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# **Universidad de Valladolid**

Departamento de Ingeniería Química y Tecnología del Medio  
Ambiente

TESIS DOCTORAL

## **ANAEROBIC DIGESTION OF LIVESTOCK WASTES: VEGETABLE RESIDUES AS CO-SUBSTRATE AND DIGESTATE POST-TREATMENT**

Presentada por Beatriz Molinuevo Salces para  
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**Dirigida por:**  
María Cruz García González and Xiomar Gómez Barrios

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## TABLE OF CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

ABSTRACT

RESUMEN

---

1. GENERAL INTRODUCTION.....	5
1.1. WASTES OVERVIEW .....	7
1.2. ANAEROBIC DIGESTION OF LIVESTOCK WASTES AND VEGETABLE PROCESSING WASTES.....	11
1.3. POST-TREATMENT: NUTRIENTS DEPLETION .....	18
2. SCOPE OF THE THESIS.....	35
2.1. OBJECTIVES .....	37
2.2. THESIS OUTLINE.....	38
3. ANAEROBIC CO-DIGESTION OF LIVESTOCK WASTES WITH VEGETABLE PROCESSING WASTES: A STATISTICAL ANALYSIS .....	41
3.1. INTRODUCTION .....	45
3.2. METHODS .....	46
3.3. RESULTS AND DISCUSSION .....	51
3.4. CONCLUSIONS.....	60
4. ANAEROBIC CO-DIGESTION OF LIVESTOCK AND VEGETABLE PROCESSING WASTES: FIBRE DEGRADATION AND DIGESTATES STABILITY.....	65
4.1. INTRODUCTION .....	69
4.2. METHODS .....	70
4.3. RESULTS AND DISCUSSION .....	74

4.4. CONCLUSIONS .....	84
5. VEGETABLE PROCESSING WASTES ADDITION TO IMPROVE SWINE MANURE ANAEROBIC DIGESTION: EVALUATION IN TERMS OF METHANE YIELD AND SEM CHARACTERIZATION .....	89
5.1. INTRODUCTION .....	93
5.2. METHODS .....	94
5.3. RESULTS AND DISCUSSION .....	96
5.4. CONCLUSIONS .....	103
6. ANAMMOX FOR AMMONIA REMOVAL FROM PIG MANURE EFFLUENTS: EFFECT OF ORGANIC MATTER CONTENT ON PROCESS PERFORMANCE .....	107
6.1. INTRODUCTION .....	111
6.2. METHODS .....	112
6.3. RESULTS AND DISCUSSION .....	116
6.4. CONCLUSIONS .....	123
7. PERFORMANCE COMPARISON OF TWO PHOTOBIOREACTORS CONFIGURATIONS (OPEN AND CLOSED TO THE ATMOSPHERE) TREATING ANAEROBICALLY DEGRADED SWINE SLURRY .....	127
7.1. INTRODUCTION .....	131
7.2. METHODS .....	132
7.3. RESULTS AND DISCUSSION .....	135
7.4. CONCLUSIONS .....	145
8. GENERAL CONCLUSIONS .....	149
APPENDIXES	

## INDEX OF TABLES

**Table 1.1.** Concentrations of acetate and propionate estimated to cause 50% methanogenic activity inhibition.

**Table 2.1.** Organization of this PhD thesis.

**Table 3.1.** Composition of the substrates: Vegetable processing wastes (VPW), poultry litter (PL), swine manure (SM), and anaerobic sludge (AS).

**Table 3.2.** Codified and real values, VS removal and Y responses for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

**Table 3.3.** Regression results for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

**Table 3.4.** Liquid matrix analyses for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

**Table 4.1.** Chemical characterization of substrates and anaerobic sludge.

**Table 4.2.** Proportion of the different mixtures (C1-C9) used in batch digestion tests. % expressed in dry weight (DW).

**Table 4.3.** Biogas and methane yields, methane content, VS Removal,  $\text{N-NH}_4^+$  concentrations,  $\text{N-NH}_3$  concentrations and pH for C1 to C9. i and f stands for initial and final data, respectively.

**Table 4.4.** NDF, ADF, ADL, CE and HC measurements for initial and final stages of C3, C5 and C8.

**Table 5.1.** Composition of the primary substrates (SM, VPW), inoculum (AS) and R1 and R2 inlet concentration for the different HRT studied.

**Table 5.2.** OLR, time of operation, methane yield and methane content for R1 and R2 during the different HRT applied.

**Table 5.3.** pH, ammonium and ammonia for R1 and R2 during the different HRT applied.

**Table 6.1.** Chemical characteristics of the pig manure effluents.

**Table 6.2.** Participation of different processes for ammonium removal from effluent after UASB post-digestion.

**Table 6.3.** Participation of different processes for ammonium removal from effluent after partial oxidation.

**Table 7.1.** Biomass growth under both photobioreactor types.

**Table 7.2.** Organic matter concentration and removal efficiencies through the different loads obtained under both photobioreactor types. Standard deviation of the replicate analyses stands in brackets.

**Table 7.3.** Ammonium concentration of the influent, total removal efficiencies, ammonium removed by stripping and by nitrification through the different loads obtained under both photobioreactor types.

**Table 7.4.** Soluble P concentration and removal efficiencies through the different loads obtained under both photobioreactor types.

**Table 7.5.** Biomass uptake in terms of C, N and P through the different loads obtained under both photobioreactor types.

## INDEX OF FIGURES

**Figure 1.1.** Pig and laying hen distribution in Spain and Castilla y León.

**Figure 1.2.** Green pea, carrot, leek and maize production in Castilla y León.

**Figure 1.3.** Anaerobic degradation of complex organic matter.

**Figure 3.1.** Accumulated methane production for: A) SM-VPW, B) PL-VPW.

**Figure 3.2.** VFA concentrations during the time for: A) SM-VPW, B) PL-VPW.

**Figure 3.3.** Response surfaces plots for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

**Figure 4.1.** Results from co-digestion of batch tests: TVFA ( $\text{mg COD} \cdot \text{L}^{-1}$ ) for SM-VPW system (A) and PL-VPW system (B). Accumulated methane production for SM-VPW system (C) and PL-VPW system (D).

**Figure 4.2.** SEM images for fresh (F) and digested (D) samples from batch C3 (SM-VPW), C5 (VPW) and C8 (PL-VPW). All the pictures were taken with a magnification of 45x.

**Figure 4.3.** TG and DTG profiles of VPW, SM and PL co-digestion systems.

**Figure 4.4.** (a) DSC profile mass signals (b)  $\text{H}_2\text{O}$ , (c)  $\text{CO}_2$  and (d)  $\text{NO}_2$  from digested PL sample.

**Figure 4.5.** TG, DTG and DSC profiles from (a,b) lignin and (c,d) humic acid sodium salt standards.

**Figure 5.1.** Performance of CSTRs R1 (SM) and R2 (SM and VPW).  $\text{CH}_4$  production (ml).

**Figure 5.2.** Performance of CSTRs R1 (SM) and R2 (SM and VPW). TVFA (mg/L).

**Figure 5.3.** SEM images for initial anaerobic sludge (AS) and final sludges for R1 and R2. The left column presents a magnification of 300x and the right one presents a magnification of 10000x. White arrows indicate bacterial morphotypes as cocci, rods or long-rod shaped bacteria.

**Figure 5.4.** Detailed SEM images of anaerobic sludge (mag. x 20000). Bacillus morphotype (A,B,C) and long-rod shaped microorganisms (D,E,F).

**Figure 6.1.**  $\text{NH}_4^+$ -N and  $\text{NO}_2$ -N removal during gradual implementation of the effluent after UASB post-digestion.

**Figure 6.2.**  $\text{NH}_4^+$ -N and  $\text{NO}_2$ -N removal during gradual implementation of the effluent after partial oxidation.

**Figure 6.3.** Effect of organic matter concentration on ammonium removal. Error bars represent standard deviation from a triplicate experiment. Dotted lines show COD threshold inhibitory levels for each effluent.

**Figure 7.1.** Dissolved oxygen measured in situ in (A) the open type photobioreactor and (B) the closed type photobioreactor.

**ABSTRACT.** The main reason why this study was carried out was to give support to livestock sector with regard to wastes treatment. Among the diverse treatment systems, anaerobic digestion was chosen as a proper biological treatment since besides organic material reduction it provides methane, which can be transformed into renewable energy. Vegetable waste addition as co-substrate in the anaerobic digestion of two livestock wastes (swine manure (SM) and poultry litter (PL)) was analysed. Those processes were studied terms of organic matter reduction and methane production following a central composite design and response surface methodology. It was concluded that in the case of SM co-digestion the vegetable addition resulted in an increase in organic matter reduction while in the case of PL co-digestion substrate concentration determined organic matter reduction registering ammonia-mediated inhibitions with volatile solids concentration above 80 g VS L<sup>-1</sup>. Organic matter degradation and more specifically lignocellulosic complex degradation during anaerobic digestion were investigated using SEM techniques and thermal analyses. Complete depletion and 50% reduction were obtained in the case of hemicelluloses and cellulose, respectively, while lignin was not degraded under anaerobic conditions. In the case of SM co-digestion, semi-continuous conditions were also investigated demonstrating the positive effect of vegetable addition on methane production. Changes in microorganisms population were studied with scanning electronic microscopy (SEM) finding that initial long rod-shaped bacteria changed to cocci and bacilli morphotypes. On the other hand, two different systems for treating nutrients in the anaerobically degraded SM were studied. First, Anammox process was examined treating two different wastes, namely anaerobically degraded SM and partially oxidised anaerobically degraded SM, obtaining ammonium removal rates around 95%. It was found that organic matter concentration determines Anammox process efficiency. The other studied process was a microalgae-bacteria consortium system. Nutrients removal and nutrients biomass uptake as well as two different photobioreactor configurations were investigated. Ammonium, phosphorus and organic matter were removed up to 100, 80 and 60%, respectively Nitrogen, Phosphorus and carbon content accounted for 10, 2 and 48% of the dried biomass, respectively. Biofilm reactor was more effective in terms of biomass harvesting since 96% of the total biomass produced was retained.



