and DSM, P was efficiently removed from the liquid streams and concentrated in the solid fractions accounting respectively for 88.5% and 92.4% of the total P contained in the digestates (Figures 2 and 3). The UF step accounted for the removal of almost the rest of P in both cases (Figures 2 and 3). Looking at E'_t for P fractionation (Table 4), the most effective steps were both DC and UF with inverse E'_t values for DCM and DSM, i.e. 0.95 and 0.57 for DC and UF respectively for DCM and 0.84 and 0.95 for DC and UF respectively for DSM. These results, again, are linked to the initial diversity of the slurries (i.e. solids characteristics). Probably, DSM was richer in smaller particles (< 10 µm) that are known to be preferentially richer in P (Massè *et al.*, 2007), so that, with membrane filtration, high P removal can be achieved. This point is crucial, as P reduction from the final effluent allows the application of correct

management practices and the recycling of P in the concentrates. Unfortunately no analyses about particle size was performed in order to support this hypothesis.

Regarding K retention, in the case of DCM, 49.1% of the element was retained in RO concentrate while the 44.1% was retained in the solids and in the UF concentrate (Figure 2). Inversely, in DSM, 63.8% of K was retained in RO concentrate and the 28.1% could be found in the solids/concentrate of the process (Figure 3). This is because K is strongly soluble and, as already observed for $N\text{-}NH_4^+$, its mass balance was linked to the wet weight mass balance. E'_t values (Table 4) confirmed RO as the most effective treatment for K fractionation.

3.3 Overall balances

The overall balances of both processes are summarized in Table 3. As main result of the process, a substantial percentage of the initial mass could be purified to clean water: approximately the 38% for DCM and the 51% for DSM (Table 3) can be easily exported from the farm as clean water and ammonium sulfate. This is the first very important result that the N-Free® system can achieve in view of a more economical sustainability of livestock farming allowing the reduction of exportable volumes.

For each batch cycle treating 14.7 ton of digestate, consistent production of concentrated ammonium sulfate was achieved (Figures 2 and 3), i.e. 182 kg at 22% as $(NH_4)_2SO_4$ (5.1% as N) and 312 kg at 31% as $(NH_4)_2SO_4$ (6.1% as N) for DCM and DSM respectively (Table 1 and 2).

In particular cold stripping resulted in RO ammonia reduction of 78% and 73% for DCM and DSM, respectively. Similar results were obtained by Bonmatì and Flotats (2003) where stripping process resulted in almost complete ammonia reduction from raw digested pig slurry operating at 80°C indipendently of a pH value of 9.5. In all reported cases, as expected, the higher the pH, the higher the removal rates. In the discussed case-study, temperature was kept by operators at ambient values to minimize plant

power consumptions and ammonia stripping was achieved operating at high pH values (12 – 12.5).

From the point of view of N management, the N captured as ammonium sulfate plus the residual N in RO permeate, can be considered as N exportable from the farm. This reduction of N resulted for DCM of 22% and 43%, respectively for TKN and $N\text{-}NH_4^+$, while for DSM 45% and 71% for TKN and $N\text{-}NH_4^+$ respectively (Table 3).

Inversely, for P and K, the overall balances pointed out how these elements are retained in the solids and concentrated streams (96% and 89% of P and K for DCM and 100% and 86% of P and K for DSM), so that they can be either used as soil fertilizer/amendment in the farm or exported from the farm with one or more of the concentrated fractions.

After two years of N-Free® functioning at full scale it was possible to evaluate process economics in terms of costs per cubic meter of treated digestate. Actually, for electrical consumptions and reagents, total costs are of 2 € m^{-3} of digestate. The complete full service cost including remote control and plant maintenance is of 1.2 € m^{-3} of digestate. Depreciation charge (10 y and interest rate of 5%) is of 1 € m^{-3} of digestate. Therefore the total cost of digestate treatment with N-Free® technology is 4.2 € m^{-3}. Practically speaking and depending by digestate management plan, from the

total price it is possible to withdraw costs relative to avoided digestate transport outside the farm, (about 1 € m^{-3} of digestate), land renting for digestate spreading (about 0.5-1.5 € m^{-3} of digestate) and incomes related to ammonium sulfate trade (about 0.5 € m^{-3} of ammonia sulfate at 6-8 % as N).

Conclusions

The N-Free® process allows the recycle of different streams and the following advantages in the overall manure management:

1. Through the cold ammonia stripping step, up to 1.8 m^3 of ammonium sulfate per each 100 m^3 of digestate treated by the plant can be produced. This product has already a commercial value (approximately 50€/ton) in the fertilizer market which can be reached by the farm through the subscription to the Fertilizers Producers Register", according to Italian legislation (Dl. 217/2006), and the product registration to the conventional fertilizers register.
2. Up to almost 50% of the initial N is easily exportable from the farm
3. Up to 49% of the initial volume is recycled as clean water. This is very important as the reduction of manures/digestates initial volumes is of

great impact in reducing management cost linked to their distribution on agricultural land

4. Around 12-32% of the initial mass is separated as solid fractions, to be considered as amendment matrixes (rich in organic matter, organic N and P) for agronomical use. Further studies should be focused on the possibility to exploit these streams for the production of organic-mineral fertilizers.
5. Around 30 - 37% of the initial mass is separated as liquid concentrates (i.e. UF concentrate and the lime residue rich in $N\text{-}NH_4^+$, P and K. They can be either exported from the farm with reduced cost or directly used for agronomical practices.

This study represents the first step to evaluate the N-Free® as an efficient treatment technology for digestates from biogas plants and its potential application to ensure economical and environmental sustainability of the agricultural sector.

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Table 1 – Chemical characterization of DCM: liquid and solid streams.

Sample	TS (g kg^{-1} w.w.*)	TKN (mg kg^{-1} w.w.)	$N\text{-}NH_4^+$ (mg kg^{-1} w.w.)	N-org (mg kg^{-1} w.w.)	$N\text{-}NH_4^+/TKN$ (*100)	P (mg kg^{-1} w.w.)	K (mg kg^{-1} w.w.)
Digestate	71.5 ± 1.7	3678 ± 146	1786 ± 86	1892 ± 61	48.5 ± 0.5	768 ± 15	2899 ± 128
Screw press liquid stream	56.7 ± 3.1	3455 ± 113	1702 ± 26	1818 ± 3	48.4 ± 0.3	693 ± 18	2935 ± 64
Screw press solid stream	227 ± 34	3266 ± 70	1326 ± 293	1900 ± 272	41.1 ± 8.8	812 ± 17	2482 ± 242
Centrifuge liquid stream	17 ± 2	2037 ± 24	1661 ± 12	390 ± 5	81.0 ± 0.3	118 ± 22	2634 ± 134
Centrifuge solid stream	192 ± 5	7307 ± 98	1340 ± 113	6023 ± 116	18.2 ± 1.5	2731 ± 160	2751 ± 128
Ultrafiltration liquid stream	8.9 ± 0.4	1460 ± 14	1420 ± 7	40 ± 7	97.3 ± 0.5	8.7 ± 1	2419 ± 153
Ultrafiltration concentrate	30.1 ± 0.2	2996 ± 204	1522 ± 48	1582 ± 70	49.0 ± 0.3	320 ± 20	2523 ± 159
Reverse osmosis liquid stream	udl	8 ± 1	7.1 ± 1.5	1.0 ± 0.9	87.5 ± 12.5	3 ± 0	17.6 ± 4.6
Reverse osmosis concentrate	27.6 ± 1.1	5987 ± 47	5690 ± 71	297 ± 117	95.0 ± 1.9	25.3 ± 1.3	9936 ± 85
Zeolites liquid stream	udl	3.6 ± 1.2	3.6 ± 1.2	udl	100	1.8 ± 0.4	11.9 ± 1.2
Ammonium sulfate	224 ± 2	51235 ± 92	51235 ± 92	udl	100	1.4	49 ± 1
Lime residue	31.8 ± 0.9	430 ± 113	395 ± 15	104	79.5	24 ± 1	9853 ± 159

All the values are intended as the grand mean of 9 samples

* On a wet weight basis

Table 2 – Chemical characterization of DSM: liquid and solid streams.

Sample	TS (g kg^{-1} w.w.*)	TKN (mg kg^{-1} w.w.)	N-NH_4^+ (mg kg^{-1} w.w.)	N-org (mg kg^{-1} w.w.)	N-NH_4^+/TKN (*100)	P (mg kg^{-1} w.w.)	K (mg kg^{-1} w.w.)
Digestate	39.2 ± 2.6	3351 ± 380	2091 ± 244	1259 ± 159	62.4 ± 3.9	620 ± 2	2524 ± 35
Screw press liquid stream	28 ± 6	3255 ± 247	2086 ± 90	1169 ± 157	64.2 ± 2.1	480 ± 91	2496 ± 19
Screw press solid stream	262 ± 8	5692 ± 165	1656 ± 360	4036 ± 373	28.8 ± 6.0	5100 ± 556	2993 ± 82
Centrifuge liquid stream	12.1 ± 0.5	2194 ± 59	1895 ± 135	374 ± 103	83.1 ± 3.3	53.1 ± 9.8	2237 ± 12
Centrifuge solid stream	196 ± 8	10179 ± 764	2585 ± 401	7594 ± 1083	25.6 ± 4.9	4702 ± 148	2546 ± 116
Ultrafiltration liquid stream	9.5 ± 0.5	1882 ± 35	1815 ± 25	47 ± 13	97.5 ± 0.7	39.6 ± 17.1	2340 ± 59
Ultrafiltration concentrate	33 ± 1	2936 ± 175	1852 ± 444	1084 ± 269	63.0 ± 10.1	255 ± 7	2230 ± 416
Reverse osmosis liquid stream	0.34 ± 0.04	88.5 ± 5.2	72.5 ± 9.1	12 ± 3.9	81.8 ± 5.5	0.85 ± 0.04	40.97 ± 0.66
Reverse osmosis concentrate	32 ± 2	7413 ± 40	7263 ± 72	150 ± 35	98 ± 0	149 ± 11	7685 ± 38
Zeolites liquid stream	0.31 ± 0.02	3 ± 1	3 ± 1	udl	100	0.71 ± 0.08	40.4 ± 1.2
Ammonium sulfate	310 ± 2	61105 ± 498	61085 ± 495	61.8 ± 2.6	100	10 ± 0	36 ± 3
Cold stripping residue	35 ± 1	436 ± 29	411 ± 22	25 ± 10	94.3 ± 1.9	141 ± 2	7649 ± 64

All the values are intended as the grand mean of 9 samples
* On a wet weight basis

Table 3 – Overall balances for DCM and DSM

	Disposable[a] kg 100 kg^{-1} of digestate		**Exportable[b] kg 100 kg^{-1} of digestate**		**Error[c] kg 100 kg^{-1} of digestate**	
	DCM	**DSM**	**DCM**	**DSM**	**DCM**	**DSM**
M[d]	62	49	38	51	-	-
TS	104	97	0	0	4	-3
TKN	68	50	22	45	-10	-4
N-NH_4^+	41	33	43	71	-16	4
TP	96	104	0	1	-4	5
TK	89	86	5	7	-7	-7

[a]Calculated as SP solid + DC solid + UF concentrate + Lime residue (Figure 2 and 3)
[b]Calculated as RO permeate + RO concentrate – Lime residue (Figure 2 and 3)
[c]Negative sign indicates missing quantities in the balance
[d]Exportable fraction is calculated as RO permeate + Ammonium sulfate (Figure 2 and 3)

Table 4 – Summary of reduced separation efficiency index for each step of the process.

Separator/Membrane	Producer		Reduced separation efficiency index (E'_t)				
			TS	**TKN**	**N-NH_4^+**	**TP**	**TK**
Screw press	Doda	DCM	0.32	0.09	0.08	0.11	0.09
		DSM	0.18	0.02	-0.01	0.22	0.01
Decanter centrifuge	Pieralisi	DCM	0.77	0.36	-0.07	0.95	-0.02
		DSM	0.46	0.16	0.02	0.84	0.00
Ultrafiltration	Orelis	DCM	0.26	0.16	-0.03	0.57	-0.01
		DSM	0.43	0.08	0.00	0.95	0.00
Reverse osmosis	Dow	DCM	0.81	1.00	1.00	0.74	1.00
		DSM	0.92	1.00	0.96	0.98	0.88

Figures

Figure 1 – Simplified scheme of the N-Free® plant; (1): Screw press separation, (2): Centrifuge separation, (3): Ultrafiltration - UF, (4): Reverse Osmosis - RO, (5): Zeolites refining, (6): Cold ammonia stripping.

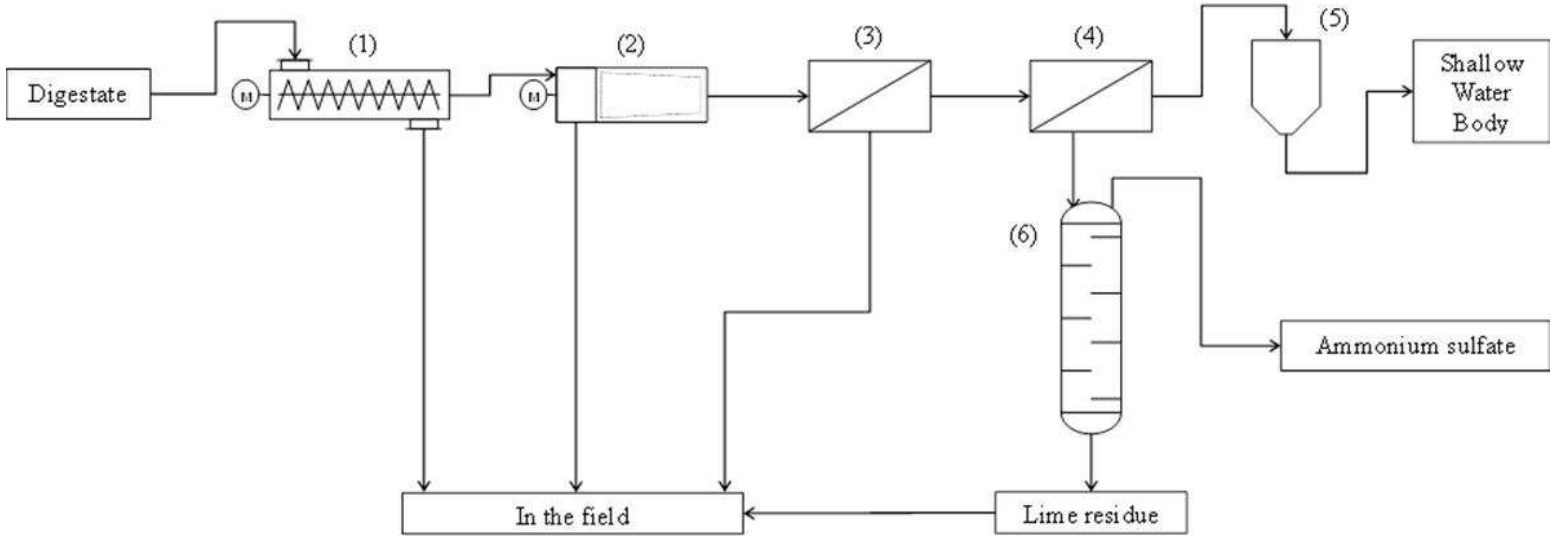


Figure 2 - Mass, TS, TKN, N-NH_4^+, P and K flow in terms of kg $cycle^{-1}$ for one batch process of DCM (1: Screw press separation, 2: Centrifuge separation, 3: Ultrafiltration - UF, 4: Reverse Osmosis - RO, 5: Zeolites refining, 6: Cold ammonia stripping)

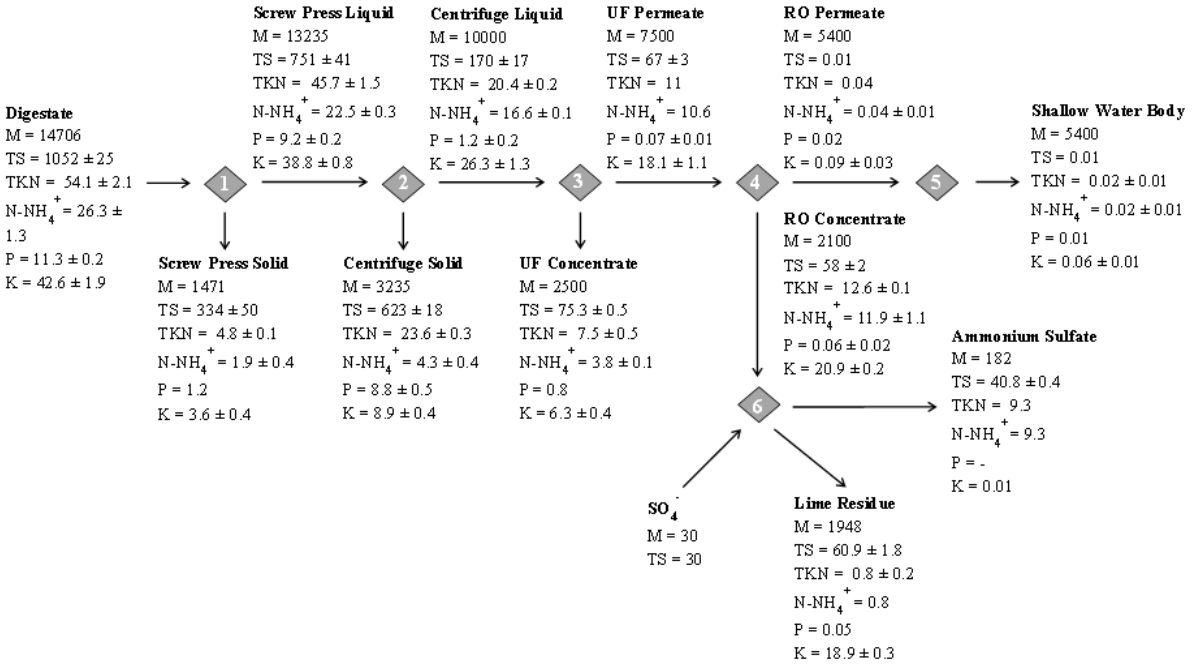
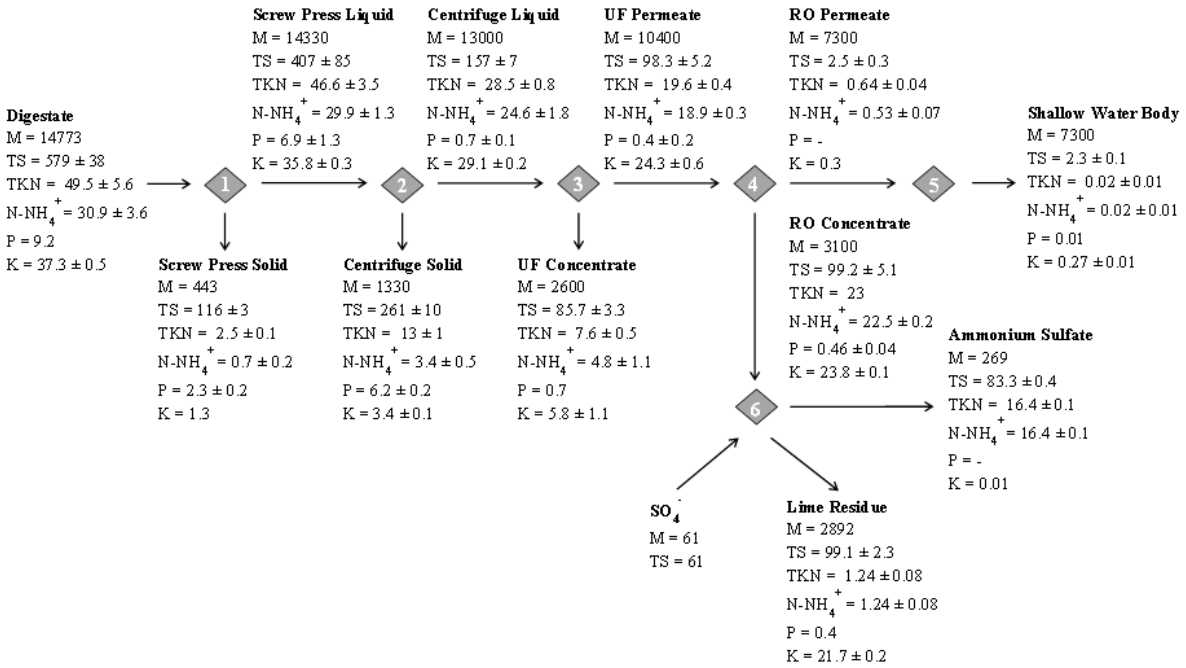


Figure 3 - Mass, TS, TKN, N-NH_4^+, P and K flow in terms of kg $cycle^{-1}$ for one batch process of DSM (1: Screw press separation, 2: Centrifuge separation, 3: Ultrafiltration - UF, 4: Reverse Osmosis - RO, 5: Zeolites refining, 6: Cold ammonia stripping)



References

- APHA, 1998. Standard Methods for the Examination of Water and Wastewater, 20th edition. American Public Health Association, Washington, D.C.
- ApSimon, H.M., Kruse, M., Bell, J.N.B., 1991. Ammonia emissions and their role in acid deposition. Atmos. Environ. 21, 1939-1961.
- ARPA Lombardia, Azienda Regionale per la Protezione dell'Ambiente, 2010. Rapporto Stato Ambiente Lombardia. Available at http://ita.arpalombardia.it.
- Bonmatì, A., Flotats, X., 2003. Air stripping of ammonia from pig slurry: characterisation and feasibility as a pre- or post-treatment to mesophilic anaerobic digestion. Waste Manage. 23, 261–272.
- Bouwman, A.F., 1990. Exchange of greenhouse gases between terrestrial ecosystems and the atmosphere. In: Soils and the greenhouse effect, ed. A.F. Bouwman, Wiley and Sons Chichester 61-127.
- Burton, C.H., 2007. The potential contribution of separation technologies to the management of livestock manure. Livest. Sci. 112, 208-216.

- Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol. Appl. 8, 559–568.
- Chambers, B.J., Lord, E.I., Nicholson, F.A., Smith, K.A., 2006. Predicting nitrogen availability and losses following application of organic manures to arable land: MANNER. Soil Use Manage. 15(3), 137–143.
- Chiou, R.J., Ouyang, C.F., Lin, K.H., Chuang, S.H., 2001. The characteristics of phosphorus removal in an anaerobic/aerobic sequential batch biofilter reactor. Water Sci. Technol. 44(1), 57-65.
- Clarisse, L., Clerbaux, C., Dentener, F., Hurtmans, D., Coheur, P.F., 2009. Global ammonia distribution derived from infrared satellite observations. Nat. Geosci. 2(7), 479–483.
- Council Directive 91/676/EEC. Protection of waters against pollution caused by nitrates from agricultural sources.
- EPA, 2007. Method EPA 3051A. Microwave assisted acid digestion of sediments, sludges, soils and oils.
- EPA, 2007. method EPA 6020A. Elements determination by inductively coupled plasma-mass spectrometry (ICP-MS)

- ERSAF, Ente Regionale per i Servizi all'Agricoltura e alle Foreste , 2009. National Strategic Nitrate Plan – Piano Strategico Nazionale Nitrati. Available at http://www.ersaf.lombardia.it.
- IRSA CNR, 1994. Metodi analitici per le acque, Quaderni, N. 100. Istituto Poligrafico e Zecca dello Stato, Rome, Italy
- Italian Ministry of Agricultural and Forestry Politics. Decreto ministeriale 7/3/2006. "Utilizzazione agronomica degli effluenti di allevamento e delle acque reflue". Available: http://www.mipaaf.it/
- Italian Parliament. Decreto Legislativo 11 maggio 1999, n. 152. " Disposizioni sulla tutela delle acque dall'inquinamento e recepimento della direttiva 91/271/CEE concernente il trattamento delle acque reflue urbane e della direttiva 91/676/CEE relativa alla protezione delle acque dall'inquinamento provocato dai nitrati provenienti da fonti agricole"; (Published on the ordinary supplement n. 172 of Italian GU October 20 1992, n.246). Available: http://www.gazzettaufficiale.it/
- Italian Parliament. Decreto Legislativo 29 aprile 2006, n. 217. "Revisione della disciplina in materia di fertilizzanti"; (Published on the ordinary supplement n. 152 of Italian GU October 20 1992, n.141). Available: http://www.gazzettaufficiale.it/

- Jorgensen, K., Magid, J., Luxhoi, J., Jensen, L.S., 2002. Phosphorus distribution in untreated and composted solid fractions from slurry separation. Journal of environmental quality 39(1), 393–401.
- Kaparaju, P., Rintala, J., 2011. Mitigation of greenhouse gas emissions by adopting anaerobic digestion technology on dairy, sow and pig farms in Finland. Renew. Energ. 36, 31–41
- Meyer, R.L., Zeng, R.J., Giugliano, V., Blackall, L.L., 2005. Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. FEMS Microbiol. Ecol. 52, 329–338.
- Masse, L., Massé, D. I., Pellerin, Y., 2007. The use of membranes for the treatment of manure: a critical literature review. Biosyst. Eng. 98(4), 371–380.
- Masse, L., Massè, D.I., Beaudette, V., Muir, M., 2005. Size distribution and composition of particles in raw and anaerobically digested swine manure. T. ASAE 48, 1943–1949.
- Nielsen, V.C., Voorburg, J.H., 2004. Odour prevention and control of organic sludge and livestock farming. Proceedings of a round-table seminar, Silsoe, UK, 15-19 April 1985.

Taylor & Francis.

- Peters, K., Hjorth, M., Jensen, L.S., Magid, J., 2011. Carbon, Nitrogen, and Phosphorus Distribution in Particle Size–Fractionated Separated Pig and Cattle Slurry. J. Environ. Qual. 40(1), 224.
- Petersen, S.O., Sommer, S.G., Béline, F., Burton, C., Dach, J., Dourmad, J.Y., Leip, A., Misselbrook, T., Nicholson, F., Poulsen, H.D., Provolo, G., Sørensen, P., Vinnerås, B., Weiske, A., Bernal, M.P., Böhm, R., Juhász, C., Mihelic, R., 2007. Recycling of livestock manure in a whole-farm perspective. Livest. Sci. 112, 180-191.
- Preez, J., Norddahl, B., Christensen, K., 2005. The BIOREK® concept: a hybrid membrane bioreactor concept for very strong wastewater. Desalination 183, 407-415.
- Rautenbach, R., Vossenkaul, K., Linn, T., Katz, T., 1996. Waste water treatment by membrane processes - New development in ultrafiltration , nanofiltration and reverse osmosis. Desalination 108, 247–253.
- Schievano, A., Adani, F., Tambone, F., D'imporzano, G., Scaglia, B., Genevini P.L., 2009. What is the digestate? In: Anaerobic

Digestion : opportunity for agriculture and environment [s.l]: Regione Lombardia, ISBN 9788890374609.

- Skiba, U., Fowler, D., Smith, K.A., 1997. Nitric oxide emissions from agricultural soils on temperate and tropical climates: sources, controls and mitigation options. Nutr. Cycl. Agroecosys. 48, 75-90.
- Smith, K.A., Chambers B.J., 1997. Nutrient losses to water following land application of farm manures. In: Proceedings of International Workshop on Environmentally Friendly Management of Farm Waste, ed. T Matsunaka, Sapporo 79-83.
- Smith, K.A., Chalmers, A.G., Chambers, B.J., Christie, P., 1998. Organic manure phosphorus accumulation, mobility and management. Soil Use Manage. 14, 156-159.
- Svarovsky, L., 1985. Solid–liquid separation processes and technology. In: Williams, J.C., Allen, T. (Eds.), Handbook of Powder Technology, vol. 5. Elsevier, Amsterdam, pp. 18–22.
- Tambone, F., Genevini, P., D'Imporzano, G., Adani, F., 2009. Assessing amendment properties of digestate by studying the organic matter composition and the degree of biological stability during the anaerobic digestion of the organic fraction of MSW. Bioresource Technol. 100(12), 3140–2.

- Vanotti, M.B., Szogi, A.A., Hunt, P.G., 2003. Extraction of soluble phosphorus from swine wastewater. T. ASAE 46(6), 1665–167

Integration of microalgae production with anaerobic digestion of dairy cattle manure

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Abstract

The aim of this work was to demonstrate the possibility to integrate microalgae production with anaerobic digestion of dairy cattle manure and digestate treatment, helping to reduce the cost of slurry treatment and improving the energy balance of the process. A real biogas and digestate-treatment units were monitored for energy, mass and nutrients balances. The existing system produces electric energy (2,182 ekWh d^{-1}) and organic fertilizers/amendments (11,655 t y^{-1}), among others. Microalgae production was integrated with this system by using untreated ultra-filtrated digestate as growth medium for the production of *Scenedesmus* sp. The tolerance of this strain to digestate was evaluated, results demonstrating that percentage of digestate upper than 10% inhibited the growth of this microalga, but below this value productivity up to 124 mg $L^{-1}d^{-1}$ could be obtained. Composition of the culture medium also influenced the biomass composition, with protein, carbohydrate and lipid content being a direct function of ammonia concentrations. Integrating microalgae production with anaerobic digestion it is possible to produce 166-190 t y^{-1} of microalgal valuable biomass. Overall energy and mass balances of the process allows demonstrating that the integration of both anaerobic digestion and microalgae production steps are positive and technically feasible.

1. Introduction

Water resources management has become an important issue due to anthropogenic effects caused by population increasing (i.e. urbanization) and, agricultural and industrial activities, that affect both quantity and quality of shallow and deep waters (Peters *et al*., 2000). As consequence of that there is the need of sustainable use of water resources by considering innovative productive processes in which a product-to-waste-to-product scheme has to be pursued.

The development of livestock production has gained attention for its environmental impact because of the production of massive volumes of polluted slurries characterized by high organic and mineral load; this latter mainly represented by nitrogen (N) and phosphorus (P) (Petersen *et al*., 2007). Nitrogen from livestock slurries contributes largely to environmental pollution through ammonia and nitrogen oxides emission to atmosphere and nitrate leaching to ground and surface water bodies (Brandjes *et al*., 1996). In order to reduce these impacts a wide range of treating techniques has been proposed although most of them are considered too expensive for livestock farming (Burton, 2007) and of difficult applicability. Livestock slurries are frequently treated by anaerobic digestion producing renewable energy. By this process the organic matter is transformed into biogas (50–

80% v/v methane), used to produce energy and heat. Anaerobic digestion modifies the composition of the slurry, particularly regarding N. Protein are degraded and ammonia is produced so that N-NH_4^+/ TKN ratio increases (Schievano *et al*., 2009). Moreover ammonia accumulation increases fertilizer properties of digestate (Schievano *et al*., 2009), but, also, can facilitate N removal from digestate by using stripping technologies.

Additionally, the recovery of nutrients from wastes (especially N, P and K), is becoming a research priority as they are finite resources concentrated in few countries (Cordell *et al*., 2009), or they require high energy input to be produced and/or transported, causing high GHG emissions (Wood and Cowie, 2004). Therefore the possibility to recover nutrients to be employed in new productive processes is becoming fascinating and important, above all for farmers that need to find new possibilities to integrate agricultural incomes because of subsidies reduction from central governments (EC MEMO/13/937). In a recent work Ledda *et al*. (2013) showed an innovative process for livestock manure treatment, coupled with anaerobic digestion, that reduced treatment costs jointly with both water and nutrient recovery, so that slurry became a resource rather than a waste.

Concerning recovery of nutrients, microalgae are photosynthetic microorganisms capable to uptake inorganic N and P, and to transform them

into valuable organic compounds using solar energy. On this sense, microalgae have been exploited due to their high productivities and the possibility to use them as single-cell bio-factories for the production of nutraceuticals, pharmaceuticals, pigments, biopolymers, chemicals and animal feed products (Pulz and Gross, 2004). Moreover, algae have been reported to effectively grow on wastewater (Oswald and Gotaas, 1957) because of their tolerance to high concentrations of nutrients, producing large amount of biomass (De la Noüe *et al*., 1992).

Among different species, green microalga *Scenedesmus* sp. has shown extraordinary vitality in urban wastewaters, with growth rates similar to those reported for complete synthetic media, so that it has been commonly used for the phytoremediation of industrial, urban and agricultural wastewaters (Xiao *et al*., 2011; Ji *et al*., 2013; Franchino *et al*., 2013).

Recently a model was proposed to develop a farm level integrated system focused on the anaerobic digestion process (Manenti and Adani, 2014). In that paper, particular attention was posed on the re-use of digestate as nutrient medium to produce microalgal biomass. Nevertheless no experimental data were reported and the proposal remained academic. This work represents a first attempt to develop a farm-level integration process that considers the production of food from farming and of energy from

anaerobic digestion, the recycling of nutrients and water from digestate treatment, and the production of algae biomass (*Scenedesmus* sp.) using the nutrient contained in the liquid streams (ultrafiltration permeate). Results from this work are of interest for the integration of livestock slurry treatment with biogas production and microalgae production, providing farm production diversification and agricultural incomes increase.

2. Methods

2.1. Biogas and digestate-treatment units

A real anaerobic digester (1,500 m^3) serves a GMO-free dairy farm with 1,200 Italian Friesian cattle, 400 of which are in lactation. The annual production of milk is about 12 t which are entirely delivered to the "Grana Padano Consortium". Farm land availability is about 200 Ha, used for cattle and biogas plant feed. The biogas plant has an installed electrical power of 180 kW and it reutilizes the cattle manure as liquid substrate but enriched by co-digesting various biomasses such as cattle manure solid fraction among others. The digestate from anaerobic is treated to separate the digestate liquid fraction from the digestate solid fraction by means of solids and concentrates and the production of ammonium sulfate (Ledda *et al*., 2013). The treatment unit operates in batch mode. The first separation is

achieved with a screw press separator after which the liquid fraction is added of a polyamide flocculant and sent to a decanter centrifuge. Then, the centrifuged liquid enters in the ultrafiltration unit, equipped with a 40 kDa grafted polyacrylonitrile membrane.

The ultra-filtrated permeate (UFP) can be used to produce microalgae but in real conditions it is subject to reverse osmosis step. Finally, permeate from reverse osmosis is refined in zeolites bed and then discharged to surface-water bodies. Besides, the concentrate from reverse osmosis enters into cold ammonia stripping unit where lime is added, raising pH up to 12-12.5. At these conditions, NH_3 is stripped in gaseous form by using a controlled air flow, which is then scrubbed with sulfuric acid, producing liquid ammonium sulfate.

2.2. Characterization of anaerobic digestion step

All the streams coming from the biogas and the digestate-treatment units were sampled within three different cycles of the treatment process and chemically characterized. Representative samples of wastes were used to carry out all analyses. Total solids (TS) and volatile solids (VS) were determined according to standard procedures (APHA, 1998). The biochemical methane potential (BMP) of the samples were determined by

using a standardized method reported in Schievano *et al.* (2008). BMP detected for both input and output material were used in joint with mass balance data to determine the bio-methane yield (BMY) of biogas plant, by using the equation 1 (Schievano *et al*., 2011b) where BMP_{in} is the bio-methane potential in the fed mixture (Nm^3 kg^{-1} TS), BMP_{out} is the bio-methane potential in the output digestate (Nm^3 kg^{-1} TS), TS_{in} are the total solids fed during the observed period (kg) and TS_{out} are the total solids output with digestate during observed period (kg).

$$BMY(\%) = \frac{(BMP_{in} \times TS_{in} - BMP_{out} \times TS_{out})}{BMP_{in} \times TS_{in}} \times 100 \quad (1)$$

Total Kjeldhal nitrogen (TKN), ammonia nitrogen ($N\text{-}NH_4^+$) and nitric nitrogen ($N\text{-}NO_3^-$) were determined using fresh material according to the analytical methods for wastewater sludge (IRSA-CNR, 1994). Elemental analysis was performed according to 3051A method (1998). Total phosphorus (P) content was determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian. Fort Collins, USA). A certified standard reference material (2782 Industrial Sludge) from the National Institute of Standards and Technology (Gaithersburg, US) was used in the digestion and

analysis. To ensure the accuracy and precision in the analyses, reagent blanks were run with samples. All analyses were performed in triplicate.

2.3. Microalga strain

The green microalga *Scenedesmus* sp. (strain number 2125) was obtained from the SAG Culture Collection of the University of Goettingen (Germany). Inoculum was maintained in Erlenmeyer flasks in Bold Basal Medium for freshwater microalgae, under continuous illumination provided by fluorescent cool white lamps (Osram L13W/840).

2.4. Experimental design on microalga growth

The influences of ultra-filtrated permeate (UFP) percentage in the culture medium and the amount of inoculum in the culture was evaluated. Different dilutions of UFP were coupled to different initial microalgal inoculum concentrations through a Box–Wilson central composite design (CCD), with the aim of optimizing biomass production rates and simultaneously modulate nutrient availability and avoid possible toxicity. It has been reported that ammonia may cause severe inhibition to microalgal communities at high concentrations (Lavoie, De la Noüe, 1985). The lowest ammonia concentration in the experimental design (39 mg L^{-1}) was chosen to be similar to available nitrogen in common freshwater microalgae

synthetic medium (Bold's Basal Medium). Besides, the concentration of inoculum can also influence the response of the culture because a higher biomass concentration enhances the robustness of the cultures to tolerate adverse growth conditions. The lowest level of inoculum (7 10^6 cells mL^{-1}) was chosen from previous literature data (Martin C. *et al*., 1985; Tam N. F. Y. & Wong Y. S., 1989). The experimental values of each factor are defined to be uniformly distributed around a center-point, according to factorial design levels coded from -1 to +1. These levels are then augmented with start points that, in a two-factor CCD, are axially placed at a coded distance of $-\sqrt{2}$ and $+\sqrt{2}$ from the center of the design. As a result, the substrate dilution and the starting inoculum were investigated at five levels, coded as ($-\sqrt{2}$, -1, 0, +1, $+\sqrt{2}$). The level code reflects the step change in the actual value chosen for the two operating parameters. All the evaluated levels were arranged in nine different treatments, corresponding to nine combinations of $N\text{-}NH_4^+$ concentration with starting inoculum.

Batch lab-scale photobioreactors were used to optimize the cultivation of *Scenedesmus* sp. Each treatment consisted of two replicated trials on 500 mL Erlenmeyer flaks. The UFP was neither filtrated nor autoclaved and diluted with deionized water thus the reliability of the results enhancing to be applied at larger scale. Cultures were submitted to constant illumination

at a light intensity of 100 μmol $m^{-2}s^{-1}$. Mixing was achieved by bubbling air at 0.5 v/v/min, no additional CO_2 being supplied. However enough carbon was supplied to the cultures as pH remained constant. Temperature was kept constant at 25°C by controlling the chamber temperature on which the experiments were performed.

2.5. Determination of microalgae growth

Dry weight of biomass into the cultures was measured at the beginning and at the end of the growth cycle by drying cells at 85 °C for 24 h after filtration through a pre-weighted GF/C filter (Whatman). Cell count was carried out daily using a Neubauer haemocytometer (Poly-Optik GmbH. Bad Blankenburg, Germany). Additionally chlorophyll-a concentration was determined; for this purpose 3 mL of culture was centrifuged at 4500 rpm and 4°C for 10 min. Supernatant was then discharged and 10 mL of pure methanol (Reagent Grade) was added to the pellet. Samples were ultrasonicated (VWR USC300T, VWR International. Radnor, USA) for 15 min at 45 kHz and then stored for 24 h at 4°C to allow maximum extraction of the chlorophyll-a. The concentration of the pigment was determined by reading the sample at an absorbance of 665 and 750 nm (Varian UV-VIS Cary 50 BIO, Varian. Fort Collins, USA) against a methanol blank, using

Mackinney (1941) empirical correlation 2 where A_{665} is the absorbance at 665 nm, after removing the sample absorbance at 750 nm, v is the volume of methanol used (mL), l is the spectrophotometric cell length (cm) and V is the volume of the sample (mL). All the analysis on cultures were done on triplicate.

$$Chlorophyll-a\left(\frac{\mu g}{mL}\right)=\frac{13.43\times A_{665}\times v}{l\times V} \quad (2)$$

Daily volumetric productivity was calculated by the equation 3 where X_m and X_0 are the concentrations of biomass at the end and at the beginning of a batch run, respectively, and t is the duration of the experiment.

$$D_P=\frac{X_m-X_0}{t} \quad (3)$$

The specific growth rate was calculated during logarithmic growth phase by the equation 4.

$$\mu=\frac{1}{t}\times\ln\left(\frac{X_m}{X_0}\right) \quad (4)$$

2.6. Biomass composition

Lowry method (Lowry *et al*., 1951) was used to measure the protein content of the biomass. Amino acids speciation was carried out as reported by Barbarino and Lourenço (2005). For this freeze dried samples with about 5 mg of protein were hydrolyzed with 1.0 mL of 6 mol L^{-1} HCl in vacuum-sealed hydrolysis vials at 110 °C for 22 h with norleucine as an internal standard. After hydrolysis the samples were dehydrated and redisolved in a suitable volume of a sample dilution Na–S® buffer pH 2.2, and analyzed for amino acids content by ion-exchange chromatography in a Beckman 7300 amino acids analyzer. Total lipids were determined using a slightly modified version of Bligh and Dyer's method (1959). An aliquot of freeze dried sample mixed with 5 mL of chloroform: methanol (2:1 v/v) was ultrasonicated (VWR USC300T, VWR International. Radnor, USA) for 15 min at 45 kHz and then stored for 24 h at 4°C to allow maximum extraction of lipids. The mixtures were then transferred into a separator funnel and shaken for 5 min. The lipid fraction was then separated from the separator funnel and gravimetrically determined after solvent evaporation using a rotary evaporator (Büchi R110, Büchi Labortechnik AG. Flawil, Switzerland). Fatty acids profiles were determined after esterification of lipids and detection by GC-MS analysis (Agilent 6850 Series, Agilent

Technologies). Quantification of fatty acids was determined injecting an external multiple standard GRAIN FAME (Supelco).

Total carbohydrates were quantified according to phenol-sulfuric acid method of DuBois (1956). Pigments were extracted in 10 mL 90 % acetone. The samples were ultrasonicated for 30 s and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.2 µm Nyaflo membrane filter (Gellman). Pigments content was determined by ion-impairing, reverse-phase HPLC.-For the analysis 700 µL of ammonium acetate are mixed with 500 µL of the pigments extract. 100 µL of the mix was then injected into a Phenomenex 5 µm C-18 column (25 cm x 46 mm id.). Dual channel detection was achieved with a Spectra-System UV detector set at 440 nm for absorbance reading. Pigments were identified by comparing their peaks and retention times with commercially available standards.

2.7. Data analysis

Data were processed by one-way ANOVA using the Tukey test to compare means. Statistical analyses were performed by using SPSS software (SPSS v19.0, IBM). The level of significant difference was set at $P<0.05$.

3 Results and discussion

3.1. Performance of anaerobic digestion and digestate treatment

The biogas plant utilizes the cattle manure as liquid substrate for a total of 42.6 t d^{-1} being processed. To enhance the yield of biogas, co-digestion of cattle manure with other residues is performed, thus liquid cattle manure constituted 77.0% of the total feeding stream whereas cattle manure solid fraction constituted 19.8%, pellets constituted 1.6% and maize flour and maize silage constitutes 1.0% and 0.7% respectively. The average daily feeding mixture is of 55.3 t d^{-1} with a hydraulic residence time of 32.3 days. On these conditions anaerobic digestion was effective; the biogas produced contained 55% of methane and allowed daily average electrical production of 2,182 ekWh d^{-1} (Table 1). The bio-methane yield resulted of 75.5 %, indicating high conversion of organic matter to biogas. Characteristics of ingestate and digestate are also showed in Table 1. The output bio-methane potential was largely reduced with respect to input one from 340 to 133 NL biogas kg^{-1} TS. The content of TS and VS decreased as consequence of degradation process. Thus due to protein degradation the $N\text{-}NH_4^+$/TKN ratio increased from 29.3% to 51.2 %.

Chemical characterization of liquid and solid fractions of digestate and mass/nutrients balances of the digestate treatment process are shown in

Table 2. All concentrations were reported on wet weight (ww) basis. The treatment unit operates in batch mode, treating about 14 m^3 of digestate for each cycle for a total daily treated digestate volume of about 50 m^3. The first separation is achieved with a screw press, then centrifugation and ultrafiltration being performed. Looking at the system balance, solid fractions after screw press and centrifuge separations represented the 32% ww/ww of the initial mass while ultrafiltration concentrated fraction accounted for the 17% ww/ww of the digestate. Both solids and concentrated streams represented the 68.7% and 98.4% of initial digestate-N and -P content, respectively. Cold stripping step allowed a reduction of digestate ammonia content of 41.6%, that resulted in the production of 182 kg of ammonium sulfate for each treating cycle (5.1% as N; 1.2% ww/ww of the digestate mass). Finally, clean water represented the 37% ww/ww of digestate, being this fraction re-used in the farm or directly disposable in shallow water. In this process, UFP fraction represented the 51% of total digestate; this stream contains 31.5% of N-NH_4^+ and 1.2% of P contained in the digestate, and could be used as growth medium for algae cultivation, instead of successive RO + stripping treatment to produce water and ammonium sulfate.

3.2. Growth of Scenedesmus sp. using UFP as culture medium

Discontinuous cultures of *Scenedesmus* sp. in diluted UFP were carried out. Composition of culture mediums (D1 to D5) prepared by diluting UFP with deionized water are showed in Table 3. The ammonia nitrogen and phosphorous content of UFP was 1130 and 17 $mg \cdot L^{-1}$ respectively, whereas in prepared culture mediums the concentrations ranged from 39 to 565 $mg \cdot L^{-1}$ of ammonia nitrogen, and from 0.6 to 8.5 $mg \cdot L^{-1}$of phosphorous. The pH of UFP was 7.9 whereas the pH of culture mediums prepared ranged from 9.4 to 8.3. According to experimental design (section 2.4) batch cultures of *Scenedesmus* sp. were performed these cultures medium with different concentrations of inoculum. Details of trials performed and results obtained are showed in Table 4. Algae growth was evident only for the first three trials of the experimental design, i.e. A1, A2 and A3. Contrarily, trials A4 to A9 did not show any relevant growth, because of inhibition caused by increased concentrations of UFP. The $N\text{-}NH_4^+$ is likely to be one of the chemical species that might have inhibited microalgal growth above 113 mg L^{-1} , more than P, that is normally not toxic for algae at the reported concentrations (Table 3 and 4). Thus no growth was measured in experiments performed at $N\text{-}NH_4^+$ concentrations higher than 113 $mg \cdot L^{-1}$. This hypothesis agrees with Park *et al*. (2010) that reported, for

Scenedesmus sp. growing on digested swine manure, the absence of inhibition below 100 mg L^{-1} of $N\text{-}NH_4^+$.

Daily variation of culture parameters on trials A1-A3 shows a rapid growth during the first 2-3 days then the growth reducing on trial A1 due to the lower nitrogen content of the culture medium on this trial (39 mg·L^{-1}); trials A2 and A3 continues growing due to the higher nitrogen content of the culture mediums used on these experiments (113 mg·L^{-1}) (Figure 1a). Variation of at $N\text{-}NH_4^+$ demonstrated as trial A1 was nitrogen limited, the nitrogen concentration reducing to zero upper 6^{th} day, whereas trials A2 and A3 were nitrogen sufficient the nitrogen concentration only reducing to zero at the end of experiment, nitrogen consumption rate being higher in trial A3 due to the higher concentration of inoculum used on this trial (Figure 1b). As a consequence of that, trial A3 resulted in the highest daily volumetric productivity of 124 mg L^{-1} d^{-1}(Table 4). Trial A2, which was characterized by the same $N\text{-}NH_4^+$ and P initial concentration of trial A3, showed a lower daily volumetric productivity (28 mg L^{-1} d^{-1}). This was probably due to the higher inoculum concentration that is known to better support algae growth at high ammonia concentration (2 mM; 51.6 mg L^{-1}) and alkaline pH (Lavoie, De la Noüe, 1985). This fact explained, also, why trial A2 presented a volumetric productivity lowers than trialA1, although the former

was characterized by a higher inoculum than trial A2. Regarding specific growth rate, the highest value was found for trial A1 (0.33 d^{-1}) significantly higher than those of A2 and A3 (0.26 d^{-1} and 0.28 d^{-1}, respectively) (Table 4). High specific growth rate of trial A1 could be explained taking into consideration the high dilution of UFP that allowed a higher light incidence inside the culture. On the other hand the low nutrient content limited successive algae growth determining the low volumetric productivity before described.

These results indicated that high ammonia concentration (113 mg L^{-1}) well supported algae productivity, if high inoculum was provided when culture started (i.e. trial A3). If diluted UFP guaranteed no inhibiting ammonia concentration at high level of inoculum, all trials were characterized by a low P concentration and, as a consequence, by a very high N:P ratio, i.e. 67 and 64 for trials A1 and A2, A3 respectively. Synthetic culture mediums are characterized by a wide range of N:P ratios typically ranging from 4:1 to 45:1 resulting in a N-deficient or P-deficient growth (Richmond, 2004); nevertheless, Yin-Hu *et al*. (2012) reported that in a P starvation batch-mode with a N:P ratio of 45:1 *Scenedesmus* sp. showed growth rates similar to those obtained in a P repletion batch-mode. These authors concluded that more consumed P (luxury uptake) did not result in more biomass production

so that its dosage should be kept low to prevent P resources waste. In addition trials A2 and A3 did not differ each other for P content but differed for total productivity. All these findings suggested that differences in productivity was not influenced by P concentration in the substrate. In any case *Scenedesmus* sp. productivities obtained in this work were comparable with those reported in literature (Park *et al*., 2010; Yoo *et al*., 2010).

3.3. Biomass composition

Biochemical composition of the biomass from trials A1, A2 and A3, is showed in table 5. The total proteins content was maximum for trials A2 and A3 (493 and 536 g kg^{-1}, respectively), and significantly higher than that found for trial A1 (294 g kg^{-1}), which was characterized by a lower concentration of $N\text{-}NH_4^+$ in the growth medium than trials A2 and A3 (Table 4). Variation in protein content between different trials could be explained by different ammonia concentration in the medium. A not-limiting ammonia content in the growth medium is well known to enhance protein accumulation in microalgae cells (Guillard, 1973), i.e. trials A2 and A3. Opposite trial A1 accumulated low proteins because from the day 9 to the end of the trial, the culture medium was characterized by an ammonia

concentration lower than 5 mg L^{-1} that is reported not optimal for proteins accumulation (Xin et al. , 2010) (Figure 1b).

Regarding lipids, trial A1 showed the highest concentration of lipids, reaching a content of 295 g kg^{-1}, while trials A2 and A3 attained a lower lipid accumulation, i.e. 148 g kg^{-1} and 151 g kg^{-1}, respectively. Again, both nitrogen and phosphorus content could be related to these differences. Nutrients availability is a well-known critic factor that triggers lipid accumulation in microalgae cells (Brennan and Owende, 2010). Substrate characterization and growth response, in terms of nitrogen consumption, are consistent with the results of Xin *et al*. (2010).; this author using *Scenedesmus* sp. reported that when initial nitrogen source was in the range of 5-25 mg L^{-1} the lipid content of the produced biomass was between 200-250 g kg^{-1}. In addition, when initial P concentration was in the range of 0.2-2.0 mg L^{-1}, the lipid content was 230-280 g kg^{-1}. Hence, in this work, A1 was the most efficient thesis in lipid accumulation due to the characteristics of the medium used (Table 2 and 3).

The carbohydrates content showed a behavior opposite to lipids, being the highest, of 398 g kg^{-1}, for trial A1 compared to trials A2 and A3 (Table 5). These data can be explained, again, taking into consideration nitrogen concentration in the growth medium. In fact, under nitrogen-depletion

conditions, many microalgae strains transform proteins or peptides to lipids or carbohydrates as the flow of photosynthetic carbon is turned from the metabolic pathway of protein synthesis to carbohydrates synthesis, resulting in an accumulation of these compounds as energy storage (Huo *et al*., 2011). In this study trial A1 showed a comparable carbohydrates accumulation to other microalgal strains. Brányiková *et al*. (2011), for example, reported that in nitrogen depleted cultures of *Chlorella vulgaris* carbohydrates accumulated up to 380-410 g kg^{-1}, while Ji *et al*. (2011) obtained about 350 g kg^{-1} in *Tetraselmis subcordiformis*. Finally the pigments profile showed similar concentrations in all trials (Table 5). Even though it was reported that nitrogen decreases the synthesis of photosynthetic pigments such as chlorophyll (Berges *et al*., 1996) and carotenoid, in this study there was not a clear correlation between initial nitrogen content and pigments biomass composition.

A more detailed analysis of aminoacids, fatty acids and pigments content of the biomass from trials A1 to A3 was also performed. Looking at amino acids speciation (Table 6) all trials showed a similar profile, although trial A3 attained almost double concentration of arginine compared to both trials A1 and A3, while cysteine was found only for trial A1. All samples showed a high concentration of glutamine plus glutamic acid, up to 25% of crude

protein. Essential amino acids contents were found similar to those reported by Becker (2007) for *Scenedesmus obliquus*. In particular in this study, trial A1 was characterized by 30.8% cp of essential amino acids, similarly to trial A2 (29.2% cp) and slightly higher than that of trial A3 (25.9% cp). Regarding fatty acids (FA) composition, trial A1 reached up to 109 g kg^{-1} of total fatty acids content (Figure 2a) significantly higher than those of trials A2 and A3 (19 g kg^{-1} and 14 g kg^{-1} respectively). Trial A1 was characterized by a significant higher saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids content (34, 54, 21 g kg^{-1} respectively) than trials A2 and A3 (Figure 2a). Regarding pigment composition no large variation were observed between trials (Figure 2b). Only in the case of cis-β-carotene, trial A1 showed a slightly higher concentration than other trials. Trials A2 and A3 conversely, were characterized by highest concentrations of neoxanthyn, astaxanthyn and total pigments.

3.4. Integration of anaerobic digestion, digestate treatment and microalgae biomass production

Anaerobic digestion produces biogas that can be used to produce renewable energy in substitution of fossil fuel-derived energy. It has been demonstrated that digestate from anaerobic digestion could be used as

feedstock to produce fertilizers (Ledda *et al*., 2013) or other products. But moreover, the results of this work demonstrate that digestate from anaerobic digestion can be also used to produce microalgae biomass. According to this fact an on-farm integrated scheme can be applied (Figure 3) to produce food, energy, fertilizers, water and algae-derived products. The obtained data indicated that from 20,201 t y^{-1} of slurry fed to the anaerobic digester it is possible to produce biogas enough to produce 2,182 ekWh d^{-1}, with a total amount of 18,787 t y^{-1} of digestate being released. This digestate is currently treated by SP, DC, UF, RO and N-S units (Option A), allowing to produce 11,655 t y^{-1} of N-P rich organic fertilizers/amendments and to recycle 6,898 t y^{-1} of clean water that can be efficiently re-used for agriculture activities or disposed in shallow water bodies. Finally, this process also produces 232 t y^{-1} of ammonium sulfate which can be used and/or sold as fertilizer. Alternatively to this option microalgae production can be performed using permeate from ultrafiltration step avoiding the utilization of reverse osmosis and ammonium sulfate production steps (Option B). On this way the digestate treatment produces 13,687 t y^{-1} of nutrients-rich UFP (11.6 t y^{-1} and 0.18 t y^{-1} of N-NH_4^+ and P respectively) that can be entirely recycled by microalgae growth.

This step must be performed using cheap production systems such as open raceway reactors, that has been demonstrated to be capable to achieve high biomass productivities up to 0.4 $g \cdot L^{-1} \cdot d^{-1}$ (40 $g \cdot m^{-2} \cdot d^{-1}$) with the same strain *Scenedesmus* sp. (Mendoza *et al*., 2013). On these reactors, the power consumption can be reduced below 5 $W \cdot m^{-3}$ of culture volume, equivalent to 0.1 $MJ \cdot m^{-2} \cdot d^{-1}$ that is ten times lower than maximum solar photosynthetic efficiency, then this system being energetically positive (Acién *et al*., 2012). Considering the combustion heat of the biomass (20 $KJ \cdot g^{-1}$) the biomass production step can sum an additional amount of energy up to 2,700 $kWh \cdot d^{-1}$. Considering a 9% of nitrogen content in the biomass obtained in this study (trial A3), the nitrogen fixation capacity of these reactors would be in the range of 3.6 g $N \cdot m^{-2} \cdot d^{-1}$, then to fix the total amount of 11.6 $Mg \cdot y^{-1}$ of $N\text{-}NH_4^+$ a total of 0.88 Ha are necessary (1,760 m^3 of culture volume),being only the 0.4% of farm land availability. This point is crucial as fertilizers, principally N and to a lesser extent P, are significant inputs of energy and finite resources if waste water is not used as a source of nutrients for microalgae production. Fertilizer production is linked directly to the cost of natural gas, the main energy source for fixing N to ammonia. In Europe (EU 25), the production of N fertilizer is falling with an increase in nitrogen price of 25% in the last five years (USDA, 2014). The price of nitrogen in

the world market is in the order of 1.4 \$ kg^{-1} (USDA, 2014) so that a facility producing microalgal biomass with the 11.6 t of N contained in UFP would allow to save up to around 17000 \$ y^{-1} representing fairly the 7% of biomass production costs on open raceway-type photobioreactors of 1.12 \$·kg^{-1} (Acién *et al*., 2014).

The integration of anaerobic digestion, digestate treatment and microalgae production has been found energetically positive.

The anaerobic digestion step was capable of producing energy for a total amount of 1,440 MWh y^{-1} (assuming 180 kW of power for 8,000 working hours).

Energy consumptions of the digestate treatment unit was around 115 MWh y^{-1} with reverse osmosis plus ammonia stripping processes being the main energy-requiring step accounting for up to 80% of digestate treatment process consumptions.

Microalgae production energy consumptions, being 100 MWh y^{-1}, was calculated summing the energy required by the hypothesized production facility (77 MWh y^{-1}) and the energy required by the UF process (23 MWh y^{-1}, i.e. 20% of the digestate treatment consumptions).

The energetic data underlined similar energy consumptions of both options A and B so that, from this point of view, there is not an optimal choice

between the two options integration. The option B (i.e. microalgae production) would allow, with the same energy consumption, to produce high added-value microalgal biomass, that could be used/and or sold by the farmer; the final choice would then rely on the farmer investments possibilities and land availability.

Beside these data inconsistent nutrient composition of wastes represents a challenge for microalgal cultivation, requiring careful reformulation to provide a consistent, and so reproducible performance. In this work, assuming that all the UFP is used for microalgae production and taking into consideration the obtained biomass yield, the total dry biomass production capacity of the system is 166-190 t y^{-1}. Produced biomass contains relevant amounts of valuable compounds, i.e., 39 - 76 t y^{-1} of carbohydrates, 56-89 t y^{-1} of proteins, 25-56 t y^{-1} of lipids, 0.9- 4 t y^{-1} of PUFA and 0.25 t y^{-1} of pigments, that considering the low production costs on the hypothesized scale-up system can increase the incomes of farmers if produced.

Conclusions

Anaerobic digestion is a real option to degrade organic residues, i.e. dairy cattle manure, producing biogas and digestate. Biogas can be used to produce electricity and heat, whereas digestate can be used as fertilizer.

Nevertheless digestate after partial treatment, i.e. ultrafiltration, can be also used to produce microalgae biomass because it contains nutrients under available forms. Results of this work indicated that the ultra-filtrated digestate can be used as a growth medium for microalgae production by diluting it at nitrogen concentration up to 113 $mg \cdot L^{-1}$. The productivity and composition of biomass obtained being comparable with that obtained using standard cultures mediums. Overall energy and mass balances of the process allow demonstrating that the integration of both anaerobic digestion and microalgae production steps are positive and technically feasible.

Despite these encouraging results, more efforts will be done to bring the UF-microalgae production process outside the laboratory scale, in a pilot raceway system where microalgae growth and nutrients uptake can be optimized. Moreover the upscale would be mandatory to finely evaluate large scale process energetic and economic sustainability.

Table 1 – Ingestate and digestate characteristics, and anaerobic digestion performances.

	Biogas plant performances					
	TS (% w.w.)	**VS (% TS)**	**TKN (g kg^{-1} w.w.)**	**$N\text{-}NH_4^+$ (g kg^{-1} w.w.)**	**$N\text{-}NH_4^+$/TKN (%)**	**BMP (NL biogas kg^{-1} TS)**
Ingestate	9.32 ± 0.34	85.37 ± 0.76	3.45 ± 0.14	1.02 ± 0.01	29.3 ± 0.7	340 ± 13
Digestate	7.15 ± 0.15	75.05 ± 1.34	3.61 ± 0.12	1.83 ± 0.04	51.2 ± 0.5	133 ± 24

AD data	**CH_4[a] (% v/v)**	**HRT (d)**	**BMY (%)**	**Organic matter degradation (% of the feeding)**	**Installed power[b] (kW)**	**Electrical productivity (ekWh d^{-1})**
	55 ± 0.4	32.3 ± 2.6	75.5 ± 3.4	41.3 ± 3.4	180	2182 ± 318

[a]Methane content measured in the biogas plant during the monitoring

[b]Around 8000 working hours per year

Table 2 – Chemical characterization of digestate fractions and mass balances.

	Mass Balance		Nutrients Balance		
	kg cycle^{-1}	%	TKN (kg cycle^{-1})	N-NH_4^+ (kg cycle^{-1})	P (kg cycle^{-1})
Digestate	14706	100%	53.06 ± 1.70	26.95 ± 0.62	11.17 ± 0.06
Screw press liquid stream	13235	90%	45.17 ± 1.61	22.52 ± 0.34	9.18 ± 0.23
Screw press solid stream	1471	10%	4.84 ± 0.11	1.95 ± 0.43	1.19 ± 0.01
Centrifuge liquid stream	10000	68%	20.32 ± 0.32	16.61 ± 0.12	1.26 ± 0.24
Centrifuge solid stream	3235	22%	23.54 ± 0.38	4.34 ± 0.37	8.98 ± 0.65
Ultrafiltration liquid stream	7500	51%	9.08 ± 0.01	8.48 ± 0.04	0.13 ± 0.01
Ultrafiltration concentrate	2500	17%	7.25 ± 0.42	3.81 ± 0.12	0.78 ± 0.06
Reverse osmosis liquid stream	5400	37%	0.06 ± 0.02	0.04 ± 0.01	0.02 ± 0.00
Reverse osmosis concentrate	2100	14%	12.77 ± 0.38	11.95 ± 0.15	0.06 ± 0.01
Zeolites liquid stream	5400		0.02 ± 0.01	0.02 ± 0.01	
Ammonium sulfate	182	1.2%	9.38 ± 0.06	9.35 ± 0.02	
Lime residue	1918	13.0%	0.82 ± 0.22	0.76 ± 0.03	0.04 ± 0.01
Disposable[a]	9124	62%	36.46 ± 0.45	10.85 ± 0.23	10.99 ± 0.36
Exportable[b]	5582	38%	12.01 ± 0.38	11.23 ± 0.76	0.04 ± 0.01
Error[c]			-4.59 ± 0.91	-4.87 ± 0.54	-0.14 ± 0.01

[a]Calculated as SP solid + DC solid + UF concentrate + Lime residue

[b]Calculated as RO permeate + RO concentrate – Lime residue

[c]Negative sign indicates missing quantities in the balance

u.d.l: under detection limit

Table 3 - UFP and diluted UFP chemical characterization

	Dilution					
Parameter	**Raw**	**D1**	**D2**	**D3**	**D4**	**D5**
pH	7.95 ± 0.23	9.4 ± 0.12	8.88 ± 0.21	8.55 ± 0.09	8.33 ± 0.11	8.25 ± 0.14
TKN (mg L^{-1})	1211 ± 1	42 ± 2	121 ± 2	313 ± 5	504 ± 24	606 ± 16
N-NH_4^+ (mg L^{-1})	1130 ± 6	39 ± 2	113 ± 3	292 ± 2	470 ± 9	565 ± 28
P (mg L^{-1})	17 ± 1	0.6 ± 0.12	1.7 ± 0.32	4.4 ± 0.4	7.1 ± 1.1	8.5 ± 0.8

Table 4 - CCD experimental design and growth parameters of A1, A2 and A3 trials.

		Codified levels of factors		Real values of factors		Growth parameters		
Trials	**Dilution**	**N-NH_4^+**	**Inoculum**	**N-NH_4^+ (mg L^{-1})**	**Inoculum (n° cells mL^{-1})**	**μ (d^{-1})**	**DM (g L^{-1})**	**D_p (mg L^{-1} d^{-1})[b]**
A1	D1	-√2	0	39	$7.55\ 10^6$	0.33 ± 0.04 [c]	1.13 ± 0.01 [c]	49 ± 1 [c]
A2	D2	-1	-1	113	$3.59\ 10^6$	0.26 ± 0.02 [b]	0.63 ± 0.03 [b]	28 ± 2 [b]
A3	D2	-1	+1	113	$1.15\ 10^7$	0.28 ± 0.03 [a]	2.38 ± 0.05 [a]	124 ± 11 [a]
A4	D3	0	-√2	292	$1.95\ 10^6$	*n.d.*	*n.d.*	*n.d.*
A5	D3	0	0	292	$7.55\ 10^6$	*n.d.*	*n.d.*	*n.d.*
A6	D3	0	+√2	292	$1.32\ 10^7$	*n.d.*	*n.d.*	*n.d.*
A7	D4	+1	-1	470	$3.59\ 10^6$	*n.d.*	*n.d.*	*n.d.*
A8	D4	+√2	0	470	$1.15\ 10^7$	*n.d.*	*n.d.*	*n.d.*
A9	D5	+1	+1	544	$7.55\ 10^6$	*n.d.*	*n.d.*	*n.d.*

[b] D_p (Daily volumetric Productivity)
DM (Dry Matter)
[c] Different letters in the columns mean significant differences ($p<0.05$).
n.d.: not determined

Table 5 – Macromolecular characterization of algae growth during A1,A2 and A3 trials.

Trial	Proteins		Total Lipids		Total Carbohydrates		Pigments	
	g kg^{-1} DM		g kg^{-1} DM		g kg^{-1} DM		g kg^{-1} DM	
A1	294 ± 8	[b]	295 ± 3	[b]	398 ± 8	[c]	1.28 ± 0.03	[c]
A2	493 ± 6	[a]	148 ± 2	[a]	201 ± 5	[b]	1.42 ± 0.04	[b]
A3	536 ± 6	[a]	151 ± 3	[a]	233 ± 12	[a]	1.51 ± 0.00	[a]

[a]Different letters in the columns mean significant differences ($p<0.05$).
DM (Dry Matter)

Table 6 – Amino acids composition of algae for A1,A2 and A3 trials.