**RESUMEN.** Este estudio se llevo a cabo con el objetivo de dar soporte al sector ganadero en cuanto al tratamiento de los residuos. Entre los tratamientos posibles se eligió la digestión anaerobia ya que además de reducir el material orgánico produce metano, pudiendo revalorizarlo en forma de energía renovable. Se estudió el efecto de la adición de vegetal en la digestión anaerobia de dos residuos ganaderos (purín de cerdo (SM) y gallinaza de ponedora (PL)) evaluando la reducción de materia orgánica y la producción de metano mediante un diseño central compuesto seguido de la metodología de superficie de respuesta. En el caso de la co-digestión de SM, se detectó un incremento en el porcentaje de eliminación de materia orgánica a medida que la cantidad de vegetal presente en el substrato se incrementaba. Sin embargo, en la co-digestión de PL se encontró que la concentración de substrato determinaba el porcentaje de eliminación de materia orgánica y que a concentraciones superiores de 80 g SV L<sup>-1</sup> se producían inhibiciones por amonio. Se estudió la degradación de material lignocelulósico mediante microscopía electrónica de barrido (SEM) y la estabilidad del digestato mediante análisis térmico. Las hemicelulosas y la celulosa se degradaron al 100% y 50%, respectivamente, mientras que la lignina no se degradó durante el proceso anaerobio. Además, en el caso de la co-digestión de SM se estudió el proceso en condiciones semi-continuas demostrando el efecto positivo de la adición de vegetal sobre la producción de metano. Además, se estudiaron los cambios en las poblaciones de microorganismos mediante SEM observando un cambio hacia formas más cocoidales. Por otro lado, se evaluaron dos sistemas para el tratamiento de nutrientes en SM digerido anaeróbicamente. Mediante el primero de ellos, el tratamiento anammox, se trataron dos efluentes (SM degradado anaeróbicamente y SM degradado anaeróbicamente y parcialmente aireado) y se obtuvieron eliminaciones de amonio y nitrito en torno al 96%. Se observó además que la concentración de materia orgánica determinaba la eficiencia del proceso. El segundo proceso estudiado fue un sistema de tratamiento con microalgas. Se estudió la eliminación de nutrientes así como la asimilación de dichos nutrientes por parte de la biomasa algal en dos tipos de reactor. Se obtuvieron eliminaciones de amonio, fósforo y materia orgánica del 100, 80 y 60%, respectivamente. La composición de la biomasa obtenida fue del 10, 2 y 48% para N, P y C, respectivamente. Por otro lado, se observó que a

la hora de cosechar la biomasa, el reactor cerrado resultó más eficiente ya que el 96% de la biomasa producida se encontraba retenida en las paredes del reactor.

# *Chapter 1*

## **General Introduction**



## 1.1. WASTES OVERVIEW

### 1.1.1. Swine manure (SM)

**Porcine population:** Spain is the second European country with the highest swine production after Germany. The Spanish porcine population is 24,639,000 heads (animals), which represents 16% of the total European population (EUROSTAT, 2008). As can be seen in Fig. 1.1, Catalonia, Aragón and Castilla y León are the main breeders accounting for 26, 21 and 14% respectively of the total Spanish pig population (MAPA, 2008). Regarding the different provinces in Castilla y León, Segovia has the major swine population with 37% of the total.

**Swine manure production:** As a result of the high pig production in Spain, an important amount of waste is generated; of which swine manure is the most important. Swine manure production depends mainly on the practices used (feed composition, drinking system applied, manure collection system...) and pig category (Burton and Turner, 2003). An estimation of 49,340,020 m<sup>3</sup> of swine manure is produced in Spain each year (data calculated from MAPA, 2008 and RD 324/2000). Since Castilla y León is the third most important region in pig population, this region produces 6,936,686 m<sup>3</sup> per year and 28% of this volume is produced in Segovia.

**Present status and perspectives:** 80% of swine manure produced in Castilla y León has been traditionally used for agricultural application (MAPA, 2008). An increase in livestock farm size and their location in concentrated areas have led to an increase in swine manure production in small, localised areas, mainly in Segovia. Thus, continuous agricultural application to fields close to the farm is not possible and transportation costs make land application economically unfeasible due to the high water content of the manure (Flotats et al., 2009). For that reason, some proposals have been suggested by the PNIR (2007-2015) with the aim of reducing water and nitrogen content and consequently optimizing land application of swine manure. Due to the livestock farm concentration problems mentioned

above and the designation of some areas of the region, 21 in Valladolid and 28 in Segovia, as Nitrate Vulnerable Zones (Directive 91/676/EEC), the 20% of swine manure production calculated to be used for energetic valorisation is increasing.

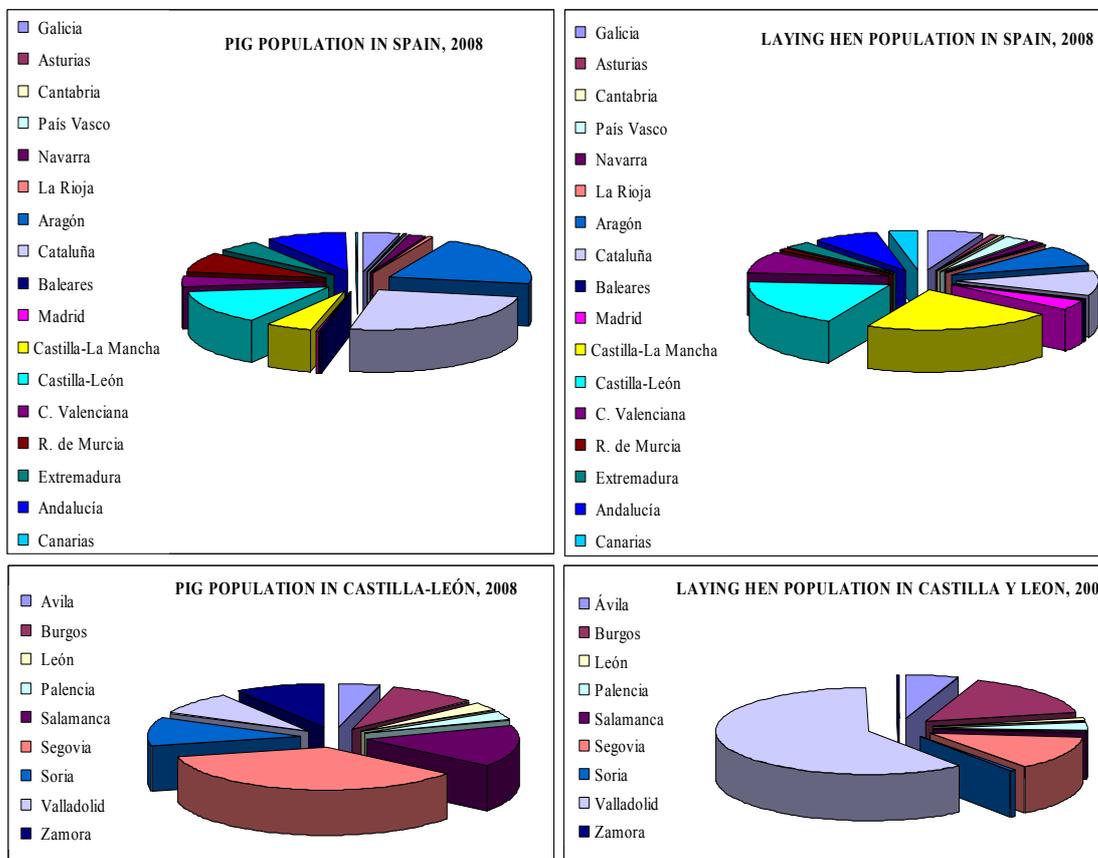


Figure 1.1. Pig and laying hen distribution in Spain and Castilla y León.

### 1.1.2. Poultry litter (PL)

**Laying hen population:** Spain and France are the European countries with the highest laying hen density. The population of laying hens in Spain is 49,994,952 heads (animals), representing 13% of the hen population in Europe (MARM, 2009). Fig. 1.1 shows the

distribution of laying hens in Spain and, more specifically, in Castilla y León. Castilla y León and Castilla-La Mancha are the main laying hen producers in Spain, corresponding to 19 and 22% of the total amount, respectively. Considering the provinces of Castilla y León, Valladolid has most of the laying hen farms (61%), as can be seen in Fig. 1.1 (MAPA, 2008).

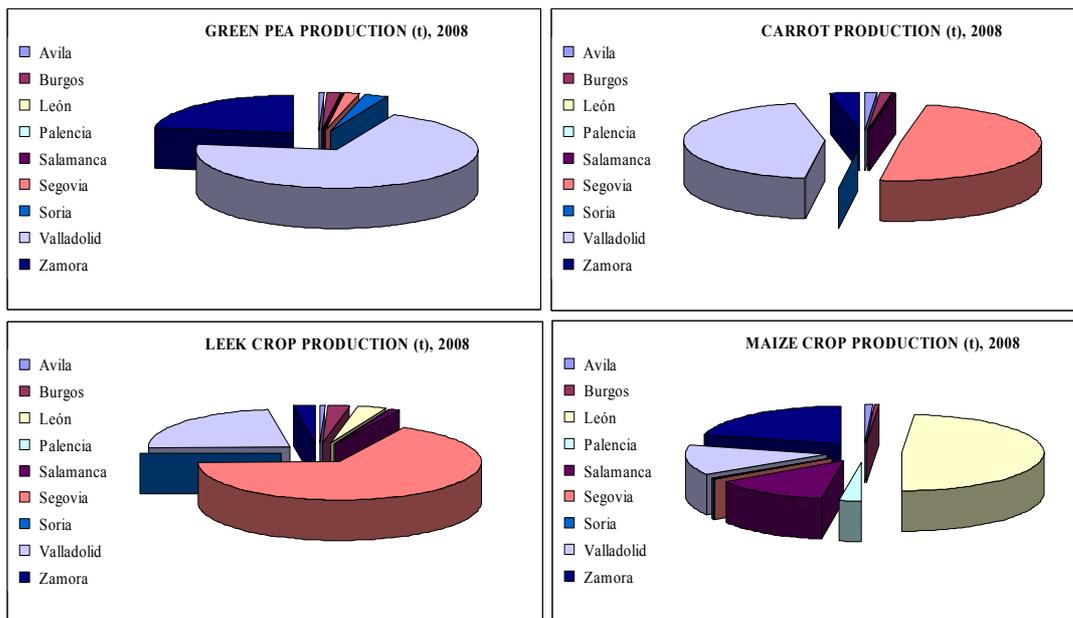
**Poultry litter production:** Different types of poultry manure are produced depending on housing systems, the way of collecting manure, feed type and poultry breeds (Burton and Turner, 2003). The most common laying hen housing system used in Spain is battery-belt scrapers. In this sense, each bird place is calculated to produce 55 kg year<sup>-1</sup> (BAT, 2001). Therefore, an amount of 2,749,722 t of laying hen droppings was estimated to have been produced in 2008, with 474,085 t produced in Castilla y León and 289,192 t in Valladolid.