	Amino acids content (g 100 g^{-1} crude protein)		
AA	**A1**	**A2**	**A3**
Ala	10.0 ± 0.3 [a]	9.3 ± 0.2 [b]	9.2 ± 0.2 [b]
Arg	6.0 ± 0.2 [a]	6.0 ± 0.1 [a]	12.1 ± 0.3 [b]
Asx	13.4 ± 0.5 [a]	12.2 ± 0.3 [b]	10.3 ± 0.2 [c]
Cys	0.2 ± 0.1	*u.d.l.*	*u.d.l.*
Glx	25.2 ± 0.4 [a]	23.7 ± 0.2 [b]	25.7 ± 0.2 [a]
Gly	0.3 ± 0.1 [a]	6.3 ± 0.2 [b]	5.1 ± 0.1 [c]
His	1.2 ± 0.2 [a]	1.2 ± 0.1 [a]	1.1 ± 0.1 [a]
Ile	3.2 ± 0.3 [a]	3.0 ± 0.2 [a]	2.6 ± 0.1 [b]
Leu	7.3 ± 0.6 [a]	7.0 ± 0.3 [a]	5.5 ± 0.3 [b]
Lys	6.2 ± 0.2 [a]	5.5 ± 0.1 [b]	5.8 ± 0.2 [a]
Met	0.8 ± 0.1 [a]	0.8 ± 0.1 [a]	0.5 ± 0.1 [b]
Phe	3.6 ± 0.2 [a]	3.7 ± 0.3 [a]	2.9 ± 0.1 [b]
Pro	5.2 ± 0.3 [a]	5.2 ± 0.3 [a]	4.4 ± 0.1 [b]
Ser	4.7 ± 0.2 [a]	4.3 ± 0.1 [a]	4.5 ± 0.1 [a]
Thr	5.0 ± 0.2 [a]	4.7 ± 0.1 [a]	4.6 ± 0.1 [a]
Tyr	2.9 ± 0.1 [a]	2.7 ± 0.2 [a]	1.8 ± 0.1 [b]
Val	4.7 ± 0.2 [a]	4.5 ± 0.2 [a]	4.0 ± 0.1 [b]

[a]Different letters in the columns mean significant differences ($p<0.05$).

u.d.l.: under detection limit

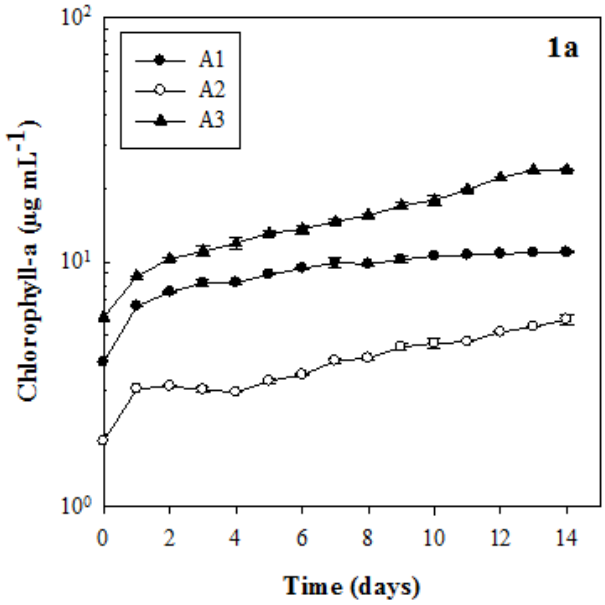
Figures caption

Figure 1 – Chlorophyll-a (1a) and N-NH_4^+ content (1b) variation during A1, A2 and A3 trials growth

Figure 2 - Fatty acids (2a) and pigments (2b) profile on a dry weight basis of A1, A2 and A3 trials (Total FA- Total Fatty Acids, SAFA-Saturated Fatty Acids, MUFA-Monounsatured Fatty Acids, PUFA-Polyunsatured Fatty Acids). Different letters on the bars mean significant differences ($p<0.05$).

Figure 3 – An integration approach for the dairy farm; SP (screw press separator), DC (decanter centrifuge), UF (ultrafiltration), RO (reverse osmosis), N-S (ammonia stripping).

Figure 1


1a
A1
A2
A3
Chlorophyll-a (µg mL-1)
Time (days)

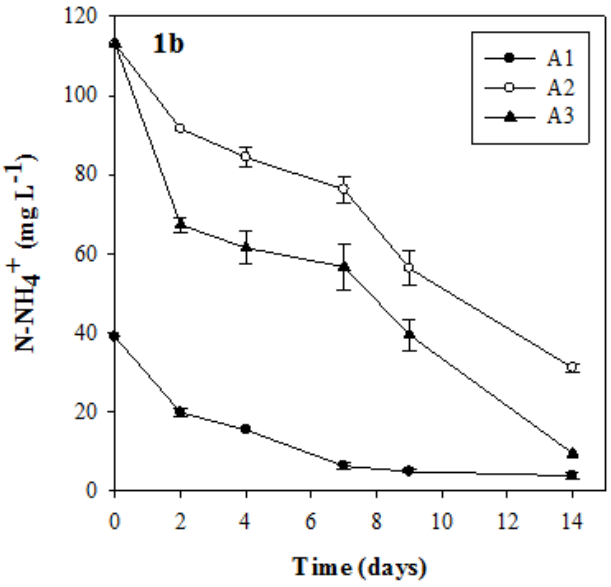

1b
A1
A2
A3
N-NH4+ (mg L-1)
Time (days)

Figure 2

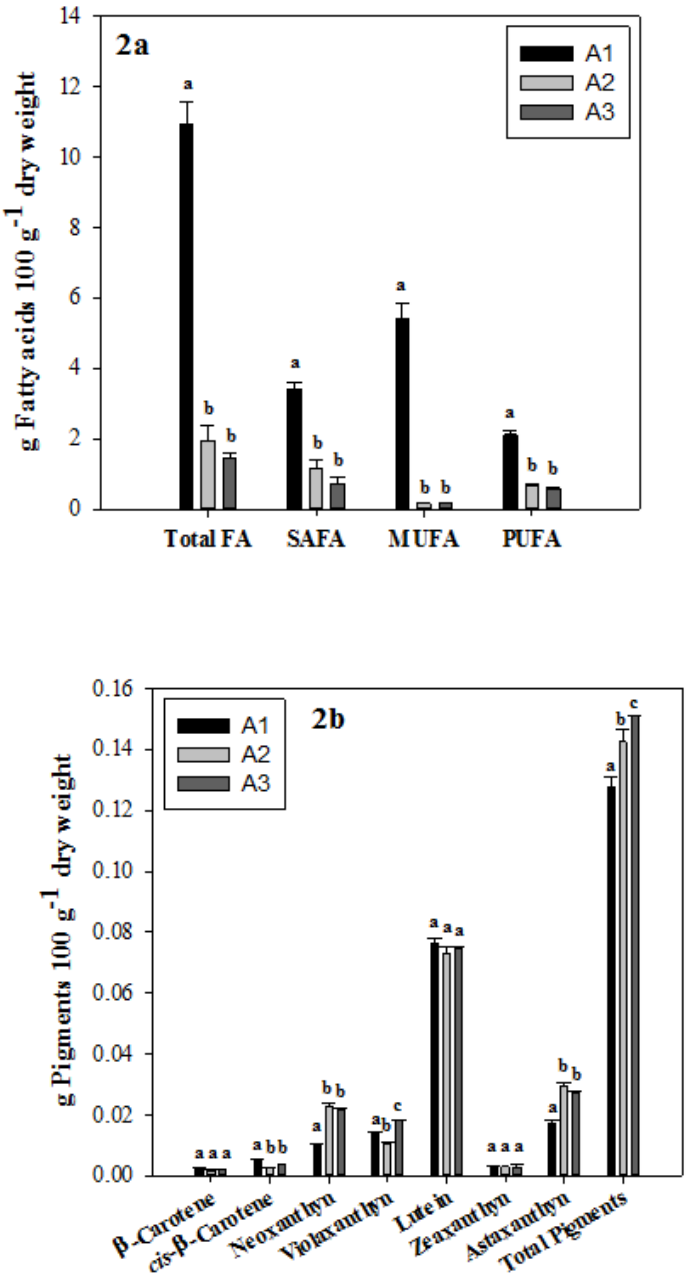
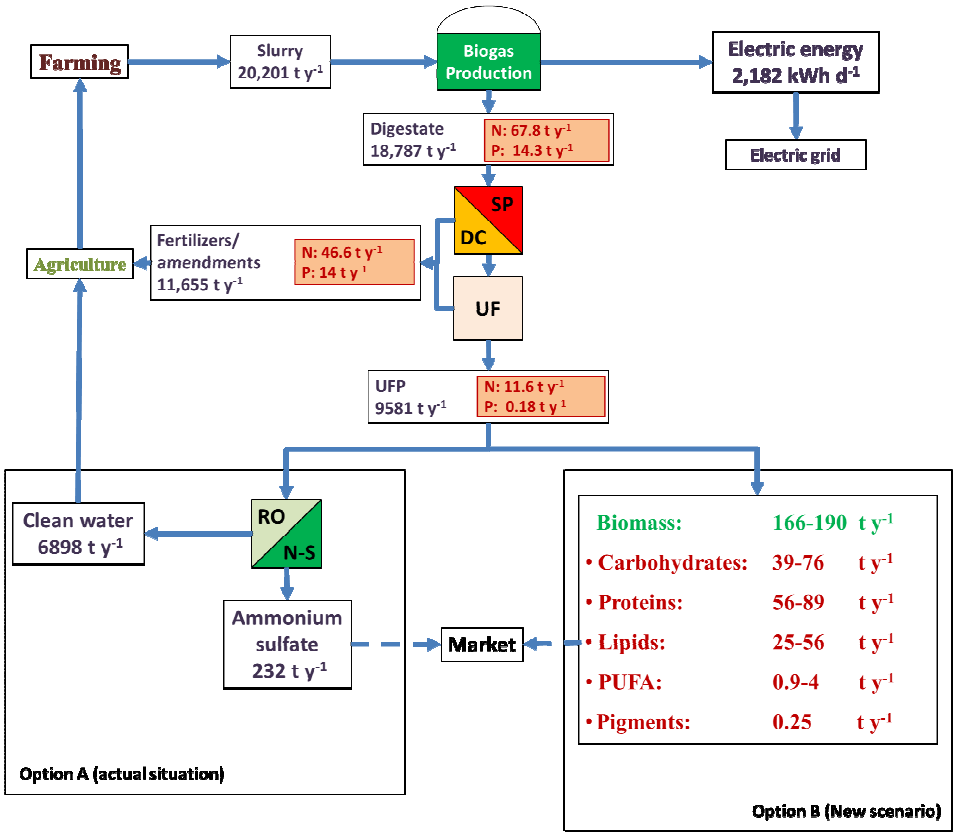

2a
A1
A2
A3
g Fatty acids 100 g-1 dry weight
Total FA
SAFA
MUFA
PUFA
2b
g Pigments 100 g-1 dry weight
β-Carotene
cis-β-Carotene
Neoxanthyn
Violaxanthyn
Lutein
Zeaxanthyn
Astaxanthyn
Total Pigments

Figure 3


Farming
Slurry
20,201 t y-1
Biogas
Production
Electric energy
2,182 kWh d-1
Digestate
18,787 t y-1
N: 67.8 t y-1
P: 14.3 t y-1
Electric grid
SP
DC
UF
Agriculture
Fertilizers/
amendments
11,655 t y-1
N: 46.6 t y-1
P: 14 t y-1
UFP
9581 t y-1
N: 11.6 t y-1
P: 0.18 t y-1
Clean water
6898 t y-1
RO
N-S
Ammonium
sulfate
232 t y-1
Market
Option A (actual situation)
Biomass: 166-190 t y-1
• Carbohydrates: 39-76 t y-1
• Proteins: 56-89 t y-1
• Lipids: 25-56 t y-1
• PUFA: 0.9-4 t y-1
• Pigments: 0.25 t y-1
Option B (New scenario)

References

- Acién, F.G., Fernández-Sevilla, J.M., Magán, J.J., Molina-Grima, E., 2012a. Production cost of a real microalgae production plant and strategies to reduce it. Biotechnol. Adv. 30 (6), 1344–1353
- Acién, F.G., Fernández-Sevilla, J.M., Molina-Grima, E., 2014. Economics of Microalgae Biomass Production.
- American Public Health Association - APHA, 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. APHA, Washington, DC.
- Barbarino, E., Lourenço, S.O., 2005. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J. Appl. Phycol. 17, 447–460.
- Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207-210.
- Berges, J.A., Charlebois, D.O., Mauzerall, D.C., Falkowski P.G., 1996. Differential effects of nitrogen limitation on photosynthetic efficiency of photosystems I and II in microalgae. Plant. Physiol. 110, 689-696.
- Bligh, E.G., Dyer, W.J., 1959. A rapid method for total lipid extraction and purification. Can. J. Biochem. Phys. 37, 911-917.

- Brandjes, P.J., De Wit, J., Van Der Meer, H.G., Van Keulen, H., 1996. Environmental impact of animal manure management. FAO Agriculture and Consumer Protection, available at FAO Corporate Document Repository.
- Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011. Microalgae-novel highly efficient starch producers. Biotechnol. Bioeng. 108, 766-776.
- Brennan, L., Owende, P., 2010. Biofuels from microalgae – a review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. 14, 557-577.
- Burton, C.H., 2007. The potential contribution of separation technologies to the management of livestock manure. Livest. Sci. 112, 208-216.
- Cordell, D., Drangert, J.O., White, S., 2009. The story of phosphorus: Global food security and food for thought. Global Environ. Chang. 19 (2), 292-305.
- De la Noüe, A., Laliberte, G., Proulx, D., 1992. Algae and wastewater. J. Appl. Phycol. 4, 247-254.
- DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356.

- EC MEMO/13/937, 2013. CAP Reform – an explanation of the main elements. Available at www.europa.eu.
- EPA, 1998. Method EPA 3051. Microwave assisted acid digestion of sediments, sludges, soils and oils.
- Franchino, M., Comino, E., Bona, F., Riggio, V.A., 2013. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. Chemosphere 92, 738–744.
- Guillard R.R.L., 1973. Culture Methods and Growth Measurements, Division Rates in Handbook of Phycological methods Stein JR (Ed.). Chambridge University Pres, Chambridge. 289-311.
- Huo, Y.X., Cho, K.M., Rivera, J.G.L., Monte, E., Shen, C.R., Yan, Y.J., Liao, J.C., 2011. Conversion of proteins into biofuels by engineering nitrogen flux. Nat. Biotechnol. 29, 346-351.
- IRSA CNR, 1994. Metodi analitici per le acque, Quaderni, N. 100. Istituto Poligrafico e Zecca dello Stato, Rome, Italy
- Ji, C-F., Yu, X-J., Chen Z-A., Xue, S., Legrand, J., Zhang, W., 2011. Effects of nutrient deprivation on biochemical compositions and photo-hydrogen production of *Tetraselmis subcordiformis*. Int. J. Hydrog. Energ. 36, 5817-5821.

- Ji, M., Abou-Shanab, R., Hwang, J., Timmes, T., Kim, H., Oh, Y., Jeon, B., 2013. Removal of nitrogen and phosphorus from piggery wastewater effluent using the green microalga *Scenedesmus obliquus*. J. Environ. Eng. 139 (9), 1198–1205.
- Lavoie, A., De la Noüe, J., 1985. Hyperconcentrated cultures of *Scenedesmus obliquus*. A new approach for wastewater biological tertiary treatment?. Water. Res. 19, 11: 1437-1442.
- Ledda, C., Schievano, A., Salati, S., Adani, F., 2013. Nitrogen and water recovery from animal slurries by a new integrated ultrafiltration, reverse osmosis and cold stripping process: A case study. Water Res. 47 (16), 6157-6166.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
- Mackinney, G., 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140, 315-322.
- Manenti, F., Adani, F., 2014. Integrating the Concept of Bio-Refinery onto the Biogas Field: the BIOREFILL Strategy. Jiří Jaromír Klemeš, Petar Sabev Varbanov and Peng Yen Liew (Editors) Proceedings of the 24th European Symposium on Computer Aided

Process Engineering – ESCAPE 24, June 15-18, 2014, Budapest, Hungary.

- Martin C., De la Noüe J., Picard G., 1985. Intensive cultivation of freshwater microalgae on aerated pig manure. Biomass, 7: 245-259.
- Mendoza, J.L., Granados, M.R., de Godos, I., Acién, F.G., Molina, E., Heaven, S., Banks, C.J., 2013. Oxygen transfer and evolution in microalgal culture in open raceways. Bioresour. Technol. 137 (2013), 188–195
- Oswald, W.J., Gootas, H.B., 1957. Photosynthesis in sewage treatment. Trans. Am. Soc. Civ. Eng. 122, 73-105.
- Park, J., Jin, H.F., Lim, B.R., Park, K.Y., Lee, K., 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. Bioresource Technol. 101 (22), 8649-8657
- Peters, N.E., Meybeck, M., 2000. Water Quality Degradation Effects on Freshwater Availability: Impacts of Human Activities. Water Int. 25 (2), 185-193.
- Petersen, S.O., Sommer, S.G., Béline, F., Burton, C., Dach, J., Dourmad, J.Y., Leip, A., Misselbrook, T., Nicholson, F., Poulsen, H.D., Provolo, G., Sørensen, P., Vinnerås, B., Weiske, A., Bernal, M.P., Böhm, R.,

Juhász, C., Mihelic, R., 2007. Recycling of livestock manure in a whole-farm perspective. Livest. Sci. 112, 180-191.

- Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol. 65, 635-648.
- Richmond, A., 2004. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Amos Richmond (Editor), Wiley-Blackwell**.**
- Schievano, A., Pognani, M., D'Imporzano, G., Adani, F., 2008. Predicting anaerobic biogasification potential of ingestates and digestates of a full-scale biogas plant using chemical and biological parameters. Biores. Technol. 99, 8112–8117.
- Schievano, A., Adani, F., Tambone, F., D'imporzano, G., Scaglia, B., Genevini, P.L., 2009. What is the digestate? Anaerobic Digestion: Opportunity for Agriculture and Environment [s.l]: Regione Lombardia, 9788890374609.
- Schievano, A., D'Imporzano, G., Orzi, V., Adani, F., 2011. On-field study of anaerobic digestion full-scale plants (Part II): New approaches in monitoring and evaluating process efficiency. Biores. Technol. 102: 8814–8819.

- Tam N.F.Y., Wong Y.S., 1989. Wastewater nutrient removal by *Chlorella pyrenoidosa* and *Scenedesmus* sp.. Environmental Pollution, 58: 19-34.
- USDA 2014
- Wood, S., Cowie, A., 2004. A review of greenhouse gas emission factor for fertilizer production. IEA Bioenergy Task. Available at leaftc.co.uk.
- Xiao, R., Chen, R., Zhang, H.Y., Li, H., 2011. Microalgae *Scenedesmus quadricauda* grown in digested wastewater for simultaneous CO_2 fixation and nutrient removal. J. Biobased Mater. Bio. 5, 234–240.
- Xin, L., Hong-Ying, H., Ke, G., Ying-Xue, S., 2010. Effects of different nitrogen and phosphorus concentrations pn the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technol. 101, 5494-5500.
- Yin-Hu, W., Yin, Y., Xin, L., Hong-Ying, H., Zhen-Feng, S., 2012. Biomass production of a *Scenedesmus* sp. under phosphorus-starvation cultivation condition. Bioresource Technol. 112, 192-198
- Yoo, C., Jun, S.Y., Lee, J.Y., Ahn, C.Y., Oh, H.M., 2010. Selection of microalgae for lipid production under high levels carbon dioxide. Bioresource Technol. 101 (1), S71–S74.

Nutrients reduction and nitrogen budget in a wild *Chlorella* sp. semi-continuous culture on digested swine manure liquid fractions.

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Abstract

Microalgae are known to be a useful biological tool for remediation of agricultural wastewaters, which if uncorrectly managed seriously degrade acquatic and soil environments due to their high organic and mineral load. In this work a wild *Chlorella* sp. has been isolated from a manure storage tank and grown semi-continuously on a digested swine manure and its

liquid fractions after centrifugation and ultrafiltration with 40 kDa membrane, in order to evaluate the depuration capacity. The microalga was capable of growing fast producing up to 0.21 g L^{-1} d^{-1} of biomass and to uptake up to 98% of ammonia nitrogen, and 70% of COD in all the substrates. Moreover also micronutrients were almost completely removed from the cultures.

Besides these encouraging data it has been found that most of the ammonia nitrogen was lost in the atmosphere due to stripping caused by aeration and high pH during *Chlorella* growth. Only 30% of the nitrogen were successfully incorporated in the microalgal biomass.

The microalga proved to be a strong strain, capable of reduce all the main macro and micronutrients although ammonia stripping is an issue that poses serious environmental concerns on the process.

1. Introduction

The developments and increase of livestock farming activities are arising environmental sustainability concerns, as water bodies eutrophication, air pollution by ammonia volatilization and soil degradation due to over-fertilization (Godos *et al*., 2010; Clarisse *et al*., 2009)

In Northern Italy, this sector is well established and developed, comprising almost 36% of Italian livestock sector and, more specifically, 70% and 80% of Italian cattle and swine heads respectively, causing over 70% of Utilized Agricultural Area (UAA) being defined as Nitrate Vulnerable Zone (National Strategic Nitrate Plan, 2009) while 81% of total ammonia emission in the atmosphere is directly correlated with manure management (ARPA, 2010). Besides, slurries improper land application causes a lack in soil nutrients availability (Chambers *et al*., 1999), thus the integration of agriculture fertilizing with chemical products and the increase of agronomical practices economic and environmental costs (Ledda *et al*., 2013).

In this context, anaerobic digestion (AD) is a useful technology to produce renewable energy from manure encouraging its proper treatment, recycling and disposal. AD treatment of animal slurries results, also, in the production of a biologically stable and partially hygienized organic product, i.e. the digestate, that could be utilized as fertilizer and/or organic amendment (Tambone *et al*., 2009).

However, farm land availability is often insufficient to cope with nutrients load limits (Nitrate Directive guidelines 91/676/CEE, 1991) so that it is mandatory to reduce and/or transport materials/nutrients. To achieve this,

digestate or slurry must be concentrated or fractionated to be cost-effectively exported from the farm (Ledda *et al*., 2013).

Regarding nutrients recovery, microalgae ability to uptake inorganic N and P is well recognized as an efficient bioremediation tool for agricultural wastewater treatment. Among these, swine slurry is typically characterized by high N and P concentrations and good balance of other nutrients, that are suitable for microalgae cultivation (Travieso *et al*., 2006): consequently, the use of microalgae for nutrient removal from this kind of wastewater has been considered to be practical, economical and promising (Olguín, 2012; Rawat *et al*., 2011). Moreover, as an added value of this process, microalgae would be capable to produce valuable organic compounds that could be extracted for several applications in feed, pharmaceutical, green chemistry and bioenergy sectors (Pulz and Gross, 2004).

In many studies, microalgae have been cultivated using digested swine slurries secondary effluents or diluted primary piggery wastewater (Travieso *et al*., 2006; Wang *et al*., 2010) above all for uptake of N, mainly in the ammonia form, and P. An important aspect of microalgae-based treatment is that N can not only be removed by cell metabolism (as ammonium), but also by stripping as ammonia, where significant amounts can be volatilized at increased pH and temperature conditions (Cai *et al*., 2013). Although many

works concluded that microalgae are able to reduce almost 100% N in agricultural wastewaters, few of them focused on the fractions volatilized during microalgae growth (Garcia *et al.*, 2000; Nuñez *et al*., 2001)

Another key-issue of this process is the detrimental effects that bacteria may have on microalgal biomass quality, so that slurry should be pretreated by sterilizing it (Cai *et al*., 2013); however, this is a costly and high energy-requiring process, which leads to an important constrain in cultivation scale-up of microalgae using wastewaters, underlying that this promising approach is still at a preliminary phase (Zhu *et al*., 2013).

This issue could be overcome by using and/or combining different strategies: first, it would be of great importance to isolate wild microalgae strains that tolerate complex substrates utilization, such as livestock slurries, and easily become predominant in the culture environment; second, it would be necessary to find existing technologies, applicable at farm scale and at a low cost, ensuring the sterilization of the slurry. Doing this it would be possible to achieve the remediation of the waste stream by recovering the nutrients into a high quality biomass.

The aim of this work was to isolate a wild microalga strain from a digested pig slurry and to cultivate it in different digestate liquid fraction sampled from a full-scale digestate treatment plant based on sequential membrane

technology (Ledda *et al*., 2013) equipped with a 40kDa (0.012 µm) ultrafiltration unit that would ensure sterilization of the digestate.

Moreover, during microalga growth nutrients reduction will be assessed and a nitrogen balance will be performed to determine the total amount of ammonia lost during the cultivation in order to evaluate sustainability of the process.

2. Material and methods

2.1. Substrates sampling and chemical characterization

Digestate (DIG), centrifuge liquid fraction (CLF) and ultrafiltrated liquid fraction (ULF) were sampled from a biogas plant equipped with a full-scale digestate treatment unit already described by Ledda *et al*. (2013). More specifically, DIG is the effluent of the biogas plant, CLF is the liquid fraction after a centrifugation of the digestate and ULF is the permeate after ultrafiltration of CLF.

Samples were stored in 1 L bottles at 4°C overnight and then analyzed.

Ammonia nitrogen ($N\text{-}NH_4^+$) and chemical oxygen demand (COD) were determined using fresh material according to the analytical methods for wastewater sludges (IRSA-CNR, 1994).

Total Kijeldahl Nitrogen (TKN) on dry biomass was determined using lyophilized pellet according to the analytical methods for wastewater sludges.

Total phosphorus (TP), Na, Mg, K, Ca, Zn, Fe, Mn, Cu, Co, Ni, Cr, As contents were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian. Fort Collins, USA) according to 3051A and 6020A EPA methods (EPA, 2007).

To close the nitrogen balance both determination of nitrogen content in the biomass and an acid trap to measure ammonia stripping were used. For the latter 250 mL Erlenmeyer flasks with 10 mL of saturated boric acid solution and 100 mL deionized water were prepared for each trial and connected to the air outlet of the cultures. The amount of ammonia nitrogen in the flask was then determined by acid titration with 0.001 N sulfuric acid. All analyses were performed in triplicate.

2.2. Chlorella sp. isolation and molecular identification.

The isolation of indigenous microalgal specie was performed according to Doria *et al*. (2012); briefly 25 mL LF samples were collected from a storage tank in the farm and incubated for 2 weeks in 300 mL Erlenmeyer flasks under 6400 °K coolwhite fluorescent tubes with an average photons flux of 1000 µmol m^{-2} s^{-1}. After that, 100 µL of the supernatant were plated in Petri

dishes with 40 mL agarized Bold's Basal Medium (Bold, 1949) and 200 µg mL^{-1} ampicillin.

Plates were incubated for 3 weeks at 25°C with a 12 h photoperiod at an average irradiance of 50 µmol photons m^{-2} s^{-1}.

Single colonies were transferred to a new Petri dish. After 2 to 3 weeks new single colonies were transferred again to a new Petri dish. After an additional week, each single colony was inoculated in 250 mL liquid 3-fold Nitrogen Bold's Basal Medium (3N-BBM), for a total of 5 isolates. Algal growth was then monitored daily by spectrophotometric optical density measurement at 560 nm (Jenway 7300, Bibby Scientific, UK). Among the isolates, a single culture grown on 3N-BBM showing a high growth rate was further isolated and the strain was identified by microscopic examination as belonging to the Chlorella genus.

To confirm microscopic identification a molecular analysis was performed: briefly culture pellets were subjected to five cycles of freeze-thawing and grinded in liquid N_2, then DNA was extracted by using GeneMATRIX Plant and fungi DNA purification kit (EURx) according to the manufacturer's instructions.

Two primers were used for PCR amplification that amplify regions of the 18S rRNA: the 18F GTCAGAGGTGAAATTCTTGGATTTA and the 18R AGGGCAGGGACGTAATCAACG.

Each PCR reaction was performed in a total volume of 25 µL containing approximately 25 ng of chromosomal DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10× buffer) supplemented to give a final concentration of 1.5 mM $MgCl_2$, 1 U of *Taq* polymerase (Bioline), and 0.5 pmol of each primer.

Amplification were run in a GeneAmp PCR system 2700 (Applied Biosystem) as follows: 1 cycle of 3 min at 94 °C; 37 cycles of 50 sec at 92 °C, 50 sec at 57 °C and a 50 sec extension step at 72 °C plus an additional 10 min cycle at 72 °C. The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide. Successful amplifications were purified from the agarose gel, using the Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI). The PCR products were sequenced on both strands and after that were manually checked obtaining a 650bp sequence. A comparative analysis was made by comparing the sequence with others from GenBank collected using BLAST giving a perfect match with a *Chlorella* sp. SSU rRNA sequence.

2.3. Microalga cultivation

According to previous experimental data and to the literature (Collos and Harrison, 2014) all the substrates were diluted with distilled water to reach an initial nitrogen concentration, in the culture, of 124 mg L^{-1} N; in fact, although ammonia is the preferred form of nitrogen for microalgae, higher concentrations are toxic because it is lipid soluble and so it easily diffuses through the membranes, thus inhibiting growth. The digested liquid streams were neither autoclaved or filtered in order to simulate a scale-up process.

Moreover all the substrates were compared to 3N-BBM standard medium which contains the same amount of nitrogen, although in the nitrate form.

Growth and biomass productivity on 3N-BBM and digested liquid streams were measured during a semi-continuous cultivation; the dilution rate was kept constant throughout the experiment and was determined by a preliminary batch cultivation, in all the tested substrates, after which a starting biomass concentration of around 0.15 g L^{-1} was set for the following trials in order to maintain the microalga in the exponential growth phase for two days of cultivation.

A linear correlation was found, in all cases, between absorbance of the cultures at 560 nm and biomass dry weight.

All the assays were performed in 500 mL Erlenmeyer flasks with an operative culture volume of 300 mL for a total of eight trials. Cultures were submitted to constant illumination with two 250 W daylight CFL bulbs with an average irradiance impinging the flasks of 150 μE $m^{-2}s^{-1}$.

Air was supplied by a commercial compressor with an average air flux of 0.3 L L^{-1} culture min^{-1}.

Growth and dry weight were determined by spectrophotometric measurements at 560 nm. Nevertheless three control dry weight determinations were performed during the experiment to confirm the data obtained through absorbance measurements. To do this 10 mL of each culture were filtered through pre-weighed Whatman GF-C filters subsequently dried at 80°C for 24 h.

The variation between indirect and direct dry weight measurements was, in all cases, lower than 5 %.

2.4. Calculations

Daily volumetric productivity (P_b) was calculated by the equation 1:

$$P_b = \frac{C_b - C_0}{t} \quad (1)$$

The specific growth rate (μ) was calculated by the equation 2:

$$\mu = \frac{1}{t} \times \ln\left(\frac{C_b}{C_0}\right) \quad (2)$$

Where C_b and C_0 are the concentrations of biomass at the end and at the beginning of a run, and t is the duration of the run.

3. Results and discussion

3.1. Chlorella sp growth

Microalga semi-continuous growth is shown in Figure 1. A result of the batch trials is that DIG could not support *Chlorella* growth at 124 mg L^{-1} N, therefore for successive semi-continuous assays the substrate has been further diluted until reaching a final concentration of 60 mg L^{-1} N.

After this, also the culture grown on DIG showed a positive growth, although it did not reach the same biomass concentration of the other cultures.

Both 3N-BBM and ULF cultures showed the same specific growth rate during the semi-continuous cultivation, i.e. 0.67 ± 0.06 d^{-1} and 0.65 ± 0.03 d^{-1} respectively.

Growth on SLF, on the other hand, resulted in a lower specific growth rate (0.52 ± 0.05 d^{-1}) while the assay on DIG reached the lowest value of 0.39 ± 0.03 d^{-1} but with a higher dilution and half the N concentration.

Regarding biomass productivity *Chlorella* grown on ULF showed the same behaviour of 3N-BBM culture achieving both the same average productivity throughout the experiment (0.21 ± 0.02 and 0.21 ± 0.01 g L^{-1} d^{-1} for 3N-BBM and ULF respectively, on a dry weight basis).

SLF culture showed a lower productivity of 0.17 g L^{-1} d^{-1}, while DIG achieved the lowest productivity of 0.10 g L^{-1} d^{-1} of dry biomass.

A main result of the semi-continuous cultivation is that DIG medium was not able to support a sustained growth even at a high dilution. In this case the preliminary batch trials (data not shown) concluded that the alga was not able to grow at 124 mg L^{-1} DIG, probably because the liquid phase of digestate is commonly characterized by high turbidity (Noike *et al*., 2004) and may be responsible for microalgal growth inhibition (Kallqvist and Svenson, 2003). Moreover this is further supported by characterization data (Table 1): infact DIG, as an expected result from the fractionation process, was characterized by a high total solid content in respect to the other streams, 39 g L^{-1} of dry matter, versus 12 and 9.5 g L^{-1} for SLF and ULF respectively.

Diluting the medium to 60 mg L^{-1} N overcame in some way this issue but was not sufficient to support *Chlorella* growth as in the other trials.

Indeed the total solid content was probably one of the main factor affecting the results in terms of growth; as in the case of DIG, also SLF did not achieve the same growth rate and biomass productivity of ULF even if it was not necessary to further dilute the stream. In this case the TS content of SLF was not much higher than that of ULF but the former was characterized by a much higher COD concentration (17767 mg O_2 L^{-1}) which was most probably correlated with medium turbidity (Nguyen *et al*., 2014)

ULF on the contrary, showed the same performance of the synthetic medium, as stated above, and confirmed that an initial ammonia nitrogen concentration of 124 mg L^{-1} is not toxic for the tested strain.

3N-BBM nitrogen source is in the form of nitrate which, although is the predominant form of nitrogen available to most plants, requires high energy input to be metabolized. Nitrate, in fact is reduced to ammonia before being available for assimilation. The reduction is divided into two steps: nitrate is reduced by a cytoplasmic NADH-dependent nitrate reductase to nitrite, which is further reduced to ammonia by a chloroplast-located NADPH-linked nitrite reductase (Yang *et al*., 2000).

This fact may explain why both 3N-BBM and ULF achieved the same growth performances: ULF nitrogen was in the form of ammonia, which was easily and energy-effective assimilated by microalgal cells, while growth on 3N-BBM required higher energy input implying a reduction of growth rate and productivity in respect to the potential performance. Unfortunately no trials were performed to confirm this hypothesis.

N:P ratio of the substrates ranged from 3 to 51 (3 for DIG, 38 for SLF and 51 for ULF); this values were expected from the treatment of the digestate, infact they reflect P behavior in solid-liquid separation processes as, in manures and slurries, it is mainly present in the solid-phase fractions (Smith *et al*., 1998) and, with membrane filtration, high P removal can be achieved. With solid/liquid separations and membrane filtration, P is then preferentially accumulated in the solid/concentrate thus decreasing its concentration in the liquid fraction.

Kaplan and Aslan (2008) reported an optimal N:P ratio of 8 for *Chlorella vulgaris* so that in this study DIG medium was characterized by a very good N:P ratio in respect to both SLF and ULF that were found to be P-limited substrates.

A wide range of N:P ratios (ranging from 4:1 to 45:1) characterize synthetic culture mediums , resulting in a N-deficient or P-deficient growth

(Richmond, 2004); in this case N:P ratio of 3N-BBM was 4, much lower than that of SLF and ULF. Nevertheless, some authors (Yin-Hu *et al*., 2012) reported that a P starvation growth (N:P ratio of 45:1) was similar to that with P repletion, concluding that more consumed P (luxury uptake) did not result in more biomass production.

Besides all these data, SLF and ULF showed better perfomances of DIG confirming how trials results were influenced by light availability in the cultures.

3.2. Nitrogen budget and nutrients reduction.

Nutrients reduction during cultures growth are summarized in Table 2.

All the assays resulted in a complete depletion of organic and mineral compounds of the substrates.

As a main result of the depuration process ammonia nitrogen was almost completely removed from the cultures, achieving reduction rate of 95% and 98% for DIG and SLF and ULF respectively.

These results are perfectly in accordance with all the previous works on microalgae-based wastewaters treatment: ammonia is easily assimilated by microalgal cells then high reductions can be achieved in more or less diluted

waste streams (Wang et al., 2009; Kobayashi *et al*., 2013; Franchino *et al*., 2014).

Besides, looking at nitrogen budget throughout the experiment, it was possible to conclude that of the total ammonia nitrogen removed only 31.3%, 26.3% and 34.4% of the nitrogen was incorporated in DIG, SLF and ULF cultures while most of it was lost by stripping (Figure 2).

These results are in accordance with the work of Nuñez *et al.* (2001) dealing with artificial wastewater. They concluded that only between 25% to 33% of nitrogen missing from the medium was actually recycled into microalgal biomass, pointing out that ammonia stripping is mainly due to mixing and aeration, favoured by high pH values of the medium and the following increase in unionized ammonia concentration.

This point is crucial as the loss of ammonia in the atmosphere is not environmentally acceptable as it may promote the formation of particulate matter (PM) having an important role in the acidification and the eutrophication processes (Clarisse *et al*., 2009).

The stripping process occurred in this study may have been caused also by the unbalanced N:P ratio in the substrates: in fact, as stated before, SLF and ULF were P-limited substrates, implying that microalgae cells were not capable to assimilate more nitrogen than that imposed by their biomass N:P

ratio. This result is in agreement with Seung-Hoon *et al*., (2013) that working with an unbalanced N:P wastewater concluded that N and P removal efficiencies were higher with a balanced N:P ratio, based on the addition of phosphate.

On the other hand in DIG culture, even though digestate had a more balanced N:P ratio, stripping occurred probably to the low *Chlorella* growth caused by light limitation.

Regarding the other nutrients, P was almost completely removed from all the trials (Table 2): in SLF and ULF the initial concentration was low (P-limited susbtrates) and microalgae assimilated all the available P in the culture medium.

In the case of DIG, a high reduction percentage was achieved probably due to precipitation of P caused by the high pH of the culture (9.2).

Moreover DIG was a more degradable substrate, as can be seen from OD_{20} and COD data (Table 1), so that probably during microalgae growth, also bacteria contributed to nutrients reduction.

This explained also the high reduction of COD, in fact DIG trial resulted in the higher COD abatement during microalgal growth (73%) while SLF and ULF showed a lower reduction percentage (68% and 61% respectively).

This, again, was probably correlated to the degradability of the digestate in respect to the centrifuge and ultrafiltration liquid fraction.

In the case of ULF most of the COD was retained in the ultrafiltration concentrate so that initial COD concentration was much lower than the other streams.

In this case, even though degradability of the substrate was low (16 mg O_2 g^{-1}dw) bacteria played a role in degradation of organic matter. Moreover probably also microalgae assimilated short-chain volatile fatty acids through mixotrophic metabolism (Hu *et al*., 2012), increasing the performance of the degradation. At the end of *Chlorella* growth on ULF, the effluent had a COD concentration of 71 mg O_2 L^{-1}, far below the acceptable limit for discharging in surface water bodies (160 mg L^{-1}).

Looking at micronutrients and heavy metals uptake, all the trials showed similar behavior, with high reductions of all the compounds in all the trials (Table 2).

In any case all the substrates did not contain high concentrations of nutrients so that *Chlorella* was capable of growing without any sign of inhibition.

4. Conclusions

A full scale digestate treatment plant produces two liquid fractions of the digestate, CLF (Centrifuge liquid fraction) and ULF (Ultrafiltration liquid fraction) that were all used as a growth medium for a wild *Chlorella* sp. isolated from the CLF storage tank and were compared to a synthetic medium (3N-BBM).

The microalga was capable of growing semi-continuously in all the trials, in particular reaching the highest growth rate and biomass productivity in ULF, with the same values obtained using 3N-BBM medium. On the other hand DIG supported growth only when initially diluted to 60 mg L^{-1} of ammonia nitrogen, because of light availability in this substrate was very low.

In all the trials, *Chlorella* growth resulted in the almost complete depuration of the substrates above all regarding nitrogen, macro- and micro-nutrients.

Also COD was removed from the cultures but in a less extent with only effluent from ULF culture that could be discharged in the environment for its low COD content.

A critical point of the process was the huge amount of ammonia nitrogen released in the atmosphere during the experiment, in fact only 30% of the removed nitrogen could be fixed into the microalgal biomass. This was

almost certainly due to aeration of the cultures and high pH (around 9 in all the trials). Increasing P content in SLF and ULF may have improved nitrogen uptake by balancing N:P ratio of the substrates but this is not a feasible option, as P is a finite non-renewable mineral.

Table 1 – Chemical characterization of digestate (DIG), centrifuge liquid fraction (CLF) and ultrafiltration liquid fraction (ULF)

	Digested liquid streams		
	DIG	**CLF**	**ULF**
pH	7.97 ± 0.13	8.06 ± 0.08	8.61 ± 0.12
TS (g kg^{-1})	39 ± 2	12 ± 1	9.5 ± 0.5
OD_{20} (mg O_2 g^{-1}dw)	37 ± 4	27 ± 5	16 ± 1
NH_4^+-N (mg L^{-1})	2055 ± 21	2018 ± 11	2013 ± 13
COD (mg O_2 L^{-1})	37643 ± 234	17767 ± 187	2900 ± 82
Na (mg L^{-1})	772 ± 157	495 ± 161	399 ± 63
Mg (mg L^{-1})	213 ± 3	4.6 ± 2.3	2.5 ± 1.0
K (mg L^{-1})	2524 ± 35	2237 ± 12	2340 ± 59
Ca (mg L^{-1})	437 ± 304	29 ± 11	25 ± 1
P (mg L^{-1})	620 ± 2	53 ± 10	40 ± 17
Zn (mg L^{-1})	28.9 ± 12.4	1.9 ± 1.0	0.9 ± 0.5
Fe (mg L^{-1})	169.2 ± 80.3	15.1 ± 10.4	8.4 ± 2.7
Mn (mg L^{-1})	4.1 ± 0.2	0.5 ± 0.1	0.3 ± 0.1
Cu (mg L^{-1})	10.6 ± 1.5	3.1 ± 0.3	3.1 ± 0.3
Co (mg L^{-1})	3.8 ± 0.6	0.7 ± 0.1	0.6 ± 0.1
Ni (mg L^{-1})	4.8 ± 1.1	0.5 ± 0.2	0.5 ± 0.1
Cr (mg L^{-1})	1.0 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
As (mg L^{-1})	7.9 ± 0.7	3.3 ± 0.2	2.8 ± 0.1
N:P	3	38	51

Table 2 – Average nutrients reduction during *Chlorella* sp. growth in digestate (DIG), centrifuge liquid fraction (CLF) and ultrafiltration liquid fraction (ULF), (n=5).