**Present status and perspectives:** Due to the low water content, 82% of the poultry waste generated in Castilla y León is used for land application as soil amendment, since it is an easier matrix regarding management and transportation, thus freeing 18% for energetic uses.

### 1.1.3. Vegetable processing wastes (VPW)

**Vegetable production:** Spain is one of the main European vegetable producers. Leek, carrot and green pea crop field surfaces were 2,633, 7,936 and 12,415 ha, respectively in 2007; resulting in productions of 70,510, 426,074 and 73,937 t (MAPA, 2008). The surface of maize fields was 361,000 ha producing 3,611,000 t of maize. The surface of Castilla y León is approximately 9.4 million ha, with 37% of this surface being covered by agricultural land. The surfaces of green pea, carrot, leek and maize crops accounts for areas of 1,652, 2,270, 1,309 and 112,586 ha, respectively. León is the main producer of maize with 59,000 ha, Valladolid and Zamora are the main green pea producers, while Segovia registered the highest carrot and leek productions (Junta Castilla y León, 2008). Regarding vegetable production, Fig. 1.2 presents green pea, carrot, leek and maize productions (t) registered during 2008.

**Waste production:** There are up to 1,400 fruit and vegetable processing factories spread across Spain, which are producing around 1,000,000 t of vegetable residues per year (MARM, 2010). Estimated vegetable processing waste production in Castilla y León is 233,428 t per year (ROB Inventory, 2009). Among the diverse uses for the above mentioned materials, fruit and vegetable transformation represents 9.4, 9.3 and 82% for leeks, carrots and green peas, respectively. Castilla y León presents 235 fruit and vegetable processing industries, half of them located in Segovia (48) and Valladolid (45).



**Figure 1.2.** Green pea, carrot, leek and maize production in Castilla y León.

**Present status and perspectives:** According to the ROB Inventory (2009), most of the vegetable processing wastes in Castilla y León are either used for animal feed (35%) or they are processed by an authorized management company (40%). However, there is a great variety in statistical data regarding the different uses of these wastes; as an example, 76% of VPW generated in Valladolid is used for animal feed. It may be estimated that of all the VPW produced in Castilla y León, 26% (61,092 t) may be available for energy production.

## **1.2. ANAEROBIC DIGESTION OF LIVESTOCK WASTES AND VEGETABLE PROCESSING WASTES**

### **1.2.1. Anaerobic digestion process (AD)**

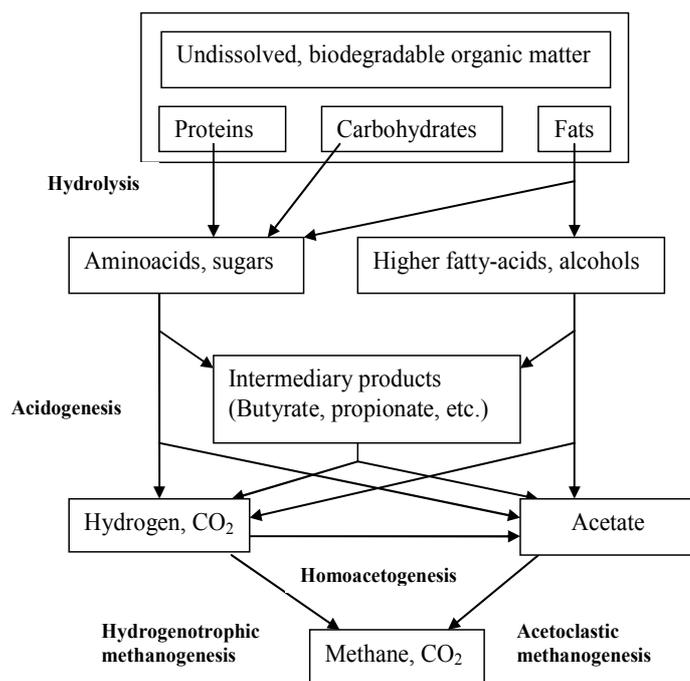
The anaerobic digestion process could be defined as the breaking-down of organic material in the absence of oxygen (Burton and Turner, 2003). Several reactions and microorganisms are involved in the process to carry out the different transformations. Anaerobic conversions occur in a variety of environments, such as marine and fresh water sediments or in the intestinal tract of animals. Mankind has used this process in order to obtain such benefits as energy or the cleaning of effluents from either anaerobic wastewater treatment plants or the digestion systems of solid wastes. Fig. 1.3 represents a global scheme and the different steps of the anaerobic digestion process with four main phases being distinguishable in this process (Zeeman, 2005):

**Hydrolysis:** Undissolved biodegradable organic matter is converted by exoenzymes (cellulases, lipases and proteases) excreted by fermentative bacteria into different compounds, which can be transported through the cell membrane. In the case of complex polymers like lignocelluloses, hydrolysis will be a rate-limiting step, since exoenzymes are not able to attack such complex compounds.

**Acidogenesis:** Acidic bacteria transform the dissolved compounds into fermentation products (volatile fatty acids (VFA), ethanol, lactic acid, hydrogen and carbon dioxide).

**Acetogenesis:** Fermentation products are oxidized to acetate, carbon dioxide and hydrogen, which are indeed the substrates for methanogenic bacteria.

**Methanogenesis:** Methane can be produced by two different routes. Hydrogenotrophic methanogenesis (30%), where hydrogen and carbon dioxide are converted into methane and acetoclastic methanogenesis (70%), where acetate is converted into methane and carbon dioxide.



**Figure 1.3.** Anaerobic degradation of complex organic matter, adapted from Zeeman, G. (2005).

### 1.2.2. Parameters affecting anaerobic digestion

**pH:** Optimum pH for hydrolytic and acidogenic bacteria is 6, whilst methanogenic bacteria have an optimum pH in the range of 7-8 (Chen and Hasimoto, 1996). pH can also affect the dissociation of other compounds such as ammonia, sulphide and organic acids. Ammonia produced during protein degradation results in an increase of the pH while VFA and carbon dioxide production during the acidogenesis stage might reduce the pH (Angelidaki and Ahring, 1993).

**Temperature:** Most of the studies have been carried out at mesophilic range (30-40 °C) due to the higher process stability and the lower amount of energy needed for heating. However, thermophilic anaerobic digestion (45-60 °C) is considered as a more efficient process in terms of organic matter removal and energy production, with the additional benefit of

reducing pathogen content to a greater extent than its mesophilic counterpart. The microbial growth rate in anaerobic digestion is dependent on temperature and the rate increases with increasing temperature (Van Lier, 1995), thus explaining the higher efficiency of the thermophilic process. Methanogenesis is also possible under psychrophilic conditions (10-20 °C), but at low degradation rates, which indeed result in low methane productions (Massé et al., 2003).

**Mixing:** It is known that a good mix of substrate and biomass is needed for good methane production. Nevertheless, it is important to find the proper mode and intensity of the mixing to assure homogenization and sludge settling but not the break-down of bacterial aggregates (Kaparaju et al., 2008).

**Macro and micro nutrients:** Several nutrients should be present and available in the medium for bacterial growth. Scherer et al. (1983) studied the element composition needed for methanogenic bacteria growth reporting minimum needs of 65, 15, 10, 10, 4, 3 and 1.8 g kg ds<sup>-1</sup> for N, P, K, S, Ca, Mg and Fe, respectively. In addition to those macro-elements, a number of micro-nutrients (Ni, Co, Mo, Zn, Mn, Cu...) should be present in smaller amounts (below 0.1 g kg ds<sup>-1</sup>). In general terms, when working with livestock wastes, the addition of micro and macro nutrients is not necessary since they are usually present with the substrate.

**Toxic compounds:** Due to the characteristics of the substrates evaluated in this PhD Thesis, namely livestock wastes and VPW, volatile fatty acids and ammonia are considered the main toxic compounds in anaerobic digestion.

***Volatile fatty acids:*** The acetate producing bacteria have a lower growth rate than fermentative bacteria resulting in slow recovery of acetogenics after a disturbance. A process imbalance results in total VFA (TVFA) accumulation. In the case of well buffered systems, ammonia inhibits acetogenic bacteria resulting in TVFA build-up. However,

regarding low buffered systems, the low alkalinity resulting from the high concentration of TVFA translates into a pH drop and consequently process failure (Murto et al., 2004).

The toxic effect of TVFA is dependent on pH and acids composition. Table 1.1 shows acetate and propionate concentrations, which cause 50% inhibition in methanogenic bacteria with respect to pH, indicating that low pH increases TVFA toxicity, thus decreasing methanogenic activity (Zeeman, 2005). Acetate is the main intermediate (Pind et al., 2003) and its accumulation reduces the metabolic activity of butyrate and propionate degrading bacteria. However, some authors have reported propionate as the main inhibitor (Nielsen et al., 2007).

**Table 1.1.** Concentration of acetate and propionate estimated to cause 50% methanogenic activity inhibition (Zeeman, G. 2005).

pH	50% Inhibiting concentration	
	Acetate mg COD L <sup>-1</sup>	Propionate mg COD L <sup>-1</sup>
5.0	44	13
5.5	100	30
6.0	300	80
6.5	912	241
7.0	2851	745
7.5	8976	2358
8.0	28368	7398

**Ammonia:** Ammonia is an important inhibitor in anaerobic digestion when treating nitrogen-rich substrates, such as livestock wastes. As previously explained, nitrogen is one of the essential elements, with a concentration in the range of 50-200 mg N L<sup>-1</sup> being reported as necessary for anaerobic bacterial growth (McCarty, 1964). Proteins are broken down during anaerobic digestion, increasing ammonium concentration inside the reactor. Free ammonia is the nitrogen species which in fact causes toxicity and it is in equilibrium with ammonium. The free ammonia concentration can be calculated according to Hansen et al. (1998).