	DIG			CLF			ULF		
	Initial	**Final**	**Reduction (%)**	**Initial**	**Final**	**Reduction (%)**	**Initial**	**Final**	**Reduction (%)**
NH_4^+-N (mg L^{-1})	60.00 ± 0.61	2.85 ± 0.05	95%	124.03 ± 0.68	2.84 ± 0.10	98%	124.00 ± 0.80	2.58 ± 0.26	98%
COD (mg L^{-1})	1099.07 ± 6.83	296.75 ± 8.2	73%	1092.00 ± 11.49	349.44 ± 8.2	68%	181 ± 13	71 ± 4	61%
P (mg L^{-1})	18.11 ± 0.06	2.72 ± 0.63	85%	3.26 ± 0.60	0.08 ± 0.02	97%	2.44 ± 1.05	0.02 ± 0.01	99%
Na (mg L-1)	22.53 ± 4.59	6.38 ± 0.63	72%	30.40 ± 9.89	6.38 ± 0.63	79%	24.56 ± 3.90	18.43 ± 2.21	25%
Mg (mg L^{-1})	6.21 ± 0.10	0.37 ± 0.12	94%	0.29 ± 0.14	0.08 ± 0.12	72%	0.15 ± 0.06	0.03 ± 0.09	81%
K (mg L^{-1})	73.68 ± 1.02	13.47 ± 0.70	82%	137.49 ± 0.76	21.35 ± 0.70	84%	144.15 ± 3.60	20.84 ± 1.75	86%
Ca (mg L^{-1})	12.75 ± 8.87	1.96 ± 0.30	85%	1.80 ± 0.65	0.64 ± 0.30	65%	1.54 ± 0.04	0.75 ± 0.14	51%
Co (mg L^{-1})	0.11 ± 0.04	*u.d.l.*	100%	0.04 ± 0.01	*u.d.l.*	100%	0.04 ± 0.01	*u.d.l.*	100%
Zn (mg L^{-1})	0.84 ± 0.36	0.08 ± 0.02	91%	0.12 ± 0.06	0.04 ± 0.02	65%	0.05 ± 0.03	0.02 ± 0.01	63%
Fe (mg L^{-1})	4.94 ± 2.35	0.02 ± 0.00	100%	0.93 ± 0.64	0.02 ± 0.00	98%	0.52 ± 0.17	0.01 ± 0.00	98%
Mn (mg L^{-1})	0.12 ± 0.02	*u.d.l.*	100%	0.03 ± 0.01	*u.d.l.*	100%	0.02 ± 0.02	0.01 ± 0.00	50%
Cu (mg L^{-1})	0.31 ± 0.04	0.01 ± 0.00	97%	0.19 ± 0.05	0.01 ± 0.00	95%	0.19 ± 0.03	0.01 ± 0.00	95%
Ni (mg L^{-1})	0.14 ± 0.02	*u.d.l.*	100%	0.03 ± 0.00	*u.d.l.*	100%	0.03 ± 0.01	0.01 ± 0.00	67%
Cr (mg L^{-1})	0.03 ± 0.01	*u.d.l.*	100%	0.02 ± 0.00	*u.d.l.*	100%	0.02 ± 0.01	0.01 ± 0.00	50%
As (mg L^{-1})	0.23 ± 0.07	0.01 ± 0.00	96%	0.20 ± 0.07	0.01 ± 0.00	95%	0.17 ± 0.06	0.13 ± 0.03	24%

Figures caption

Figure 1 – *Chlorella* sp. growth on 3N-BBM, digestate (DIG), centrifuge liquid fraction (CLF) and ultrafiltration liquid fraction (ULF)

Figure 2 – Nitrogen balance during *Chlorella* sp. cultivation on digestate (DIG), centrifuge liquid fraction (CLF) and ultrafiltration liquid fraction (ULF)

Figure 1

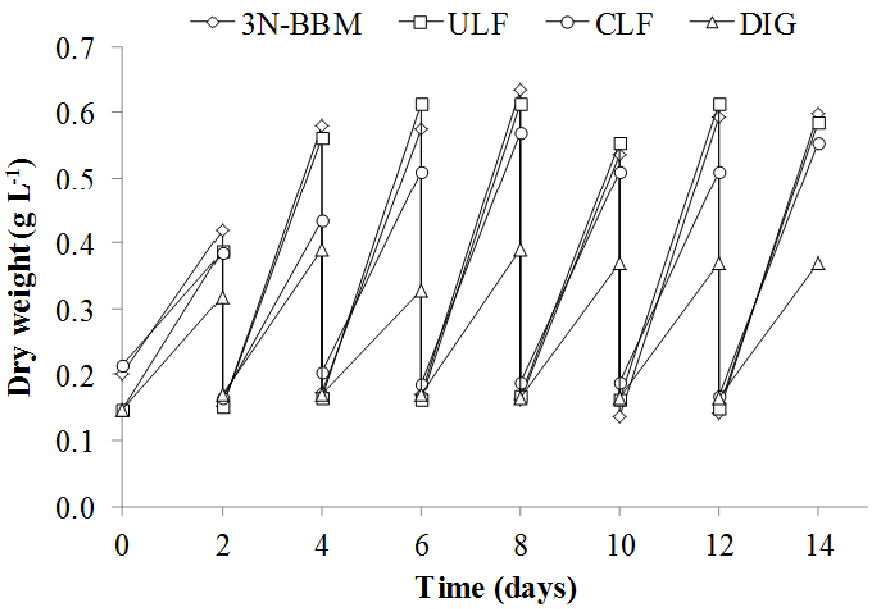
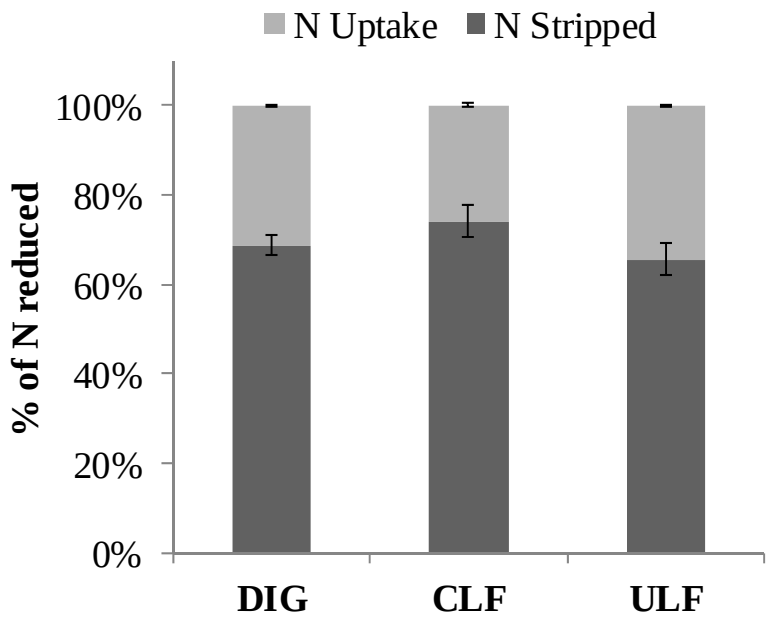

3N-BBM
ULF
CLF
DIG
Dry weight (g L-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
2
4
6
8
10
12
14
Time (days)

Figure 2



Rerences

- ARPA Lombardia, Azienda Regionale per la Protezione dell'Ambiente, 2010. Rapporto Stato Ambiente Lombardia. Available at http://ita.arpalombardia.it.
- Bold, H.C. 1949. The morphology of *Chlamydomonas chlamydogama* sp. nov. Bull. Torrey Bot. Club. 76, 101-8.
- Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renewable and Sustainable Energy Reviews 19: 360–369
- Chambers, B.J., Lord, E.I., Nicholson, F.A., Smith, K.A., 2006. Predicting nitrogen availability and losses following application of organic manures to arable land: MANNER. Soil Use Manage. 15(3), 137–143.
- Clarisse, L., Clerbaux, C., Dentener, F., Hurtmans, D., Coheur, P.F., 2009. Global ammonia distribution derived from infrared satellite observations. Nat. Geosci. 2(7), 479–483.
- Collos, Y., Harrison, P.J., 2014. Acclimation and toxicity of high ammonium concentrations to unicellular algae. Marine Pollution Bulletin, 80(1–2):8–23.

- Doria, E., Longoni, P., Scibilia, L., Iazzi, N., Cella, R., Nielsen, E., 2012. Isolation and characterization of a *Scenedesmus acutus* strain to be used for bioremediation of urban wastewater Journal applied Phycology 24(3), 375-383.
- EPA, 2007. Method EPA 3051A. Microwave assisted acid digestion of sediments, sludges, soils and oils.
- EPA, 2007. method EPA 6020A. Elements determination by inductively coupled plasma-mass spectrometry (ICP-MS)
- ERSAF, Ente Regionale per i Servizi all'Agricoltura e alle Foreste , 2009. National Strategic Nitrate Plan – Piano Strategico Nazionale Nitrati. Available at http://www.ersaf.lombardia.it.
- Garcia, J., Mujeriego, R., Hernandez-Marine, M., 2000. High rate algal pond operating strategies for urban wastewater nitrogen removal. Journal of Applied Phycology 12:331–339.
- Godos, de I., Vargas, V.A., Blanco, S., García González, M.C., Soto, S., García-Encina, P.A., Becares, E., Muñoza, R., 2010. A comparative evaluation of microalgae for the degradation of piggery wastewater under photosynthetic oxygenation. Bioresour Technol 101(14):5150-5158.

- IRSA CNR, 1994. Metodi analitici per le acque, Quaderni, N. 100. Istituto Poligrafico e Zecca dello Stato, Rome, Italy
- Kallqvist, T., Svenson, A., 2003. Assessment of ammonia toxicity in tests with the microalga, Nephroselmis pyriformis, Chlorophyta. Water Res. 37, 477–484.
- Kapdan, I. K. and S. Aslan. 2008. Application of the Stover–Kincannon kinetic model to nitrogen removal by *Chlorella vulgaris* in a continuously operated immobilized photobioreactor system. J. Chem. Technol. Biotechnol. 83: 998-1005.
- Ledda, C., Schievano, A., Salati, S., Adani, F., 2013. Nitrogen and water recovery from animal slurries by a new integrated ultrafiltration, reverse osmosis and cold stripping process: A case study. Water Res. 47 (16): 6157-6166.
- Nguyen, L.A.T., Ward, A.J., Lewis, D., 2014. Utilisation of turbidity as an indicator for biochemical and chemical oxygen demand. Journal of Water Process Engineering 4:137–142
- Noike, T., Goo, I.S., Matsumoto, H., Miyahara, T., 2004. Development of a new type of anaerobic digestion equipped with the function of nitrogen removal. Water Sci. Technol. 49, 173–179.

- Nuñez, V.J., Voltolina, D., Nieves, M., Piña, P., Medina, A., Guerrero, M., 2001. Nitrogen budget in Scenedesmus obliquues cultures with artificial wastewater. Bioresour Technol 78:161-164.
- Olguín, E.J., 2012. Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. Biotechnol Adv 30(5):1031-1046.
- Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol. 65, 635-648.
- Richmond, A., 2004. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Amos Richmond (Editor), Wiley-Blackwell.
- Smith, K.A., Chalmers, A.G., Chambers, B.J., Christie, P., 1998. Organic manure phosphorus accumulation, mobility and management. Soil Use Manage. 14, 156-159.
- Tambone, F., Genevini, P., D'Imporzano, G., Adani, F., 2009. Assessing amendment properties of digestate by studying the organic matter composition and the degree of biological stability during the anaerobic digestion of the organic fraction of MSW. Bioresource Technol. 100(12):3140–3142.

- Travieso, L., Benítez F., Sánchez, E., Borja, R., Martín, A., Colmenarejo, M.F., 2006. Batch mixed culture of Chlorella vulgaris using settled and diluted piggery waste. Ecological Engineering 28(2):158-165.
- Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., Ruan, R., 2010. Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnol 162(4):1174-86.
- Yin-Hu, W., Yin, Y., Xin, L., Hong-Ying, H., Zhen-Feng, S., 2012. Biomass production of a *Scenedesmus* sp. under phosphorus-starvation cultivation condition. Bioresource Technol. 112, 192-198
- Zhu, L., Wanga, Z., Takalab, J., Hiltunenb, E., Qina, L., Xua, Z., Qinc, X., Yuana, Z., 2013. Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production. Bioresource Technol 137: 318–325

Primary filtration and *H. pluvialis* cultivation to treat swine slurry: a preliminary approach toward valuable astaxanthin production.

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Abstract

Livestock farming produces large amounts of nutrients-rich slurries that, if incorrectly managed, may threat soil and aquatic environments. In this work a double stage filtration-microalgae cultivation process is proposed to both depurate swine farming wastewater and to produce *H. pluvialis* astaxanthin-rich biomass. The first step is achieved by a low-cost filtration system that is capable of reducing up to 66% of ammonia nitrogen, 7% of total phosphorus and 19% of chemical oxygen demand. The increase of nitric nitrogen after the filtration is further exploited in the second step by *H. pluvialis* discontinuous cultivation in 2, 4 and 8-fold diluted wastewater. This step causes a drastic reduction in macro and micronutrients concentration (up to 99% for NO_3-N and NH_4-N, 98% for TP and 26% for COD). All the trials showed a comparable astaxanthin accumulation (up to 1.27% on a dry weight basis) after 14 days in nutrients deprived conditions.

These results indicate the possibility to couple low-cost filtration and microalgae production to recycle nutrients from swine wastewaters and to valorize them by providing high-added value astaxanthin.

1. Introduction

Managing the input of organic and mineral nutrients derived from livestock activities into the atmosphere and water-bodies poses both technical and economic challenges to the agricultural sector. Storage and land application of animal slurries cause the loss of large amounts of N in the air due to volatilization of ammonia (Clarisse *et al*., 2009), nitrate leaching and accumulation of P and K compounds in agricultural soil (Ledda *et al*., 2013). On farm environmentally sustainable manure management is thus crucial to minimize losses of valuable nutrients and to prevent contamination and/or eutrophication of the surrounding environment (Kebede-Westhead *et al*., 2006).

Wastewater-based mass cultivation of algae is a promising solution to contribute to the protection of freshwater ecosystems, providing a more sustainable approach reducing the toxicity potential of pollution point sources than achieved by current treatment practices.

Compared to physical and chemical treatment processes, algae based treatment can potentially achieve nutrient removal in a less expensive and ecologically safer way with the added benefits of resources recovery and recycling (Oswald, 2003). Common nitrogen removal methods such as bacterial nitrification/denitrification remove the majority of the nitrogen as

N_2 gas, whereas algal treatment retains useful nitrogen compounds in the biomass.

Usually in conventional biological wastewater treatment processes, nitrate reduction requires external carbon sources such as methanol or acetate. Additionally the produced biomass must be treated and disposed safely and in an economically feasible way increasing operating costs. On the other hand nitric nitrogen can be converted into microalgal biomass without any external carbon source, producing bioactive substances, bioenergy, or valuable chemicals (Kang *et al*., 2006)

Notwithstanding these benefits, microalgae treatment may have application as an integrative process in common nutrient removal mainly due to low rates of growth and nutrients uptake by microalgae leading to an effective difficulty for this process to be used as a principal treatment. In this sense many efforts have been done, since several decades, to study and optimize microalgae cultivation in wastewaters both in stand-alone or integrated systems, mainly regarding nutrients reduction and/or abatement in the final effluent. In particular, swine manure is characterized by high contents of nitrogen (up to 872 mg L^{-1}, Borin *et al*., 2013), organic matter (6.7%) (Roche, 1984) and suspended solids (Buelna *et al.*, 2008), so that a pre-

treatment system that can reduce these components is often a necessary solution before depuration by algae.

As a primary step, filtration could then be used as an economic solution. Traditional methods of wastewater filtration consist in trickling filters, rotation biological contactors, intermittent sand filters and infiltration percolation system (Loupasaki and Diamadopoulos , 2013). Although sand layers filters are quite common (Roseth, 2000; Liu *et al*., 2003; Nakhla and Farooq, 2003; Tao and Wang, 2009; Zheng *et al.*, 2012), the aim of the treatment can be different, depending on the used substrate. Biological filter filled with semi-soft plastic media can give good result in COD removing (Wei *et al*., 2010). Yasuda *et al*. (2009) demonstrated that rock wool could be a possible substrate for ammonia removal from manure composts. Also laterite material has shown to be a suitable medium to reduce COD, BOD, ammonia, nitrite and turbidity (Kadam *et al*., 2009). Saliling *et al.* (2007) suggested wood chips and wheat straw as alternative biofilter media to treat high nitrate content wastewaters.

Among different genera of algae, Chlorella, Scenedesmus, Athrospira and Clamydomonas have been commonly used for the phytoremediation of industrial, urban and agricultural wastewaters (Ji *et al*., 2013; Franchino *et al*., 2013) producing a widely chemically diversified biomass which have

claimed to sustain potential applications above all in biofuels and animal feed sectors.

Within the algae-derived products, the ketocarotenoid astaxanthin from green alga *H. pluvialis* is a high-value carotenoid with applications in nutraceuticals, cosmetics, food and feed industries. Astaxanthin sells for US$ 2,500 per kg with an annual worldwide market estimated over US$ 200 million (Del Rìo *et al.,* 2005). Although most of this market is based on synthetically-derived astaxanthin, consumers demand for natural products provides an opportunity for the natural molecule. In this sense, the microalga *H. pluvialis* represents the richest source of natural astaxanthin and it is now cultivated at large scale (Olaizola, 2000; Olaizola and Huntley, 2003),

Therefore, *Haematococcus* derived astaxanthin has high application potential in the nutraceutical, pharmaceutical, cosmetics, food, and feed industries (Del Rìo *et al.,* 2005).

The cultivation of *H. pluvialis* represents a highly sensitive process, in the way that vegetative growth phase, prior to astaxanthin accumulation in cells bodies, is susceptible to contamination by fast-growing unicellular green and/or blue-green algae due to *Haematococcus* relative slow growth (Bubrick, 1991; Orasa *et al*., 2000).

For this reason, commercial production of Haematococcus-derived astaxanthin has been commonly reported by using a two-step culture where the first stage is done photoautotrophically under highly controlled culture condition in either tubular, bubble column or airlift photobioreactors and the reddening stage, less prone to contamination, is done in open cultivation ponds (Hata *et al*., 2001).

Moreover, many works have been conducted on the development of an optimal synthetic growth medium (Gong & Feng, 1997; Fábregas et al., 2000;) but, as far as we are aware, few studies focused on the possibility to use agricultural wastewaters for *H. pluvialis* and subsequent astaxanthin production (Kang *et al*., 2006).

In this work, a raw pig slurry has been treated by a low-cost filtration system at an experimental plant aimed to phytodepuration, located in Padova. The effluent from the system, still rich in organo-mineral nutrients was further treated by using it as a nutrients source for *H. pluvialis* cultivation and astaxanthin production in a one-step batch system which is considered simple and requires low capital investment and technological know-how (Richmond and Hu, 2013).

The aim of this work is to evaluate the reduction of the raw slurry organic and mineral pollutants by a two-step filtration-microalgae system, coupled

to biomass production. As an added value of the process, astaxanthin accumulation under nutrients-deprived conditions has been evaluated.

The results of this work would be useful as a first insight on an integrated farm facility where a low cost pre-treatment and microalgae cultivation would deal with nutrients overload in animal slurries and allow on-farm high-added value productions.

2. Materials and methods

2.1. Swine wastewater pretreatment

The swine wastewater (RW) derived from an integrated pilot plant realized in 2012 and experimented by the University of Padova (North-Eastern Italy). It was located at the Experimental Farm “Lucio Toniolo” at Legnaro (8 m a.s.l., Padova, Italy; N45° 20', E11° 58') and occupied a total area of 72 m². The plant consisted of an upstream filtering system and a downstream phytodepuration system, the former to pre-treat the wastewater before the depuration by plants to reduce salinity, organic matter and chemical elements potentially dangerous for plants growth. It treated a wastewater portion coming from a piggery stable made of feces, urine, and fresh water used daily for the stable cleaning. The filtering system was composed of six filters with iron structure and a 0.5 m^3 volume capacity (0.8

m x 0.8 m x height 0.8 m). They were filled with different substrates, some already used as filter media for swine wastewater treatment such as sand (Zheng *et al*., 2012), gravel and zeolite (e.g. Nguyen and Tanner, 1998; Nikolaeva S., 2002), the others never used previously for filtration purposes in swine treatment but interesting for their low cost such as bamboo,(*Phyllostachys pubescens*, Pradelle, Mazel ex J.Houz.), giant reed (*Arundo donax* L) and plastic tops;. The filters presented one or a mix of different substrates (Table 1). The filtering system worked in parallel, so each filter was fed simultaneously with wastewater moving vertically on filling substrate. A daily load volume of 30 L of swine wastewater coming from the storage tank of the stable (5L per filter) with 5 re-circulations (one every three hours) was applied to the system. All effluents coming from the filters were finally mixed (FW) and afterwards sent to the phytodepuration system (not studied in this work).

The filtering system was tested in 2012 for 35 days, from August to October. The monitoring was carried out at inlet (RW) and at outlet (FW) of the system and regarded the physical and chemical parameters of the wastewater.

For *H. pluvialis* cultivation test a sample of the FW was picked up in October to be used as growth medium.

2.2. H. pluvialis seed cultures and cultivation

Green microalga *H. pluvialis* (strain number 34-1d) was obtained from the SAG Culture Collection of the University of Goettingen (Germany). Pre-cultures were maintained in 200 mL Erlenmeyer flasks in Bold Basal Medium for freshwater microalgae.

Constant illumination during seed cultures was provided by fluorescent cool white lamps (Osram L13W/840) with an average irradiance of 50 µE $m^{-2}s^{-1}$. FW was filtered with 0.45 Whatman GF/C filters and diluted with deionized water to obtain a final growth medium containing 50% FW, 25% FW and 12.5% FW. A preliminary test with 100% FW did not result in any microalgal growth (data not shown).

Each trial was inoculated with seed cultures of *H. pluvialis* to reach a seed dry biomass concentration of around 0.05 g L^{-1}.

For each thesis, cells were grown in discontinuous mode, photoautotrophically, in two 500 mL Erlenmeyer flaks containing 300 mL of cell suspension. The flasks were continuously illuminated using two 30W OSRAM-Sylvania GRO-LUX 6400 °K lamps, with an average irradiance on the flasks surface of 150 µE $m^{-2}s^{-1}$. The temperature of the culture was maintained constant at 25°C.

Mixing and CO_2 feeding were achieved by air bubbling with a commercial aquarium pump with a nominal flow rate of 250 L air h^{-1}. The specific flow rate for each culture was 2.3 L air L culture^{-1} min^{-1}.

Cultures growth was monitored with optical density (OD) measurement through spectrophotometric analysis at 750 nm wavelength. A linear relationship was previously found between absorbance at 750 nm and dry weight of the culture.

Dry weight of biomass in the cultures was measured by drying cells at 85 °C for 24 h after filtration through a pre-weighted GF/C filter (Whatman).

Astaxanthin accumulation did take place in the same experimental conditions of the growth trials. It's content was spectrophotometrically determined according to Boussiba *et al*. (1992) after 14 days from the beginning of the stationary phase and after the depletion of nitrogen compounds.

All the analysis on cultures were done on triplicate.

2.3. Wastewater physical and chemical analyses

pH of the wastewater were measured on-site using a Hach Lange HQD 40d multi-parameter with interchangeable probes according to standards

methods (APHA, 1998). Turbidity (NTU) was measured using a portable HI83414 (HANNA Instruments) turbidimeter.

Chemicals concentrations of the wastewater were analyzed in a laboratory immediately after the sample collection. Chemical Oxygen Demand (COD), total nitrogen (TN), ammonia nitrogen (NH_4^+-N) and nitric nitrogen (NO_3-N) were determined using Mackerey-Nagel NANOCOLOR® kit for COD (COD 1500), Total Nitrogen (TN_b 220), Ammonia (Ammonium 10, Ammonium 50) and Nitrate (Nitrate 50, Nitrate 250).

Total phosphorus (TP), Mg, K, Mn, Ca, Cl, Zn, Fe, Na, Cu contents were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian. Fort Collins, USA) according to 3051A and 6020A EPA methods (EPA, 2007): briefly a representative sample of 3 mL was digested in 10 ml of concentrated nitric acid using microwave heating with a suitable laboratory microwave unit.

For the algae growth tests, the sample and the acid were placed in a fluorocarbon (PFA or TFW) microwave vessel which is capped and heated in the microwave unit. After cooling, the vessel content was diluted to 50 mL with deionized water, filtered with 0.45 μm cellulose acetate filters and then analyzed. A certified standard reference material (2782 Industrial Sludge) from the National Institute of Standards and Technology

(Gaithersburg, US) was used in the digestion and analysis. Average recovery was 92 ± 4% for all the determinations. To ensure the accuracy and precision in the analyses, reagent blanks were run with samples.

All analyses were performed in triplicate.

2.4. Calculations

Nutrients concentration reduction in both filtering system and *H. pluvialis* cultivation assays were calculated as percentage reduction (*R*) and daily removal rate (D_r) by using equation 1 and 2:

$$R(\%) = \frac{C_{in} - C_{out}}{C_{in}} \times 100 \quad (1)$$

$$D_r = \frac{C_{in} - C_{out}}{t} \quad (2)$$

where C_{in} is inlet concentration, C_{out} is outlet concentration and t is the duration of the run;

Specific growth rate (μ) and daily volumetric productivity of *H. pluvialis* (P_b) were calculated by the equations 3 and 4 respectively:

$$\mu = \frac{1}{t} \times \ln\left(\frac{Cb}{C0}\right) \quad (3)$$

$$Pb = \mu \times Cb \quad (4)$$

Where C_b and C_0 are the concentrations of biomass at the end and at the beginning of a growth phase, and t is its duration.

2.5. Data analysis

Data were processed by one-way ANOVA using the Tukey test to compare means. Statistical analyses were performed by using SPSS software (SPSS v19.0, IBM). The level of significant difference was set at $P<0.05$.

3. Results and discussion

3.1. Pretreatment performance in treating swine wastewater

The physic-chemical composition of the swine wastewater at inflow (RW) changed over the experimental time (Figure 1).

The system showed a not constant behavior for the first 10 days of the experiment, for every parameter studied: after that the filtering performance was stable until the end of the assay. Probably this was due to two main factors: changing in wastewaster composition during the first days of the experiment and sampling differences caused by a different stratification between the solid and liquid phase inside the swine wastewater storage tank.

The results of the filtering system, during the constant phase, are shown in Table 2. The pH of the wastewater ranged between 7.66 and 9.46 (mean 8.56 ± 1.27), with higher values in the first two weeks (August 28th - September 12th). After the filtration the pH of the wastewater was higher, in the range of 7.88-9.71 (mean 8.69 ± 0.91). The turbidity of the RW oscillated between 3718 NTU and 1145 NTU, higher in the first week and decreasing over time, until a quite constant value of 1145 NTU. After the filtering treatment it decreased by 29% on average, achieving a mean value at outlet of 808 NTU.

The RW average total nitrogen (TN) concentration was 543 ± 32 mg L^{-1}, while the TN concentrantion in FW was lower throughout the period. At the end of the process TN concentration was reduced by 26%, till 403 ± 41 mg L^{-1}.

As expected, after the filtration the NO_3-N concentration increased, from 51.9 ± 16.3 mg L^{-1} in RW to 250 ± 30 mg L^{-1} in FW with an average increase of 381%.

On the other hand ammonia nitrogen (NH_4-N) concentration was strongly reduced during the filtering varying from 226 ± 16 mg L^{-1} in RW to 76 ± 9.9 mg L^{-1} in FW, with a reduction of 66%.

Also the TP concentration declined after filtration, even though with a lower reduction percentage, 7%: as several authors reported (e.g. Billore *et al*., 1999), there are different processes by which phosphates compounds may be removed from wastewater: adsorption, ionic exchange, and absorption. Some authors (Mann and Bavor, 1993) reported that substrate is the main sink for phosphates in the long term. The chemical oxygen demand (COD) of the wastewater at inlet was particularly high in the first experimental week afterwards it remained stable to about 2160 ± 61 mg L^{-1}. The COD concentration was reduced by 19% after filtration, with an average outlet concentration (FW) of 1745 ± 185 mg L^{-1}.

These results proved that the filtering system has been able to reduce all chemical compounds present in the swine wastewater, except for the nitrogen nitric form. The NO_3-N increment after filtration derived from the oxidation of the ammonia-nitrogen and the organic nitrogen. The effect of

the oxygen due to recirculation resulted also in the abatement of the COD by oxidizing the organic content of the raw wastewater. Besides other phenomena like media adsorption, ionic exchange, biological activity and environmental factors played an important role on chemical removals of the filters.

The filtration of FW before *H.pluvialis* cultivation resulted in a decrease in turbidity which was lowered from 808 NTU to 732 NTU and a higher decrease in COD concentration which was reduced by 0.45 µm filtration from 1745 to 812 mg L^{-1}. Whereas the turbidity remained constant, COD decrease could represent an advantage for microalga growth: in this case lowering carbon content may avoid inhibition and/or competition with bacteria for the nutrients.

3.2. H. pluvialis growth and nutrients uptake

H. pluvialis showed a positive growth on all the trials (Fig. 2a) in terms of dry weight increase throughout the experiment with 25% FW and 12.5% FW showing a faster growth reaching stationary phase in 13 days with an average dry weight concentration of 0.97 and 0.81 g L^{-1} for 25% FW and 12.5% FW respectively. On the other hand, 50% FW reached the stationary

phase after 24 days, after a lag phase of at least three days after inoculum, with a final dry weight concentration of 1.31 g L^{-1}.

The maximum specific growth rate (μ_{max}) was registered during 12.5% FW assay (0.37 day $^{-1}$) significantly higher than that obtained in 25% FW trial (0.33 day $^{-1}$) (Figure 2b). On the other hand 50% FW achieved the lowest maximum specific growth rate of 0.26 day $^{-1}$ (Figure 2b).

Although 25% FW and 12.5% FW trials showed a faster response in terms of cultures growth, regarding biomass productivity all the trials showed a similar average daily volumetric productivity,

with the 25% FW trial that was capable of producing up to 63 mg L^{-1} of dry biomass per day of cultivation significantly different from 50% FW and 12.5% FW assays which showed a lower productivity, 45 and 49 mg $L^{-1}d^{-1}$ of dry biomass respectively

Looking at maximum biomass productivity (Figure 2c) all the trials achieved almost the same value, 110 mg $L^{-1}d^{-1}$ for 50% FW and 25% FW and 100 mg $L^{-1}d^{-1}$ for 12.5% FW with no significant differences between the assays..

The differences in these results are likely to be correlated with the dilution of FW. In fact, although macro and micronutrients concentrations (Table 3) were not found inhibiting or limiting, if compared with a synthetic medium,

a strong difference between the trials was represented by light incidence in the culture. In fact raw FW, probably, did not support *H.pluvialis* growth due to its high turbidity (Table 3); consequently, diluting 2, 4 and 8 fold the FW could have overcome this issue, improving light availability in the culture. The result of 50% FW trial supports this hypothesis, showing a much slower growth than that obtained in the other trials although the initial nitrogen and phosphorus concentration would have otherwise improved *H. pluvialis* performance in 50% FW (Figure 2a).

Beside, these results are also linked with nutrients uptake, above all nitrogen, in the trials; nitrogen compounds were almost completely removed from the cultures by cell growth with high reduction percentages at the stationary phase (Table 4). In particular 25% FW and 12.5% FW assays did result in the highest nitrate reduction, up to about 99% of removal at the end of the trials with no significant difference with 50% FW trial.

Similarly all the cultures were capable of uptaking all the ammonia nitrogen which was totally removed from the medium (Table 4).

Nitrogen removal in the cultures is strongly related to cultures biomass productivity (Figure 2): 50% FW initial biomass productivity is much related to ammonium uptake even though there is not a direct proportionality between them implying, probably, a certain degree of

ammonia stripping from the culture. In this case the culture showed a more affinity for the ammonia form even if light availability at the beginning of the cultivation was probably a limiting factor for cells growth.

After ammonia depletion and change in cells metabolism, nitric nitrogen uptake started to increase reaching the highest value when microalga showed the highest biomass productivity at day 13 (Figure 2, 50% FW) after seven days of constant nitrogen uptake.

On the other hand when light was not limiting, as in the case of 25% FW and 12.5% FW, both nitrogen form were utilized, with no preference, from the beginning of the cultivation, with a parallel strong increase in biomass productivity, indicating the absence of a limiting environment for cells growth (Figure 2, 25% FW; Figure 2, 12.5% FW). In this cases, according to FW characterization, nitrogen removal was found faster than 50% FW.

Although the N:P ratio in the cultures varied between 12 (25% FW) and 13 (50% FW and 12.5% FW) phosphorus was almost completely removed from the culture mediums (Table 4). In this case 50% FW showed a slightly higher efficiency (98.08%) than 25% FW (97.74%) and 12.5% FW (96.95%) even though these data did not shown any significant differences. These results are similar to those obtained by Yin-Hu *et al*. (2013) that reported high N and P efficiency removal by *H. pluvialis* in a domestic

secondary effluent with a slightly higher N:P ratio of 15. In that case both nitrogen (93.8%) and phosphorus (97.8%) were efficiently removed when the stationary phase was reached, but initial NO_3-N plus NH_4-N concentration was much lower (5.3 mg L^{-1}) than that in the present study (Table 3).

Moreover, the same authors reported a daily dry weight productivity that was from 1.8 to 2.4 fold lower than the productivities obtained in this study.

Cell growth, in all cases, was sustained until the nitrogen concentration was depleted. Probably, as already reported by Kang *et al*. (2006), working with *H. pluvialis* grown in primary-treated piggery wastewater, phosphate concentration in the culture decreased rapidly in the first days of cultivation, so that growth was possible due to phosphate accumulation in polyphosphate granules which have been commonly observed in microalgal cells (Sawayama *et al*., 1992). Unfortunately no analyses about phosphorus trend during cultivation were performed in order to support this hypothesis.

According to this, 50% FW culture had the higher dry biomass concentration at the stationary phase than the others assays because of the higher availability of nitrate (Figure 2 and Table 3). On the contrary in 25% FW and 12.5% FW assays, *H. pluvialis* achieved the maximum growth rate,

but sustainable growth was rapidly limited by the early exhaustion of nitrogen compounds.

Regarding carbon degradation in the cultures, results showed that in all cases no inhibition of the growth was observed. Travieso *et al.* (2006) concluded that a COD concentration in the range of 250-800 mg L^{-1} did not affect the growth of *Chlorella vulgaris*, a much more resistant and fast-growing microalga, in piggery waste. In this study, 50% FW was characterized by 410 mg L^{-1} of COD and did not suffer any inhibition phenomena. In all trials, at the stationary phase, up to 26.4% of COD was removed (50% FW) (Table 4).

Looking at micronutrients removal (Figure 3) all the assays behaved similarly with high reductions rate, in particular in the case of Mg, K, Ca, Fe, Na (> 90% of reduction).

A lower removal percentage was obtained in the case of Mn, Zn and Cu.

These results showed that *H. pluvialis* grown in a primary-treated swine manure could enhance its remediation with high removal percentages in all cases, except for the COD. In the latter case, the actual Italian legislation consider a COD limit of 160 mg L^{-1} for discharging in surface waters (Dlgs 152/2006); 25% FW and 12.5% FW assays did results in a final COD

concentration below this limit so that exhausted culture medium can effectively and sustainably be discharged in surface water bodies.

3.3. Astaxanthin accumulation

As already reported in material and methods section, astaxanthin accumulation did take place in the same experimental conditions of the growth trials.

This was intended to evaluate the possibility to use a system that could reduce the costs of *H. pluvialis* cultivation and astaxanthin production, above all considering the final users of this hypothesized system, i.e. small farmers.

The encystment of the cells was spontaneously induced by nitrogen depletion at the stationary phase and was followed by the accumulation of astaxanthin during induction period without any reformulation of the culture medium. Microscopic observations confirmed that green vegetative cells were transformed into red cyst cells during this incubation. Between the trials, 50% FW showed the highest percentage of the carotenoid in the biomass (1.27 ± 0.02 %), slightly higher than that observed in 12.5% FW (1.17 ± 0.02 %) and 25% FW (0.92 ± 0.03 %). The obtained values are much lower than those reported by Kang *et al*. (2006), where cultures

accumulated up to 5.9% of astaxanthin in the cells, although experimental conditions during induction period were substantially different, i.e. the use of high irradiance and CO_2-mixed gas.

In this work high concentrations of astaxanthin in *H.pluvialis* biomass were obtained without any reformulation of the culture medium, a constant low light irradiance and with no use of CO_2 gas for induction of the pigment accumulation, thus improving economic viability of the process.

3.4. Low-cost filtering coupled to H. pluvialis continuous culture

Coupling preatreament with low cost filter system and *H. pluvialis* growth would allow to strongly reduce organic and mineral nutrients contained in the swine wastewater.

In this case, a main result of the process was a net increase in nitric nitrogen concentration, which has not been seen as negative, on the contrary it represented a positivity for the depuration by plants and algae, which require macronutrients like nitrate for their growth, avoiding ammonia nitrogen toxicity at high concentrations.

Concerning ammonia a strong reduction was observed during pretreatment (66% of reduction) due to nitrification processes and absorption of the filters. The FW finally contained low levels of ammonia which were

assimilated by *Haematococcus pluvial*is and/or loss in the air due to alkaline pH reached during the growth and continuous aeration of the culture.

The treatment with *Haematococcus* pluvialis resulted in a more efficient P reduction, causing almost the complete depletion of the element in the cultures. On the other hand the pretreatment resulted in a low abatement (7% of reduction); in this case probably the filter system was not capable of a high adsorption rate of P.

Beside this, COD content revealed a similar behavior to that of P. High reductions was achieved by microalga trials (up to 26%) but a lower reduction percentage was observed throughout the filtering assay (19%).

According to the results of the batch cultures it was possibile to theorize a continuous culture of *H. pluvialis* using FW as a growth medium coupling it with the filtration system for nitrogen reduction in swine slurry (Figure 4).

Looking at specific growth rate trend throughout the trials (Figure 1b), the optimal dilution rate (D_{opt}) was determined: in continuous cultures, at steady state, it equals to the specific growth rate (Yuan-Ku *et al*., 2013) in respondance to the maximum biomass productivity (Figure 1b, 1c). In this case the obtained D_{opt} was 0.25, 0.21 and 0.24 d^{-1} for 50% FW, 25% FW and 12.5% FW respectively.

From maximum biomass productivity (Pb) using equation 4 (see Materials and methods section), it was calculated the optimal working biomass concentration (Cb_{opt}) which was 0.45, 0.51 and 0.41 g L^{-1} for 50% FW, 25% FW and 12.5% FW respectively.

According to the daily load in the filtering system (30 L d^{-1}) and the dilution of FW, three photobioreactors with a working volume of 227 L (50% FW), 363 L (25% FW) and 645 L (12.5% FW) were hypothesized to treat all the FW coming from the filtration system.

A main result of theorized continuous system is that in 50% FW reactor it would not be possible to achieve a good reduction of nitrate, working with the optimal dilution rate; in fact the outlet medium would contain still 80.2 mg L^{-1} of nitric nitrogen, meaning the impossibility to discharge the effluent.

In this case lowering dilution rate to 0.1 d^{-1} would allow to achieve higher reduction (down to 16 mg L^{-1}, below the discharge limit) but it would imply also a reduction of around 50% of the maximum biomass productivity (down to 60 mg $L^{-1}d^{-1}$).

This system is capable of reducing up to 44%, 99% and 34% of TN, NH_4-N and NO_3-N respectively, producing around 25 g biomass d^{-1}.

The other two systems would behave differently; infact both reactors (25% FW and 12.5% FW) would allow to reduce nitric nitrogen down below the discharging limit (18.7 mg L^{-1} and 13.2 mg L^{-1} for 25% FW and 12.5% FW respectively) at the optimal dilution rate.

This last point being fundamental, as the maximal biomass productivity would be maintained, allowing the production of 40 g and 65 g of dry biomass per day of cultivation.

Regarding nitrogen consumption 25% FW reactor would be the most performant with 74%, 98%, and 70% of TN, NH_4-N and NO_3-N reduction respectively.

4. Conclusions

Wastewater treatment and astaxanthin production were performed by a primary treatment filtering system and culture and subsequent carotogenesys induction of *H. pluvialis* on swine wastewater. High content of nitric-nitrogen in the wastewater coming from the filtering system seem to have been successful in alga growth. Inorganic wastes in the wastewaters, above all nitrogen and phosphorus, were removed successfully by *Haematococcus* cultivation, after which green vegetative cells were

transformed by photoautotrophic induction to red aplanospores with high astaxanthin content.

The proposed method has potential as biological wastewater treatment process because of the combination of inorganic waste removal without any additives and the simultaneous

production of high-value added astaxanthin. From the viewpoint of astaxanthin production only, the culture of *H. pluvialis* on primary-treated wastewater could also make algal culture processes more economical by eliminating the need to supply nutrients. Therefore, the proposed system is expected to be an alternative treatment technology in biological wastewater treatment with the additional benefit of a highly valuable compound production.

Table 1 – Filtering system composition and characteristics

Filter number	Filling materials	Composition	Grain size/lenght/diameter range (mm)	Average filter porosity (%)	Total materials weight (kg)
1	GRAVEL AND PUMICE	pumice 64% gravel 36%	4/8- fine gravel*;12/20- medium-coarse gravel*;15/20- pumice	39	490
2	SAND AND GRAVEL	sand 44% gravel 56%	0.2/4-sand; 12/20- medium-coarse gravel*	33	688
3	GIANT REED	-	90-stems lenght; 20-stems diameter	62	97
4	TOPS	-	30/60	68	75
5	GRAVEL	-	4/8	37	677
6	BAMBOO	-	50-stems lenght; 25-stems diameter	51	157

* Grain size classification according to the *Udden-Wentworth scale* (1922)

Table 2 – Inlet and outlet main compounds concentration and total reduction (R) during pretreatment of RW with filtering system. Negative sign indicates an increase of component concentration

	RW	FW	
	IN	**OUT**	**R (%)**
pH	8.56 ± 1.27	8.69 ± 0.91	
Turbidity (NTU)	1145	808	29
TN (mg L^{-1})	543 ± 32	403 ± 41	26
NO_3-N (mg L^{-1})	51.9 ± 16.3	250 ± 30	-381
NH_4-N(mg L^{-1})	226 ± 16	76.0 ± 9.9	66
TP (mg L^{-1})	25.8 ± 3.6	24.0 ± 2.6	7
COD (mg L^{-1})	2160 ± 71	1745 ± 185	19

Table 3 – Chemical characterization of filtered wastewater (FW) used as growth medium for *Haematococcus pluvialis* growth

	100% FW	**50% FW**	**25% FW**	**12.5% FW**
pH	7.98 ± 0.02	8.25 ± 0.02	8.61 ± 0.03	8.87 ± 0.02
Turbidity (NTU)	732	361	175	91
COD (mg L^{-1})	812 ± 14	410 ± 2	200 ± 4	102 ± 3
TN (mg L^{-1})	272 ± 7	139 ± 8	70 ± 6	35 ± 7
NH_4-N (mg L^{-1})	40 ± 3	21.4 ± 1.4	11.1 ± 2	6.1 ± 0.2
NO_3-N (mg L^{-1})	235 ± 6	122 ± 2	63 ± 2	34 ± 1
TP (mg L^{-1})	22.5 ± 2.1	11 ± 0.1	6.2 ± 0.7	3.1 ± 0.1
K (mg L^{-1})	454 ± 5	222 ± 17	125 ± 5	63 ± 4
Mg (mg L^{-1})	28.4 ± 2.2	13.9 ± 0.1	7.8 ± 0.8	3.9 ± 0
Na (mg L^{-1})	131 ± 3	64 ± 4	36 ± 2	18 ± 1
Ca (mg L^{-1})	95 ± 17	46.1 ± 4.2	26.1 ± 5.3	13.1 ± 1.4
Fe (mg L^{-1})	4.4 ± 1.4	2.1 ± 0.5	1.2 ± 0.4	0.6 ± 0.2
Zn (mg L^{-1})	1.4 ± 0.6	0.7 ± 0.2	0.4 ± 0.2	0.2 ± 0.1
Cu (mg L^{-1})	0.2	0.1 ± 0	0.041	0.021
Mn (mg L^{-1})	0.5 ± 0.1	0.26 ± 0.03	0.15 ± 0.03	0.07 ± 0.02

Table 4–Daily removal rate and percentage reduction of nitrogen compounds (NO_3-N, NH_4-N), total phosphorus (TP) and carbon (COD) during *Haematococcus pluvialis* growth

	Nitrogen				**Phosphorus**		**Carbon**	
	NO_3-N		**NH_4-N**		**TP**		**COD**	
	D_r (mg L^{-1} d^{-1})	**R (%)**	**D_r (mg L^{-1} d^{-1})**	**R (%)**	**D_r (mg L^{-1} d^{-1})**	**R (%)**	**D_r (mg L^{-1} d^{-1})**	**R (%)**
50% FW	5.98 ± 0.10	97.75 ± 0.32	1.06 ± 0.07	99.31 ± 0.28	0.54 ± 0.07	98.08 ± 0.03	5.41 ± 0.07	26.40 ± 0.28
25% FW	3.10 ± 0.09	99.12 ± 0.14	0.55	98.66 ± 0.62	0.30 ± 0.02	97.74 ± 0.06	2.47	24.70 ± 0.62
12.5% FW	1.71 ± 0.07	99.06 ± 0.06	0.29 ± 0.02	97.52 ± 1.01	0.15 ± 0.03	96.95 ± 0.01	1.19 ± 0.02	23.40 ± 1.01

Figure caption

Figure 1 - Trends of chemical elements concentration (mg L^{-1}) measured at inlet (RW) and outlet (FW) of the filtering system during the filtering assay (35 days): turbidity (NTU), total nitrogen (TN), nitric nitrogen (NO_3-N), ammonia nitrogen (NH_4-N), total phosphorous (TP) and chemical oxygen demand (COD)

Figure 2 - Growth of *H. pluvialis* in 50% FW, 25% FW and 12.5% FW: dry weight (a), specific growth rate (b) and biomass productivity (c) trend during the experiment; NO_3-N, NH_4-N and TN uptake trend against biomass productivity in 50% FW, 25% FW, 12.5% FW trials

Figure 3 - Nutrients reductions (%) in 50% FW, 25% FW and 12.5% FW trials.