The free ammonia concentration depends on three factors, namely pH, temperature and total ammonia concentration. The free ammonia concentration increases with temperature and pH resulting in lower methane yields (Hansen et al., 1998). Free ammonia inhibits methanogenesis at initial concentration of 100-1100 mg N L<sup>-1</sup> (DeBaere et al., 1984; Wiegant and Zeeman, 1986; Angelidaki and Ahring, 1993) depending on the degree of adaptation of the microbial population (Nielsen and Angelidaki, 2008).

***Other toxic compounds:***

*Hydrogen:* The H<sub>2</sub> concentration influences TVFA degradation. During the acidogenesis stage, the H<sub>2</sub> produced can inhibit the process leading to TVFA accumulation. Propionate is degraded at a low H<sub>2</sub> pressure, when partial H<sub>2</sub> pressure is rising, propionate degradation is hindered (Fakuzaki et al., 1990).

*Antibiotics:* Since antibiotics are widely used in farms to prevent infections, some studies have been carried out in order to investigate the inhibition effect of antibiotics on the anaerobic digestion of livestock wastes. Álvarez et al. (2010) studied the chlortetracycline and oxytetracycline effect and Massé et al. (2000) investigated the penicillin and tetracycline effect, both reporting significant inhibitions on the anaerobic digestion of swine manure.

*Long chain fatty acids (LCFA):* Lipids are hydrolyzed to LCFA and glycerol. Long chain fatty acids (LCFA) have been described as inhibitory species (Hwu et al., 1997), but it has been demonstrated that anaerobic systems are able to recover activity and microorganisms can adapt to high levels of these compounds. Moreover, several strategies have been used to recover anaerobic activity, dilution of the reactor and the addition of adsorbents being reported as the best recovery strategies (Palatsi et al., 2009).

*Hydrogen sulphide:* Sulphate reducing bacteria (SRB) reduce the sulphates present in the substrate, producing sulphides. It has been reported that concentrations of 0.1-0.3 g L<sup>-1</sup> of

total hydrogen sulphide or 0.05-0.15 g L<sup>-1</sup> of free hydrogen sulphide caused severe inhibition of the anaerobic digestion process (Imai et al., 1998). Moreover, SRB are able to use several intermediates of the anaerobic digestion process, leading to a competition for these substrates between SRB and methanogenic bacteria.

### **1.2.3. Livestock and vegetable wastes anaerobic digestion**

Environmental concern has increased in recent years, favouring the anaerobic technology for the treatment of organic wastes, thus allowing the establishment and development of this technology. At the beginning of 2010, up to 5900 full-scale biogas plants with an installed capacity of 2300 kW were operating in Europe as reported by the study called “The Market for Biogas Plants in Europe 2010/2011”. Due to the stricter rules concerning land application, usage and storage of livestock waste, the biological treatment of these wastes is considered as one of the fields where the anaerobic process is widely used nowadays (MARM, 2010). Regarding manure treatment, Germany, Denmark, Austria and Sweden are the countries where digestion technology has been widely developed (Holm-Nielsen et al., 2009). Anaerobic treatment offers several advantages besides organic matter reduction, such as the reduction of greenhouse gas emissions, odour and pathogen reduction or the conversion of organic nitrogen into nitrogen available for plant growth. Additionally, it offers the possibility of biogas valorisation, thus allowing the production of renewable energy (Cantrell et al., 2008).

Several co-substrates have been studied in order to improve livestock methane yields, while achieving a better performance of the process. Callaghan et al. (1999) studied anaerobic co-digestion of cattle slurry with different co-substrates (brewery sludge, dissolved air flotation sludge, fish offal, chicken manure and vegetables) with fish offal being obtained as the best co-substrate. Moreover, they reported ammonia and TVFA-mediated inhibition in the case of chicken manure and vegetable co-digestion, respectively. Umetsu et al. (2006) studied the effect of the addition of sugar beet to the anaerobic digestion of dairy manure, obtaining improved methane yields with up to 40% beet addition.

Regarding continuous processes, completely stirred tank reactors (CSTR) are widely used for the anaerobic treatment of manure. Successful results have been reported when evaluating pig-manure co-digestion. Co-substrates, such as potato by-products, slaughterhouse wastes, dissolved air flotation sludge, crop residues or cassava pulp have been studied, obtaining methane yield improvements up to 12-fold (Murto et al., 2004; Kaparaju and Rintala, 2005; Alvarez and Lidèn, 2008; Creamer et al., 2010; Panichnumsin et al., 2010; Wu et al., 2010).

The anaerobic digestion of poultry litter has also been studied under continuous conditions, reporting that the minimization of ammonia levels is the key factor in achieving stable processes. The dilution of solid content to 0.5-3% TS and either surfactant or adsorbent addition have been proposed as solutions to avoid ammonia-mediated inhibitions (Kelleher et al., 2002). Recently, successful anaerobic digestion of up to 25% TS poultry litter has been obtained using ammonia stripping to avoid ammonia accumulation (Abouelenien et al., 2010). Co-digestion has been reported to improve methane yields. Callaghan et al. (2002) studied chicken manure as co-substrates for cattle manure anaerobic digestion, reporting ammonia inhibition when a proportion greater than 15% of chicken manure was added. Magbauna et al. (2001) studied anaerobic co-digestion of hog and poultry waste, demonstrating that this mixture was also viable. On the other hand, Gelegenis et al. (2007a, b) studied the effect of whey and olive oil mill water on diluted poultry litter anaerobic digestion, reporting reactor instability when more than 50% of whey or more than 30% of olive oil mill wastewater were added.

Many fruits and vegetables have been evaluated as anaerobic digestion substrates. They are characterized by high moisture and volatile solids content, as well as high biodegradability. The two main factors hindering the anaerobic digestion of fruits and vegetables are low alkalinity and high fibre content. Alkalinity should not be less than 1500 mg L<sup>-1</sup> in order to avoid process failure (Gunaseelan, 1997). Regarding fibre content, some pre-treatments have been proposed to improve biodegradability. Madukara et al. (1997) used 15 days and 6 months of ensilaging as pre-treatment, reducing fibre content and improving methane yields

during the anaerobic digestion of green peas. Bruni et al. (2010) evaluated different pre-treatments such as size reduction, CaO addition, enzymatic and partial aerobic microbial conversion or stem treatment with catalyst before the anaerobic digestion of biofibers separated from digested manure. This resulted in the chemical treatment (CaO addition) and steam treatment with NaOH giving the highest methane yield increases.

### **1.3. POST-TREATMENT: NUTRIENTS DEPLETION**

Ammonia nitrogen rises during anaerobic digestion as protein breakdown occurs, so digested effluents still have a high concentration of ammonia. As previously mentioned, the main application of livestock waste anaerobic effluents is land application, but nowadays, and due to intensive farming in reduced areas, land availability is reduced. Actually, agriculture represents approximately 62% of nitrogen load to surface water (Council Directive 91/676/EEC), which indeed contributes to water pollution.

The main forms of nitrogen are organic nitrogen, ammonia, nitrite and nitrate. Nitrate itself is not toxic, but it can be converted to nitrite. Nitrite is a potential public health hazard in water causing eutrophication. Different chemical and biological processes have been used to remove this nitrogen. Many physicochemical treatment systems have been developed but; in most of the cases, they do not solve the problem and only transfer the polluting agent from one environment to another. In order to improve the management of those effluents and make a more economically feasible and effective process, a biological nitrogen removal post-treatment is expected to be necessary (MARM, 2010).

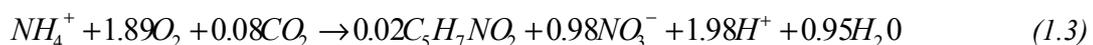
#### **1.3.1. Nitrification-Denitrification**

**Nitrification:** Nitrification implies a chemolithoautotrophic oxidation of ammonia to nitrate under aerobic conditions. It is performed by different bacterial genera which use ammonia or nitrite as the energy source, molecular oxygen as the electron acceptor and carbon dioxide as the carbon source (Madigan et al., 2003).

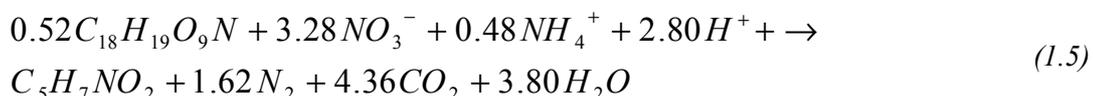
In the first step of nitrification, ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite according to Eq. (1.1). In the second step of the process, nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate according to Eq. (1.2).



The global reaction consumes oxygen (Eq. 1.3), produces biomass and very important acidification of the media because of the  $H^+$  produced.



**Denitrification:** In the denitrification process, nitrate is reduced to nitrogen gas by heterotrophic bacteria under anaerobic conditions through the steps in Eq. (1.4). Organic matter is necessary as carbon source and nitrate is used as the electron acceptor according to Eq. (1.5).

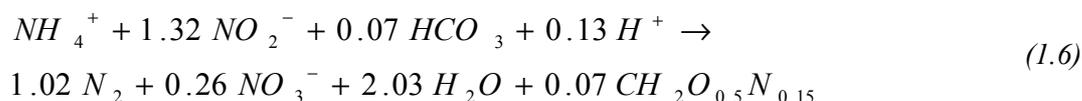


### 1.3.2. Anammox

Since many new processes to remove nitrogen have been developed over the last decade, Anaerobic Ammonium Oxidation (Anammox) has received special attention because it is an efficient and cost-effective biological alternative to conventional nitrogen removal methods from wastewater.

Anammox bacteria were discovered in the 1990s in a waste water treatment plant in Delft, the Netherlands. After that, they were found in the anoxic water column of the Black Sea (Kuypers et al., 2003), the Costa Rica shoreline, an oceanic oxygen-minimum zone, marine sediments and several estuaries. These bacteria belong to Planctomycetes, a phylum of emerging interest for microbial evolution and ecology. They have a unique organelle, called anammoxosome, the membrane of which is much less permeable than normal bio-membranes because of the presence of unique ‘ladderane’ lipids. Such a membrane is required to protect the remainder of the cell from the toxic Anammox intermediates, namely hydrazine (N<sub>2</sub>H<sub>4</sub>) and hydroxylamine (NH<sub>2</sub>OH), and to maintain concentration gradients during the exceptionally slow Anammox growth rate, where the Anammox bacteria grow exceptionally slowly, with a doubling time of 11 days (Strous et al., 1998).

Under anaerobic conditions, ammonium is oxidized to nitrogen gas with nitrite as the electron acceptor and carbon dioxide is used for growth and development. This chemolithoautotrophic nature of the Anammox bacteria has been demonstrated by the incorporation of <sup>14</sup>C-CO<sub>2</sub> into the cells, confirmed by mass balances (Strous et al., 1999). The stoichiometry of the Anammox reaction is given in Eq. (1.6).



Anammox bacteria are very sensitive. The activity is maximal at pH in the range of 6.7 - 8.3 and temperature range between 35 and 45 °C. As anaerobic microorganisms, they are inhibited by more than 0.94 mg dissolved O<sub>2</sub> L<sup>-1</sup> (Van Hulle, 2007). It has been shown that Anammox activity is not inhibited by N-NH<sub>4</sub><sup>+</sup> or N-NO<sub>3</sub> up to concentrations of 1 g N L<sup>-1</sup> (Strous et al., 1999), but it has also been found that when free ammonium concentration in the reactor increases, the ammonium removal rate decreases (Jung et al., 2007). The process is inhibited by N-NO<sub>2</sub> concentrations around 0.1 g N-NO<sub>2</sub> L<sup>-1</sup> working under continuous operation, but the addition of trace amounts of Anammox intermediates (Hydrazine or

hydroxylamine) makes it possible to restore the system (Strous et al., 1999). However, in batch experiments, the process is 50% inhibited by N-NO<sub>2</sub> concentrations of 0.36 g N-NO<sub>2</sub> L<sup>-1</sup> (Dapena-Mora et al., 2007).

The anammox process is very suitable for wastewater with low Carbon/Nitrogen (C/N) ratios. At C/N ratios above 1, the Anammox bacteria are no longer able to compete with heterotrophic denitrifying bacteria (Güven et al., 2005). Chemical oxygen demand (COD) concentrations over 0.3 g L<sup>-1</sup> were found to inactivate or eradicate Anammox communities (Chamchoi et al., 2007). NaCl, KCl, NH<sub>4</sub>Cl have an effect on Anammox at concentrations higher than 150, 100 and 50 mM, respectively (Dapena-Mora et al., 2007). It has been found that alcohols inhibit Anammox. Methanol inhibits the process immediately, while ethanol inhibits by 30% at 2 mM. However, Anammox bacteria can use acetate and propionate as an energy source for the reduction of nitrite and nitrate. The addition of glucose, starch, formate and alanine had little or no effect on Anammox bacteria (Güven et al., 2005). Sulphide concentrations between 1 and 2 Mm cause a reduction of 60% of Anammox activity and flocculant was found to increase Anammox activity due to the formation of biomass conglomerates (Dapena-Mora et al., 2007).

Anammox microorganisms have been successfully used for the treatment of livestock wastewaters. Waki et al. (2007) applied Anammox to wastewaters from an activated sludge reactor treating swine manure and from the anaerobic treatment followed by trickling filter of swine manure. They concluded with successful results and proposed partial nitrification as pre-treatment for the practical application of Anammox. Karakashev et al. (2008) employed Anammox treatment after partial oxidation in a multi-stage treatment treating swine manure, achieving nitrogen removals of up to 90%.

### **The advantages of Anammox as compared to Nitrification-Denitrification**

- Conventional nitrification and denitrification have to be separated in space or time to be effective, while Anammox is a single process, thereby less space is needed.

- The nitrification reactor consumes a large amount of oxygen to convert ammonia into nitrate ( $4,2 \text{ g O}_2 \text{ g N-NH}_4^+_{\text{removed}}^{-1}$ ), while for Anammox no oxygen is required. The combination of partial nitrification and Anammox is usually performed to improve Anammox efficiency. Complete nitrification requires 2 moles of  $\text{O}_2 \text{ mol N}_2^{-1}$  Eq. (1.3), whereas partial nitrification (50%) requires 0.75 mol of  $\text{O}_2 \text{ mol N}_2^{-1}$  (Eq. (1.7)), implying 62.5% less oxygen demand for 50% nitrification.



- Anammox bacteria do not require organic carbon. By contrast, the denitrification process does, and in some cases, the addition of an external carbon source is needed to convert nitrate into nitrogen gas.
- Biomass yield in Anammox is low, and consequently, little sludge is produced. Although this fact may be seen as an advantage, it may also be a drawback since the low biomass yield also requires an efficient system for sludge retention, and additionally long start-up times are required to obtain a sufficient biomass concentration (Jetten et al., 1999).
- Anammox reduces  $\text{CO}_2$  emissions by up to 90% (compared to conventional nitrification/denitrification) and the production of harmful compounds such as nitrous oxide ( $\text{N}_2\text{O}$ ) is avoided.

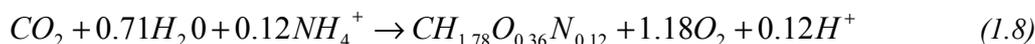
In conclusion, the Anammox process permits a simplification of nitrogen removal procedures, with a considerable reduction in energy and resources required, making the process economically feasible and more efficient than conventional treatment systems.

### 1.3.2. Microalgae-based processes

Microalgae based processes have been extensively studied due to their wide range of applications. Microalgae technology has been used for biomass and compounds production,

pollution control and wastewater treatment. The biomass obtained has been used for producing biodiesel, bio-ethanol and biogas, soil fertilization, animal feed and health food fabrication (Spolaore et al., 2006; Wang et al., 2008; Posten et al., 2009).

Most of the studies performed using microalgae for wastewater treatment are based on the symbiotic relationship between microalgae and bacteria (González et al., 2008; de Godos et al., 2010). Microalgae provide the photosynthetic oxygen required by aerobic bacteria, while bacteria supply CO<sub>2</sub> needed for microalgae growth. Autotrophic microalgae produce complex organic compounds from single inorganic molecules (carbon dioxide or bicarbonate and water) using energy from light (photosynthesis) Eq. (1.8). Therefore, they are able to recycle nutrients via uptake, which results in the conversion to macromolecules such as carbohydrates, lipids and proteins.



Microalgae are able to absorb and use light from the whole visible wavelength range, 400 to 700 nm. The photosynthetic machinery is located inside the chloroplasts of the microalgae. The photosynthetic units are fixed inside the thylakoid membranes and each unit is composed of the photosystems called photosystem I (PSI) and photosystem II (PSII) and a cytochrome complex connected by mobile electron carriers. The absorption of photons by the microalgae photosystems sets in motion the cascade of light and dark reactions ultimately resulting in growth (Madigan et al., 2003). The photosystems are complexes of proteins and pigments. Photons are absorbed by the pigments and the resulting excitation energy is channelled to a specialized chlorophyll molecule called the reaction centre of PSII.

Besides light, temperature and nutrients are factors affecting microalgae-bacteria consortia activity. The specific microalgae growth rate increases with temperature until the maximum is reached. The optimal temperature range is typically a few degrees Celsius wide, below the optimum the growth rate slowly decreases while above the optimal temperature the decrease

in growth rate is much more pronounced. The optimal temperature for microalgae growth was found within a range of 15-35 °C (Noue et al., 1994; de-Bashan et al., 2008).

The most important elements the biomass is composed of are hydrogen (H), oxygen (O), carbon (C), nitrogen (N) and phosphorus (P). Since microalgae grow in an aqueous environment, they take hydrogen and oxygen from water. Most microalgae are autotrophic, thus carbon is taken up as carbon dioxide. Nitrogen is used to create proteins; nitrogen can be taken by microalgae in the form of ammonium and nitrite, with the former being more easily assimilated. It is worth mentioning that some nitrogen can be physically removed via ammonia stripping, depending on the pH in the medium (González, 2008, de Godos et al., 2009). However, free ammonia is toxic for microalgae and previous studies have reported inhibition in a range of 11- 84 mM, depending on the microalgae species and the degree of adaptation (Ogbonna and Tnaka, 2000; González et al., 2008). In the case of phosphorus, it is mainly assimilated as soluble phosphorus; organic phosphorus has to be hydrolyzed to be used by microalgae. Depending on the pH, some phosphorus can be physically removed by precipitation with some metal ions when the pH increases (González, 2008).

Several microalgae-bacteria consortia systems have been used for livestock waste treatment mainly focused on nutrient removal efficiencies (De Godos et al., 2010., Xin et al., 2010), but also regarding the biochemical composition of biomass as well as the photosynthetic efficiency (González-Fernández et al., 2010). Among those systems, bio-films have been proven to be more effective for nutrient recovery and biomass harvesting (Muñoz et al., 2009). However, open ponds have been extensively used due to their low cost, effectiveness and ease of application (Grobelaar, 2009).

### **The advantages of microalgae-based processes as compared to Nitrification-Denitrification**

- Microalgae produce O<sub>2</sub>, thus aeration costs are reduced. A mechanical aerated pond requires 0.8-6.4 KWh Kg BOD<sup>-1</sup> removed, while photosynthetically oxygenated ponds consume 0-0.57 KWh Kg BOD<sup>-1</sup> removed, according to Oswald (1995).
- Regarding microalgae-bacteria consortia, CO<sub>2</sub> produced by heterotrophic bacteria is used by autotrophic microalgae as their carbon source. Therefore, it is not necessary to add any external carbon source.
- Microalgae are able to assimilate nutrients (nitrogen and phosphorus) in form of biomass, which is a valuable product. Algae biomass has different uses, such as soil fertilizer, animal feed or energy production.
- Lower amounts of CO<sub>2</sub> are released to the atmosphere since most of the CO<sub>2</sub> produced by heterotrophic bacteria is assimilated in the form of biomass. Therefore, microalgae based processes contribute to the mitigation of greenhouse effects

To conclude, microalgae –bacteria treatment is presented as an economically feasible system which contributes to green-house effect mitigation while producing a value-added product.