Figure 4 – Combined pretreatment-microalgae system: *Haematococcus pluvialis* continuous cultivation model and nutrients concentration throughout the process: DW (dry weight),

Figure 1

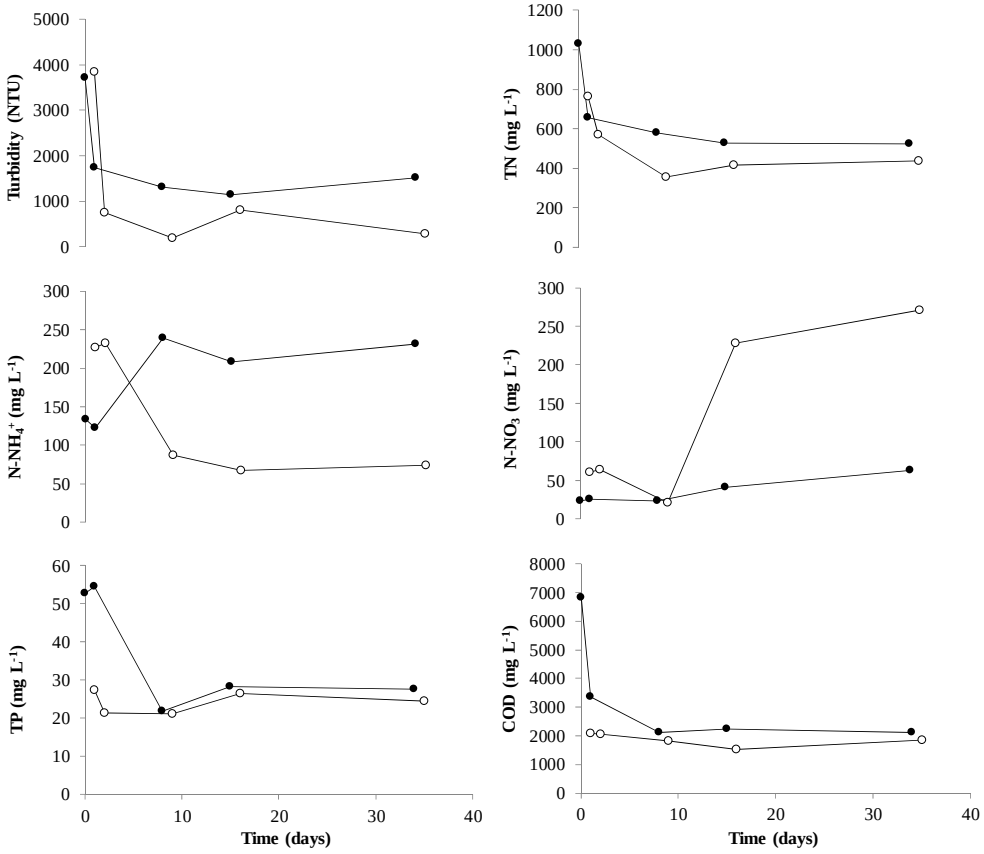

Turbidity (NTU)
TN (mg L-1)
N-NH4+ (mg L-1)
N-NO3 (mg L-1)
TP (mg L-1)
COD (mg L-1)
Time (days)
Time (days)

Figure 2

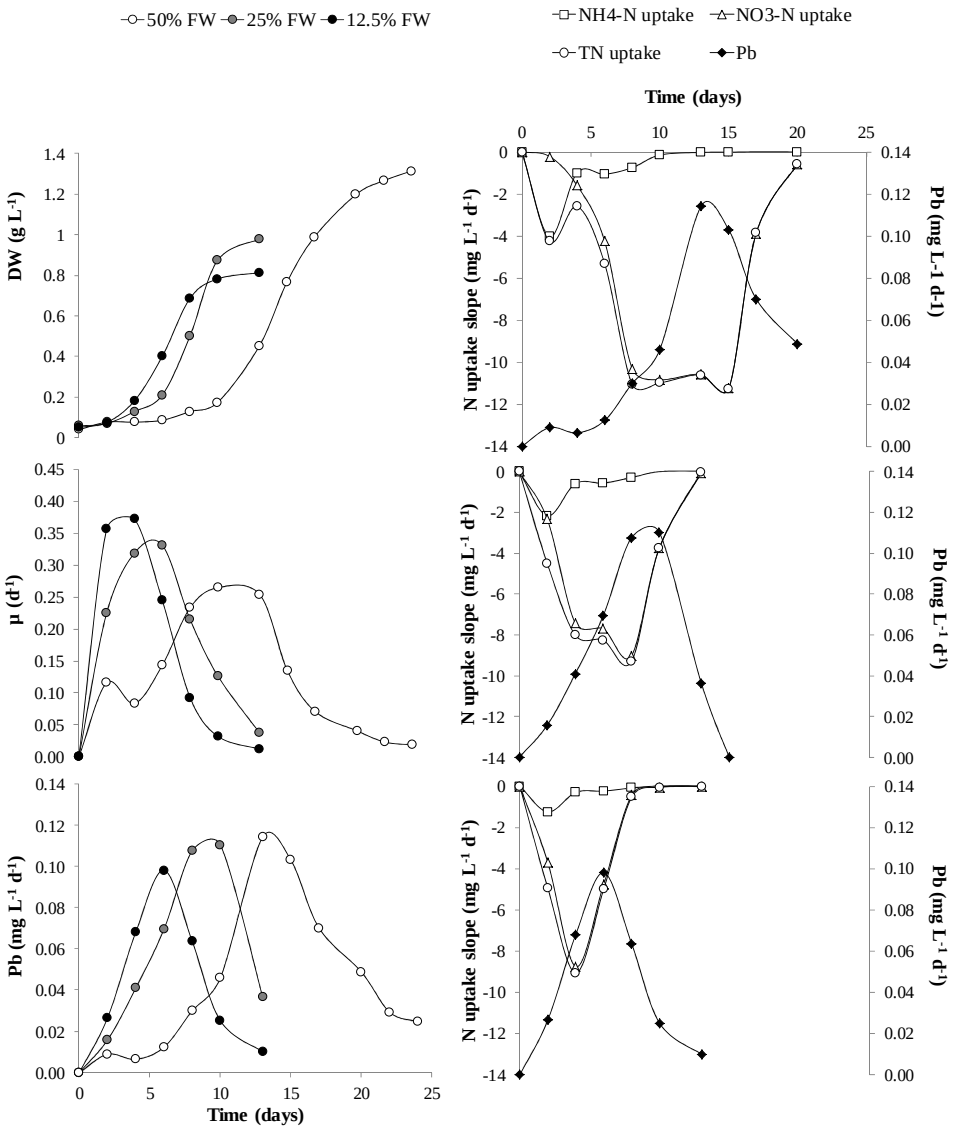

50% FW
25% FW
12.5% FW
NH4-N uptake
NO3-N uptake
TN uptake
Pb
Time (days)
DW (g L-1)
μ (d-1)
Pb (mg L-1 d-1)
N uptake slope (mg L-1 d-1)
Pb (mg L-1 d-1)

Figure 3

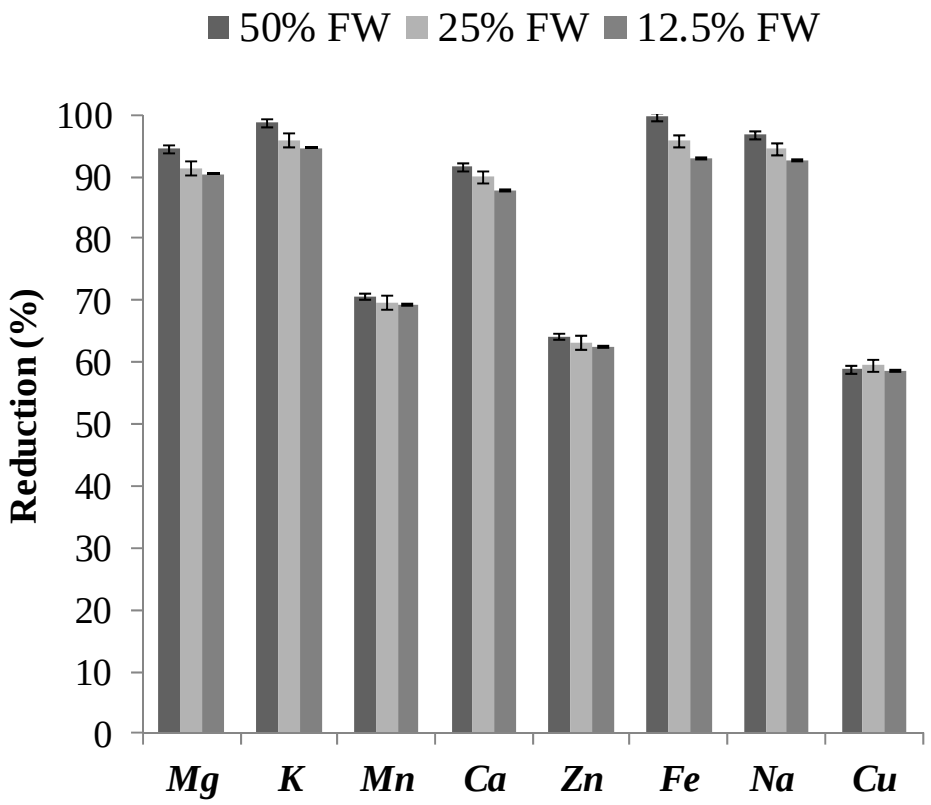
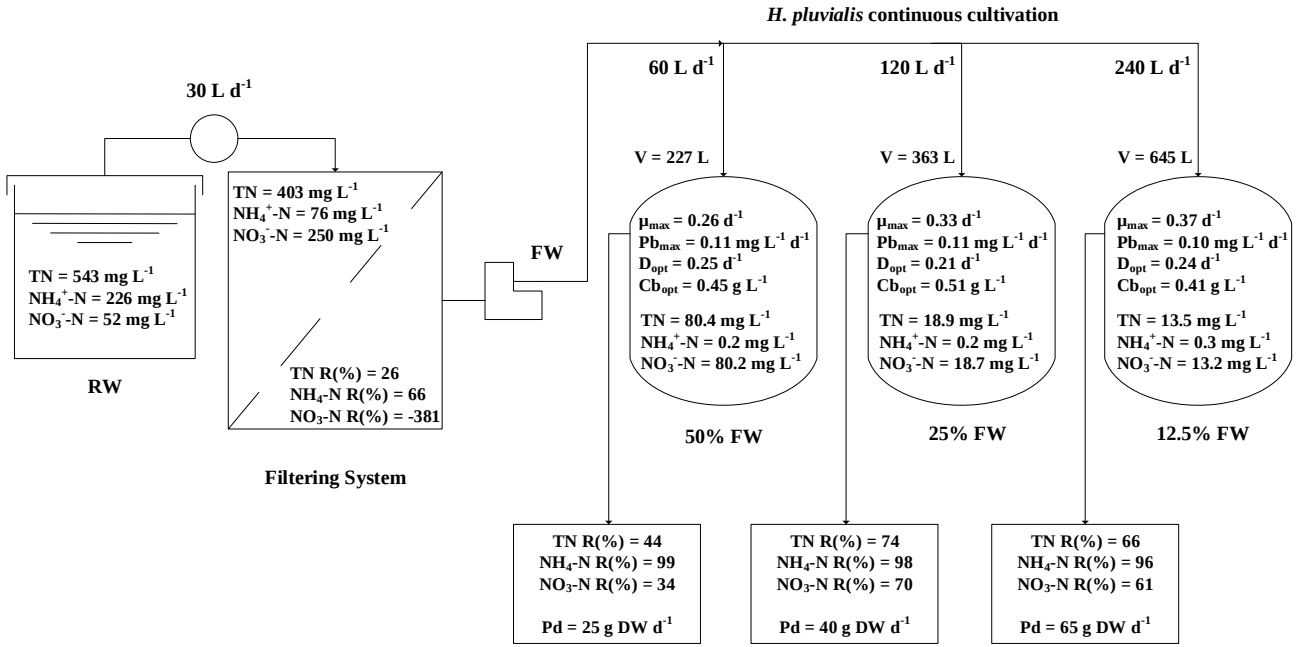

50% FW
25% FW
12.5% FW
Reduction (%)
100
90
80
70
60
50
40
30
20
10
0
Mg
K
Mn
Ca
Zn
Fe
Na
Cu

Figure 4


H. pluvialis continuous cultivation
30 L d-1
60 L d-1
120 L d-1
240 L d-1
V = 227 L
V = 363 L
V = 645 L
TN = 543 mg L-1
NH4+-N = 226 mg L-1
NO3--N = 52 mg L-1
RW
TN = 403 mg L-1
NH4+-N = 76 mg L-1
NO3--N = 250 mg L-1
TN R(%) = 26
NH4-N R(%) = 66
NO3-N R(%) = -381
Filtering System
FW
μmax = 0.26 d-1
Pbmax = 0.11 mg L-1 d-1
Dopt = 0.25 d-1
Cbopt = 0.45 g L-1
TN = 80.4 mg L-1
NH4+-N = 0.2 mg L-1
NO3--N = 80.2 mg L-1
50% FW
μmax = 0.33 d-1
Pbmax = 0.11 mg L-1 d-1
Dopt = 0.21 d-1
Cbopt = 0.51 g L-1
TN = 18.9 mg L-1
NH4+-N = 0.2 mg L-1
NO3--N = 18.7 mg L-1
25% FW
μmax = 0.37 d-1
Pbmax = 0.10 mg L-1 d-1
Dopt = 0.24 d-1
Cbopt = 0.41 g L-1
TN = 13.5 mg L-1
NH4+-N = 0.3 mg L-1
NO3--N = 13.2 mg L-1
12.5% FW
TN R(%) = 44
NH4-N R(%) = 99
NO3-N R(%) = 34
Pd = 25 g DW d-1
TN R(%) = 74
NH4-N R(%) = 98
NO3-N R(%) = 70
Pd = 40 g DW d-1
TN R(%) = 66
NH4-N R(%) = 96
NO3-N R(%) = 61
Pd = 65 g DW d-1

References

- Billore, S.K., Singh, N., Sharma, J.K., Nelson, R.M., 1999. Horizontal subsurface flow gravel bed constructed wetland with Phragmites Karka in central India. Water Science and Technology 40 (3), 163–171
- Borin, M., Politeo, M., De Stefani, G., 2013. Performance of a hybrid constructed wetland treating piggery wastewater. Ecol. Engin., 51, 229–236
- Bubrick, P., 1991. Production of astaxanthin from Haeamatococcus. Bioresour. Technol. 38: 237-239
- Buelna, G., Dubé, R., Turgeon, N., 2008. Pig manure treatment by organic bed biofiltration. *Desalination*, 231, 297-304.
- Clarisse, L., Clerbaux, C., Dentener, F., Hurtmans, D., Coheur, P.F., 2009. Global ammonia distribution derived from infrared satellite observations. Nat. Geosci. 2(7), 479–483.
- Del Río, E., Acién, F.G., García-Malea, M.C., Rivas, J., Molina-Grima, E., Guerrero, M.G., 2005. Efficient one-step production of astaxanthin by the microalga Haematococcus pluvialis in continuous culture. Biotechnol Bioeng 91(7): 808-815.

- E. Del Río, F. G. Acién, M. C. García-Malea, J. Rivas, E. Molina-Grima, and M. G. Guerrero. Efficiency assessment of the one-step production of astaxanthin by the microalga Haematococcus pluvialis. Biotechnology and Biongineering, 100(2):397–402, 2008.
- EPA, 2007. Method EPA 3051A. Microwave assisted acid digestion of sediments, sludges, soils and oils.
- EPA, 2007. method EPA 6020A. Elements determination by inductively coupled plasma-mass spectrometry (ICP-MS)
- Fábregas, J., Dominguez, A., Regueiro, M., Maseda, A., Otero. A., 2000. Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis* Applied Microbiology and Biotechnology, 53(5):530–535.
- Franchino, M., Comino, E., Bona, F., Riggio, V.A., 2013. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. Chemosphere 92, 738–744.
- Gong, X., Chen, F., 1997. Optimization of culture medium for growth of *Haematococcus pluvialis.* Journal of Applied Phycology, 9(5):437–444.
- Hata, N., Ogbonna, J.C., Hasegawa, Y., Taroda, H., Tanaka., H., 2001. Production of astaxanthin by Haematococcus pluvialis in a sequential

heterotrophic-photoautotrophic culture. Journal of Applied Phycology 13: 395–402.

- Ji, M., Abou-Shanab, R., Hwang, J., Timmes, T., Kim, H., Oh, Y., Jeon, B., 2013. Removal of nitrogen and phosphorus from piggery wastewater effluent using the green microalga *Scenedesmus obliquus*. J. Environ. Eng. 139 (9), 1198–1205.
- Kadam, A.M., Nemade, P.D., Shankar, H.S., 2009. Treatment of municipal wastewater using laterite-based constructed soil filter. Ecological Engineering 35: 1051-1061.
- Kang, C.D., An, J.Y., Park, T.H., Sim, S.J., 2006. Astaxanthin biosynthesis from simultaneous N and P uptake by the green alga *Haematococcus pluvialis* in primary-treated wastewater. Biochemical Engineering Journal, 31 (3): 234–238
- Kebede-Westhead, E., Pizarro, C., Mulbry, W.W., 2006. Treatment of swine manure effluent using freshwater algae: Production, nutrient recovery, and elemental composition of algal biomass at four effluent loading rates. Journal of Applied Phycology 18, 41–46
- Ledda, C., Schievano, A., Salati, S., Adani, F., 2013. Nitrogen and water recovery from animal slurries by a new integrated ultrafiltration,

reverse osmosis and cold stripping process: A case study. Water Res. 47 (16), 6157-6166.

- Liu, Q.W., Mancl, K., Tuovinen, O.H., 2003. Biomass accumulation and carbon utilization in layered sand filter biofilm systems receiving milk fat and detergent mixtures. Bioresource Technology 89 (3), 275–279.
- Loupasaki, E., Diamadopoulos, E., 2013. Attached growth system for wastewater treatment in small and rural communities: a rewiew. Journal of Chemical Technology and Biotechnology 88 (2): 190-204.
- Mann, R.A., Bavor, H.J., 1993. Phosphorous removal in constructed wetlands using gravel and industrial waste substance. Water Science and Technology 27, 107–113
- Moral, R., Perez-Murcia, M.D., Perez-Espinosa, A., Moreno-Caselles, J., Paredes, C., Rufete, B. 2008. Salinity, organic content, micronutrients and heavy metals in pig slurries from South-eastern Spain. Waste Management 28, 367–371
- Nakhla, G., Farooq, S., 2003. Simultaneous nitrification–denitrification in slow sand filters. Journal of Hazardous Materials 96:291–303.

- Nguyen, M.L., Tanner, C.C., 1998. Ammonium removal from wastewaters using natural New Zealand zeolites. New Zeal. J. Agr. Res., 41(3), 427–446.
- Nikolaeva, S., Sánchez, E., Borja, R., Travieso, L., Weiland, P., Milán, Z., 2002. Treatment of piggery waste by anaerobic fixed bed reactor and zeolite bed filter in a tropical climate: a pilot scale study. *Process Biochemistry*, 38, 405-409.
- Olaizola, M., 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25000-liter outdoor photobioreactors. J Appl Phycol 12:499–506.
- Olaizola, M., Huntley, M.E., 2003. Recent advances in commercial production of astaxanthin from microalgae. In: Fingerman M, Nagabhushanam R, editors. Biomaterials and bioprocessing. Enfield: Science Publishers. pp 143–164.
- Orasa, M., Torres, E., Fidalgo, P., Abalde, J., 2000. Production and analysis of secondary carotenoids in green algae. Journal of Applied Phycology. 12: 553-556.
- Oswald, W.J., 2003. “My sixty years in applied algology.” Journal of Applied Phycology, 15, 99-106.

- Richmond, A., Hu, Q., 2013. Handbook of Microalgal Culture: Applied Phycology and Biotechnology, 2nd Edition, Wiley-Blackwell, ISBN: 978-0-470-67389-8
- Roche, L., 1984. Amélioration et démonstration du procédé de compostage du lisier de porc. Environment Canada, 214.
- Roseth, R., 2000. Shell sand: a new filter medium for constructed wetlands and wastewater treatment. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 35 (8): 1335-1355.
- Saliling, W.J.B., Weaterman, P.W., Losordo, T.M., 2007. Wood chips and wheat straw as alternative biofilter media for denitrification reactors treating aquaculture and other wastewaters with high nitrate concentration. Aquacultural engineering (37): 222-233.
- Sawayama, S., Minowa, T., Dote, Y., Yokoyama, S., 1992. Growth of the hydrocarbon-rich microalga Botryococcus braunii in secondarily treated sewage. Appl. Microbiol. Biotechnol., 38 (1992), pp. 135–138
- Tao, W.D., Wang, J., 2009. Effects of vegetation, limestone and aeration on nitritation, anammox and denitrification in wetland treatment systems. Ecological Engineering 35 (5): 836–842.

- Wei, X.M., Lin, C., Duan, N., Peng, Y.X., Ye, Z.Y., 2010. Application of aerobic biological filter for treating swine farms wastewater. Procedia Environmental Sciences 2: 1569-1584.
- Wentworth, C.K., 1922. A scale of grade and class terms for clastic sediments. Jour. Geology, v. 30, no. 5, p. 377-392
- Yasuda, T., Kuroda, K., Fukumoto, Y., Hanajima, D., Suzuki, K, 2009. Evaluation of full scale biofilter with rockwool mixture treating ammonia gas from livestock manure composting. Bioresource Technology 100 (4): 1568-1572.
- Yin-Hu, W., Jia, Yang., Hong-Ying, Hu., Yin, Yu., 2013. Lipid-rich microalgal biomass production and nutrient removal by *Haematococcus pluvialis* in domestic secondary effluent. Ecological Engineering, 60:155-159.
- Yuan-Ku, L., Wei, C., Hui, S., Danxiang, H., Yantao, L., Howland, D.T.J., Jerilyn, A.T., Qiang, H., 2013. Basic culturing and analytical measurement techniques. In: Handbook of Microalgal Culture: Applied Phycology and Biotechnology, 2nd Edition, Wiley-Blackwell, ISBN: 978-0-470-67389-8
- Zheng, D., Deng, L.W., Fan, Z.H., Liu, G.J., Chen, C., Yang, H. Liu, Yi, 2012. Influence of sand layer depth on partial nitritation as

pretreatment of anaerobically digested swine wastewater prior to anammox. *Bioresourse Technology*, 104, 274-279.

Conclusions

Microalgae are considered a promising tool for the depuration of agro-zootechnical wastewaters, due to their ability of assimilating soluble organic and mineral nutrients and recycling them into a valuable biomass. In this work three microalgae strains, *Scenedesmus* sp., *Chlorella* sp., and *Haematococcus pluvialis* were tested for growing in digestate liquid clarified fractions and pretreated swine slurry showing to be capable of a fast grow with high biomass production, even though growth performance were highly dependent from light availability, i.e. dilution of the slurry.

The produced biomass was rich in proteins, lipids, carbohydrates and pigments such as astaxanthin, a strong antioxidant, underlying how these biological systems can provide bulding blocks for a biobased biorefinery improving the economical exploitation of livestock farming waste streams and the development of new production chains in the food and feed, nutraceutical and bioenergy sectors.

Besides this, the tested substrates were strongly depurated from pollutants such as ammonia, phosphorus, COD and micronutrients with high removal rate up to 100%, producing in almost all cases, a dischargeable effluent, allowing the farm to deal sustainably with its activity by-products.

Although the depuration performances were very good, a main issue was represented by ammonia stripping, caused by high pH and aeration of the cultures. This result poses technical and environmental problems to microalgae cultivation in wastewaters and should seriously be taken into account if scaling-up the process to a farm scale.