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## *Chapter 2*

### **Scope of the thesis**



## 2.1. OBJECTIVES

The main objective of this Thesis has been *to study the effect of the addition of vegetable processing wastes on the anaerobic digestion of livestock wastes and to evaluate anammox and microalgae-based systems to treat the anaerobic digestates.*

Different research works have been carried out with the purpose of achieving the main objective:

- i) Evaluating the influence of initial substrate concentration and vegetable content added as co-substrate on the final methane yield and volatile solids removal in the anaerobic digestion of livestock wastes (swine manure and poultry litter).
- ii) Studying the degradation of a lignocellulosic complex in the co-digestion of livestock wastes with vegetable processing wastes (VPW) under batch operation. Evaluating the degradation of organic matter by means of thermal analysis.
- iii) Investigating the effect of VPW addition on methane production under semi-continuous operation using completely stirred tank reactors (CSTRs).
- iv) Examining the nitrogen removal achieved by the Anammox process using a semi-continuously fed UASB reactor. Digested swine manure and partially-oxidized digested swine manure were used as influents and the effect of the chemical oxygen demand (COD) concentration on the process performance was determined.
- v) Studying the nutrients removal attained by means of microalgae-bacteria consortium using digested swine manure as influent. Comparing two configurations of reactors and evaluating nutrients uptake as algae biomass increases.

## 2.2. THESIS OUTLINE

Livestock wastes have been traditionally applied as organic amendments as a way of recycling nutrients and organic matter. However, in some cases, the high content of nutrients may cause water, soil and air pollution. Offensive odours can be generated from organic matter degradation during the management and storage of these wastes, often resulting in social and environmental problems. Additionally, stricter regulatory approaches concerning land application of manures have led to an increasing interest in biological treatment technologies. In this sense, anaerobic digestion is a viable alternative for reducing organic matter content while producing renewable energy from biogas valorisation. However, two of the main drawbacks of digesting livestock wastes are the high ammonia evolution from protein degradation, which might result in process inhibition, and the low methane yields achieved due to the high water or fibre content of the manure. In order to overcome such problems, co-digestion with carbon-rich substrates, like vegetable processing wastes (VPW), has been evaluated.

In *Chapter 1*, a general overview is presented regarding livestock and VPW waste production and current uses in Castilla y León, as well as an introduction to anaerobic digestion, Anammox and microalgae-based processes. In *Chapter 2* the objectives of the thesis and the thesis outline are presented.

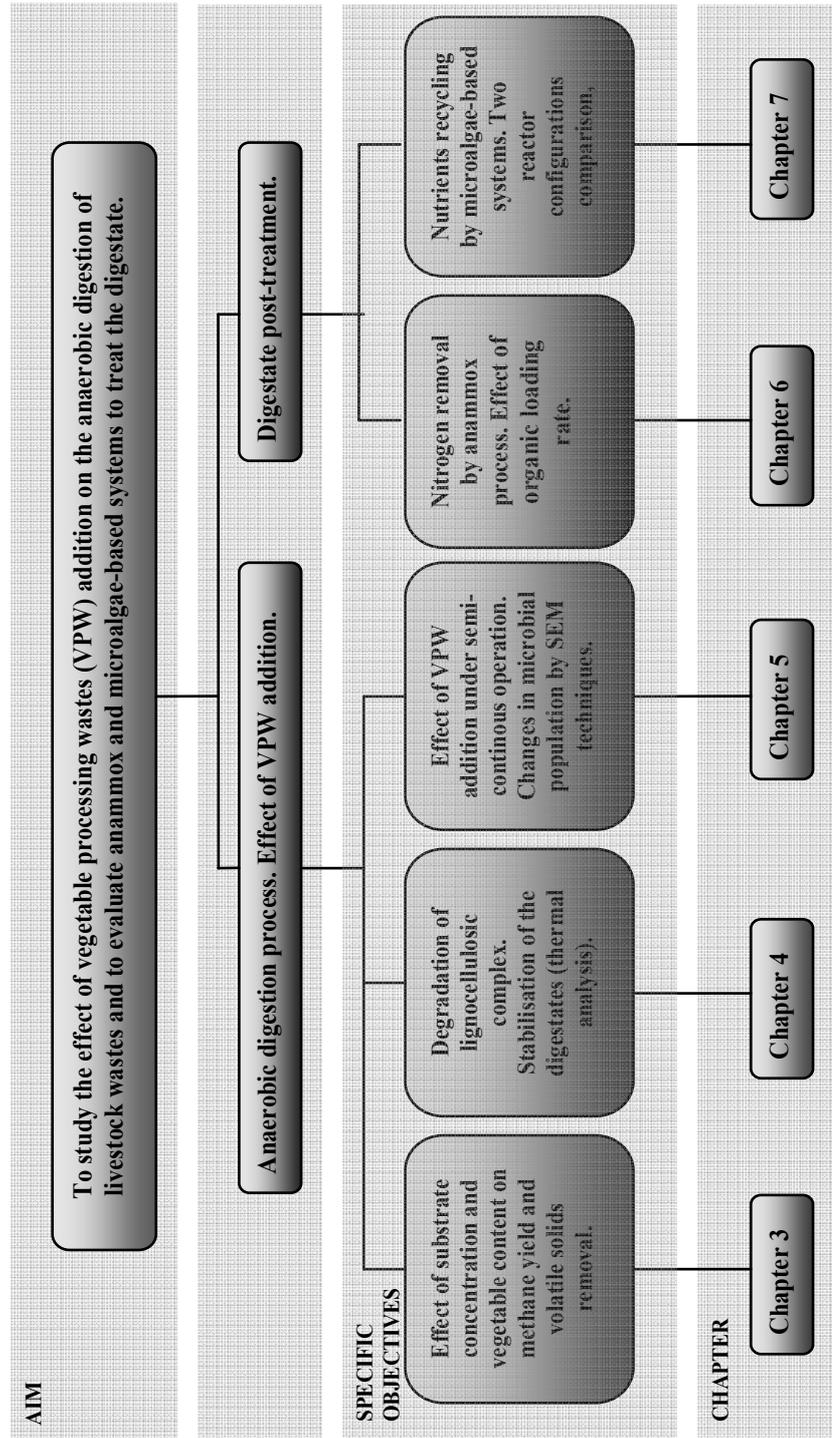
In *Chapter 3*, co-digestion of livestock wastes is studied by means of central composite design and response surface methodology. Swine manure (SM) and poultry litter (PL) are studied under batch digestion with VPW being used as co-substrate. Two factors are selected, namely the initial concentration of substrate and the proportion of VPW added to the co-digesting mixture. Evaluation of the digestion process is performed in terms of methane yield and volatile solids removal. Regarding fibre degradation, the lignocellulosic complex during the anaerobic process is studied by means of scanning electron microscope (SEM), thermal analysis and physicochemical analyses (*Chapter 4*).

After concluding that poultry manure anaerobic digestion is difficult to carry out, and taking into account that swine manure is the most problematic waste studied in the region, semi-continuous anaerobic digestion is investigated for swine manure. The effect of VPW addition to the semi-continuous anaerobic digestion of swine manure is studied in **Chapter 5**. In this context, continuous stirred tank reactors (CSTR) are employed and SM and SM-VPW mixture of 50:50 (W/W dry weights) are used as substrates. Changes in microbial populations are also studied using SEM techniques.

In **Chapter 6** and **Chapter 7**, two biological treatments for nutrients recovery are studied, namely Anammox and microalgae-based processes. In Chapter 6, the Anammox process is investigated under semi-continuous operation in an up-flow anaerobic sludge blanket (UASB) reactor treating anaerobically digested SM and evaluating the effect of organic matter on Anammox activity. In Chapter 7, the microalgae-bacteria symbiotic relationship is used for the removal of nutrients from anaerobically digested SM. The performance of two reactors: open pond and closed photobioreactor, are studied. Different nitrogen removal mechanisms and biomass compositions are also evaluated.

General conclusions are presented in **Chapter 8**.

Table 2.1. Organization of this PhD thesis.



## *Chapter 3*

# **Anaerobic co-digestion of livestock wastes with vegetable processing wastes: a statistical analysis**

**Molinuevo-Salces, B., García-González, M. C., González-Fernández, C., Cuetos, M. J., Morán, A., Gómez, X.**

*Bioresource Technology 101, (2010) 9479-9485.*



**Abstract.** Anaerobic digestion of livestock wastes with carbon rich residues was studied. Swine manure and poultry litter were selected as livestock waste, and vegetable processing waste was selected as the rich carbon source. A Central Composite Design (CCD) and Response Surface Methodology (RSM) were employed in designing experiments and determining individual and interactive effects over methane production and removal of volatile solids. In the case of swine manure co-digestion, an increase in vegetable processing waste resulted in higher volatile solids removal. However, without a proper substrate/biomass ratio, buffer capacity of swine manure was not able to avoid inhibitory effects associated with TVFA accumulation. Regarding co-digestion with poultry litter, substrate concentration determined VS removal achieved, above 80 g VS L<sup>-1</sup>, N-NH<sub>3</sub> inhibition was detected. Statistical analysis allowed us to set initial conditions and parameters to achieve best outputs for real-scale plant operation and/or co-digestion mixtures design.

**Resumen.** En este trabajo se estudió la digestión anaerobia de residuos ganaderos (purín de cerdo y gallinaza) mezclados con residuos ricos en carbono (residuos del procesado de vegetales). Para el diseño de experimentos se utilizó un diseño central compuesto y la metodología de superficie de respuesta, estudiando la producción de metano y la eliminación de sólidos volátiles. En el caso de la co-digestión de purín, en el porcentaje de eliminación de sólidos volátiles se detectó un incremento a medida que el porcentaje de residuo vegetal añadido aumentaba. Sin embargo, cuando la relación inicial sustrato/microorganismos no era la adecuada, la capacidad tampón del purín no fue suficiente para evitar la inhibición. Respecto a la co-digestión de gallinaza, se observó que el porcentaje de eliminación de sólidos volátiles era determinado por la concentración de sustrato. Por encima de 80 g SV L<sup>-1</sup>, se detectó inhibición por amonio. El análisis estadístico permitió establecer los parámetros iniciales óptimos para obtener mejores rendimientos así como establecer diseños de mezclas óptimos para co-digestión.



### **3.1. INTRODUCTION**

Castilla y León is one of the most important pig and poultry producers in Spain. In 2008, pig production was 3.7 million and laying hens production was 47.5 million (MARM, 2010). The continuous development of intensive pig and poultry production in that region has led to an increase of livestock wastes in small and located areas. Uncontrolled discharge of these wastes cause serious environmental, social and health problems, thus it is necessary to minimize the risks following the current legislation.

Anaerobic digestion is a large extended technique with several full biogas-plants under operation for organic solid waste treatment and energy recovery (Angelidaki et al., 2005; Bolzonella et al., 2006). Although composting and direct application to land as a substitute for inorganic fertilizers are also widely used treatments, specially when dealing with poultry litter (PL), the rise in environmental concerns associated with the production of energy and CO<sub>2</sub> mitigation policies has renewed interest in digestion technologies. In this context, anaerobic digestion has largely been studied in recent years as a suitable technique for the treatment of swine manure (SM) and PL allowing reduction in organic matter content and odours and producing energy (Flotats et al., 2009). Moreover, by means of anaerobic digestion, pathogens can be minimized and even removed (Sahlström, 2003).

Anaerobic digestion of SM and PL has been extensively studied often leading to low methane yields due to the high amount of water, fibres and nitrogen content of these wastes (Bujoczek et al., 2000; Magbanua et al., 2001). Furthermore, breakdown of proteins during anaerobic digestion raise ammonium concentration of the medium (Angelidaki and Ahring, 1993). This fact contributes to ammonia-mediated inhibition of the process. Depending on the adaptation degree of microbial population, unionized ammonia has been reported to inhibit methanogenesis at initial concentration of 100-1100 mg N L<sup>-1</sup> (Angelidaki and Ahring, 1993; de Baere et al., 1984; Nielsen and Angelidaki, 2008). Other relevant factors which may hinder the digestion process, and thus needing special consideration, are organic

overloading caused by high concentration of total solids (TS) and inadequate substrate to biomass ratio. When employing SM as substrate, increasing methane production is attained with increasing TS concentration, until a certain threshold after which methane production decreases due to total volatile fatty acid (TVFA) accumulation from the acidogenesis phase. More specifically, Fischer et al. (1984) reported a maximum biogas yield at solid concentration of 70 g TS L<sup>-1</sup>. Similar results have been suggested for PL digestion when different concentrations of solids were evaluated. Under mesophilic digestion of PL, Webb and Hawkes (1985) achieved a maximum biogas production at TS concentration of 40-60 g TS L<sup>-1</sup>, while higher TS concentration led to a reactor performance failure due to the increase of free ammonia concentration.

The low carbon/nitrogen (C/N) ratio characterizing the above mentioned wastes makes them suitable for co-digestion with other carbon-rich co-substrates. Hence, methane production of both substrates is enhanced by reaching a more balanced C/N ratio as well as decreasing potential ammonia or TVFA-mediated inhibition. Livestock wastes provide the nitrogen necessary for cell growth and their high buffer capacity avoids pH drops. The carbon-rich co-substrate supplies organic matter improving methane yields and avoiding toxic ammonia concentrations (Murto et al., 2004; Álvarez et al., 2010).

The aim of this work was to study the effect of two operating parameters, namely the initial concentration of substrate and the proportion of vegetable processing waste mass added as co-substrate on the anaerobic co-digestion of SM and PL under batch conditions. A central composite design followed by response surface methodology was applied in order to determine the effect of both operating parameters over the methane yield and the volatile solids removal.

## **3.2. METHODS**

### **3.2.1. Raw materials**

SM was obtained from a pig farm located in Avila (Spain) and PL from a poultry farm located in Palencia (Spain). Vegetable Processing Waste (VPW) was collected in a vegetable processing factory located in Segovia (Spain) and was composed by maize, carrots, peas and leeks (25:25:25:25 (dry weight)). This latter residue was ground to approximately 1 mm particle size. The anaerobic sludge used as inoculum was collected from an anaerobic digester in the municipal wastewater treatment plant of Valladolid (Spain). The chemical characterization of each waste and the sludge employed is shown in Table 3.1.

**Table 3.1.** Composition of the substrates: Vegetable processing wastes (VPW), poultry litter (PL), swine manure (SM), and anaerobic sludge (AS)

<b>Parameters</b>	<b>VPW*</b>	<b>PL</b>	<b>SM</b>	<b>AS</b>
<b>pH</b>	4,4	7,4	7,9	7,5
<b>VS (g L<sup>-1</sup>)</b>	114.9 (4.1)	226.5 (38.2)	45.5 (2.4)	9.1 (0.9)
<b>TS (g L<sup>-1</sup>)</b>	124.1 (4.0)	306.2 (41.4)	57.1 (1.9)	16.6 (1.6)
<b>CODs (g L<sup>-1</sup>)</b>	70.9 (1.7)	12.4 (0.9)	6.9 (3.8)	3.8 (0.1)
<b>CODt (g L<sup>-1</sup>)</b>	224.5 (49.4)	259.8 (25.7)	39.8 (3.9)	25.9 (2.5)
<b>TKN(g L<sup>-1</sup>)</b>	3.3 (0.1)	9.4 (0.9)	3.7 (0.0)	1.3 (0.0)
<b>N-NH<sub>4</sub><sup>+</sup> (g L<sup>-1</sup>)</b>	0.5 (0.0)	1.1 (0.1)	2.7 (0.1)	0.5 (0.1)
<b>TVFA (g L<sup>-1</sup>)</b>	10,5	6,5	n.d.	n.d.

\*Data are means of three replicates, exception made for pH data and total VFAs measurements. Standard Deviation is shown in brackets.  
n.d.: Not determined

### **3.2.2. Set-up**

Two set of experiments were carried out using the same methodology. From now on SM-VPW will stand for co-digestion of SM with VPW and PL-VPW for co-digestion of PL with VPW. The selected factors for the study were the initial substrate concentration (SC)

measured in terms of volatile solids (VS as g VS L<sup>-1</sup>) and the proportion of vegetable processing waste mass added as co-substrate (Veg.) measured in terms of percentage of TS of VPW in relation to the TS of the initial substrate. SC range of the SM-VPW co-digestion was selected in accordance with Campos (2001), who reported a decrease in methane production whenever the solids concentration of SM was above 100 g TS L<sup>-1</sup>. Typical values of VS/TS ratio for pig slurry are around 0.75-0.85 (Kaparaju and Rintala, 2005) usually containing less than 60 g VS L<sup>-1</sup> in Castilla and León (González-Fernández et al., 2008).

**Table 3.2.** Codified and real values, VS removal and Y responses for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

SM-VPW*						
Treatments	Codified values		Real values		Responses	
	SC (g VS L <sup>-1</sup> )	Veg(%)	SC (g VS L <sup>-1</sup> )	Veg(%)	VS removal (%)	Y <sub>SM</sub> (ml CH <sub>4</sub> g VS added <sup>-1</sup> )
					Real	Real
T1	-1	1	12.4	85.4	64 (2)	231 (45)
T2	-1	-1	12.4	14.6	61 (10)	182 (28)
T3	1	1	60.1	85.4	80 (3)	173 (7)
T4	1	-1	60.1	14.6	63 (2)	162 (38)
T5	0	1.4142	36.3	100.0	82 (1)	303 (48)
T6	0	-1.4142	36.3	0.0	61 (2)	138 (15)
T7	-1.4142	0	2.5	50.0	48 (8)	48 (9)
T8	1.4142	0	70.0	50.0	70 (1)	257 (34)
T9	0	0	36.3	50.0	72 (2)	286 (80)

PL-VPW*						
Treatments	Codified values		Real values		Responses	
	SC (g VS L <sup>-1</sup> )	Veg(%)	SC (g VS L <sup>-1</sup> )	Veg(%)	VS removal (%)	Y <sub>SM</sub> (ml CH <sub>4</sub> g VS added <sup>-1</sup> )
					Real	Real
T1	-1	1	30.5	85.4	62 (1)	412 (94)
T2	-1	-1	30.5	14.6	64 (0)	236 (21)
T3	1	1	129.5	85.4	27 (4)	5 (1)
T4	1	-1	129.5	14.6	38 (4)	315 (92)
T5	0	1.4142	80.0	100.0	50(1)	20 (1)
T6	0	-1.4142	80.0	0.0	60 (7)	251 (74)
T7	-1.4142	0	10.0	50.0	64 (1)	162 (20)
T8	1.4142	0	150.0	50.0	19 (1)	329 (4)
T9	0	0	80.0	50.0	53 (0)	426 (69)

\*Data are means of two replicates, except T9, which data are means of six replicates. Standard Deviation is shown in brackets.

Hence, in the present study the selected range for evaluating the SC in SM-VPW co-digestion was 2.5-70 g VS L<sup>-1</sup>. In the case of PL-VPW co-digestion, the SC range was selected in accordance with Bujoczek et al. (2000), who obtained successful PL digestion

with TS values up to 160 g TS L<sup>-1</sup>. Thus, the selected range was 10-150 g VS L<sup>-1</sup> for SC factor. On the other hand, the selected range for the addition of VPW as co-substrate was 0-100% for both co-digestion experiments evaluated. The experimental design is shown in Table 3.2.

All the assays were carried out in duplicate, except for the central point (T9) which was repeated six times. The anaerobic assays were conducted in 500 mL bottles filled with 100 mL of inoculum and 100 mL of the corresponding substrate mixture. Two blanks containing 100 mL of inoculum and 100 mL of distilled water were also run to determine the endogenous methane production. Bottles were closed with a septum and the headspace flushed with N<sub>2</sub>. Bottles were incubated in a thermostatic shaker at 100 rpm and 35 ± 2 °C for 80 days.

### **3.2.3. Central composite design (CCD) and data analyses**

Central composite design is a second order factorial design employed when the number of runs for a full factorial design is too large to be practical (Box and Wilson, 1951). This type of factorial design usually consists of a 2<sup>k</sup> factorial nucleus, six replications of the central point and 2\*k axial points, where *k* is the number of factors evaluated. More specifically, in the present study the two factors were SC and Veg. percentage. Factorial design levels are codified from -1 to +1. Central point is replicated six times in order to estimate experimental error. Axial points ensure design rotatability and their distance to the central point ( $\alpha$ ) is calculated according to Eq. (3.1).

$$\alpha = 2^{k/4} \tag{3.1}$$

The experimental design was analyzed using response surface methodology (RSM). RSM is a collection of mathematical and statistical techniques used to model and analyze problems in which a response of interest is influenced by several variables (Montgomery, 2005). The selected responses for analysis were VS removal, measured as percentage, and the methane

yield, measured as volume of methane produced per unit of VS added. The variables,  $X_i$ , were coded as  $x_i$  according to Eq. (3.2), such that  $X_0$  corresponded to the central value:

$$x_i = (X_i - X_i^*) / \Delta X_i; \text{ where } i=1, 2, 3, \dots, k \quad (3.2)$$

where  $x_i$  is the dimensionless coded value of an independent variable,  $X_i$  is the actual value of an independent variable for the  $i$ th test,  $X_i^*$  is the actual value of an independent variable at the centre point and  $\Delta X_i$  is the step change (Chong et al., 2009). All the evaluated levels were combined in nine different treatments. Codified and real values for both factors are presented in Table 3.2.

For predicting the optimal point, a second order polynomial function was performed Eq. (3.3):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + E \quad (3.3)$$

where  $Y$  represents the predicted response,  $\beta_0$ ,  $\beta_1$ ,  $\beta_2$ ,  $\beta_{11}$ ,  $\beta_{22}$  and  $\beta_{12}$  are the regression coefficients.  $E$  is the standard error and  $X_1$  and  $X_2$  are the evaluated factors (SC and Veg.). Coefficient of determination ( $R^2$ ) was calculated to achieve the proportion of data variability that is explained by the model, thus the quality of fit to the model. The  $p$ -values of the parameter estimation were used to validate the model. P-values less than 0.05 indicate the significant model terms.

To obtain eigenvalues, derivative of Eq. (3.3) was performed. Multiple regression analysis for the data sets collected was performed using Excel software (Excel 2003). The optimization process was carried out using software Matlab R12.

#### **3.2.4. Chemical analysis**

TS, VS, pH, soluble chemical oxygen demand (CODs), total chemical oxygen demand (CODt), total Kjeldahl nitrogen (TKN), ammonium nitrogen ( $\text{N-NH}_4^+$ ) analysis were

performed in accordance with APHA (2005) Standard Methods. For all treatments, free ammonia concentrations were calculated in accordance with Hansen et al. (1998). Samples from the beginning and the end of the experiment were analysed.

The biogas production was measured with a portable pressure transducer (Colleran et al., 1992). Measurements were recorded everyday for the first 5 days of experimentation, and twice a week in posterior days. Biogas composition was analysed using a gas chromatograph (Varian CP 3800 GC) with a thermal conductivity detector, provided by a CP-Molvsieve5A column (15m x 0.53 mm x 15  $\mu$ m) followed by a CP-Porabond Q column (25m x 0.53 mm x 10  $\mu$ m). Hydrogen (13.6 mL min<sup>-1</sup>) was used as the carrier gas. The injection port temperature was set at 150 °C and the detector temperature was 175 °C. Volatile fatty acids (VFA) were analysed using a gas chromatograph (Varian CP 3800 GC) equipped with a capillary column (from Supelco) and a flame ionization detector. The carrier gas was helium and the temperature of the injector was 250 °C. The temperature of the oven was set at 150 °C for 3 min and thereafter increased to 180 °C.

### **3.3. RESULTS AND DISCUSSION**

#### **3.3.1. Swine manure co-digestion**

The experimental design data and responses obtained from experimentation are presented in Table 3.2. When considering methane production obtained from SM and VPW co-digestion it was observed that all treatments raised the expected methane potential production, except T3 (Fig. 3.1A). This treatment was characterized by high concentration of solids and vegetable waste content; therefore TVFA concentration was the highest among all the treatments (Fig. 3.2A). Moreover, methane production for T3 seemed not to be completely stopped at the end of experimental time. An organic overload was produced in T3 resulting in TVFA accumulation. When TVFA were steady consumed, methane production started. Nevertheless, experimental time was not enough to complete TVFA conversion, leading to underestimating response values. Similar behaviour was reported by González-Fernández

and García-Encina (2009) when digesting swine manure. This research explained that increasing substrate-microorganisms ratio resulted in high acetic and propionic acids formation with a delay on methane production due to partial inhibition over methanogenic bacteria. Based on the previous statement, T3 values were excluded when adjusting data to the model. Regression analyses for both responses (VS removal and  $Y_{SM}$ ) resulted in the Eqs. (3.4 and 3.5), respectively:

$$VS\ Removal = 71.9 + 6.7*SC + 6.7*Veg - 5.7*SC^2 - 0.7*Veg^2 + 4.7*SC*Veg \quad (3.4)$$

$$Y_{SM} = 286.2 + 49.9*SC + 59.3*Veg - 55.3*SC^2 - 21.4*Veg^2 + 35.6*SC*Veg \quad (3.5)$$

In the case of VS removal response (Eq. (3.4)), the determined  $R^2$  coefficient showed that the model explained 86% of the variability data (Table 3.3). Both factors presented a significant effect over the response, as well as the interaction factor and the quadratic factor for SC. Eigenvalues were calculated resulting in values of  $\lambda_1 = -6.47$  and  $\lambda_2 = 1.47$ , which indicated the presence of a saddle point in the plot surface. Therefore, the optimum for VS removal response was outside the experimental region evaluated. Results imply that under normal operating conditions in livestock farms considered, no optimum values can be attained for achieving maximum removal of VS, indicating that only the best operational point may be selected.

From the response surface plot (Fig. 3.3), it can be observed an increase in VS removal concomitant with an increase in factors studied. In this sense, the highest values (80% VS removal) were achieved in treatments with high vegetable content (T3, T5). Similar tendency was followed by T5, T6 and T9. In these treatments the SC factor was constant; however the increase of Veg. was proven to enhance VS removal concomitantly with this factor. Results obtained were in accordance with those reported by Habiba et al. (2009) who digested VPW and activated sludge in different proportions achieving 65-88% VS removal, reporting a positive effect of VPW addition over VS removals.

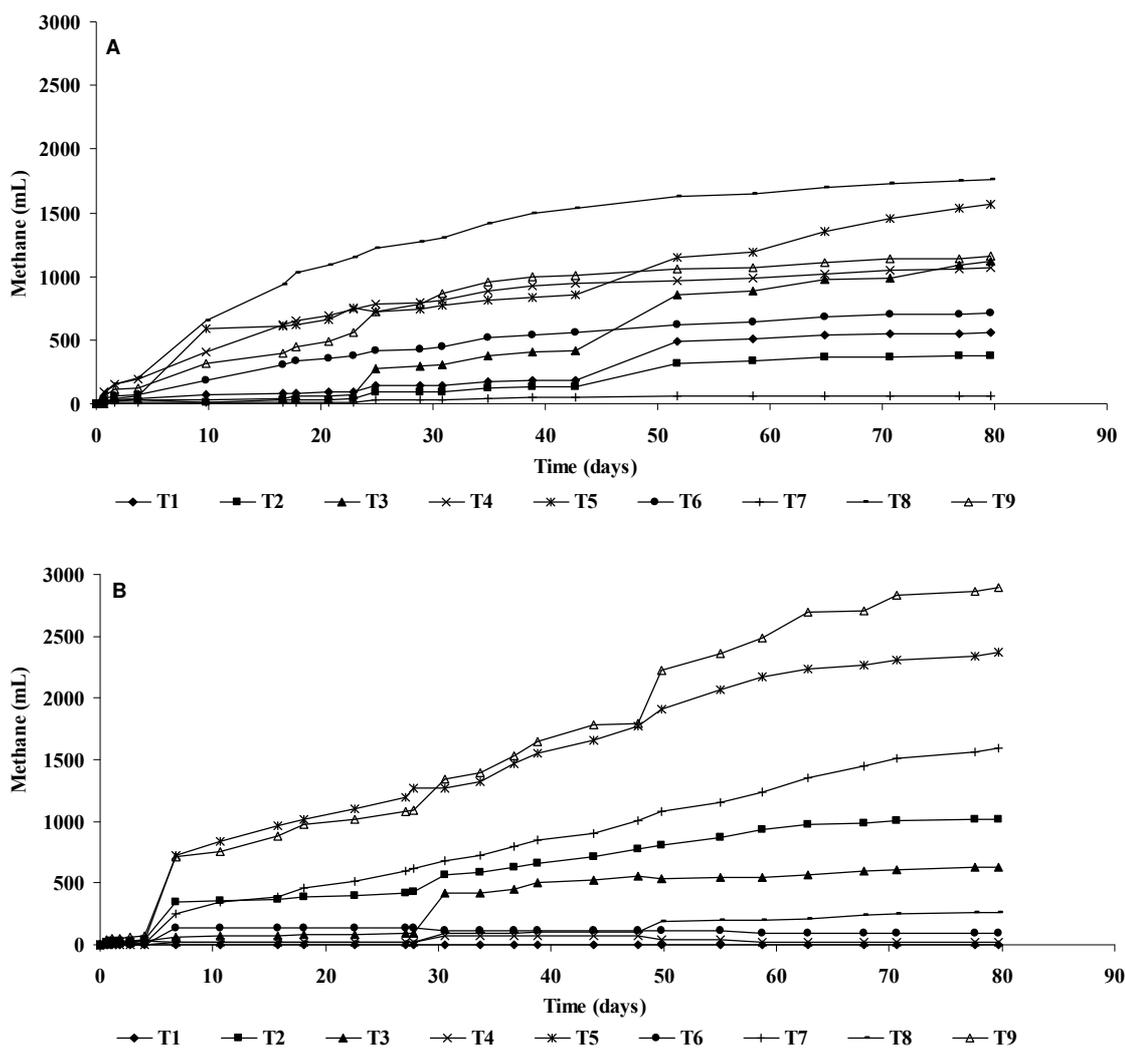


Figure 3.1. Accumulated methane production for: A) SM-VPW, B) PL-VPW.

The response  $Y_{SM}$  (Eq. (3.5)) presented a  $R^2$  coefficient of 0.65, therefore the capacity for predicting the response was not satisfactory. As previously stated, the response did not take into consideration data obtained from treatment T3. The values of methane yield obtained were in a similar range to those reported by Kaparaju and Rintala (2005), employing potato

processing wastes as co-substrate for anaerobic digestion of pig manure. Methane content was above 65% and a reduction of VS higher than 50% was registered for all treatments, exception made for T7 treatment with a poor performance. This treatment presented a methane yield of 48 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup> with a methane content of only 32%. The small amount of VS added as substrate (2.5 g VS L<sup>-1</sup>) at the initial stage of the digestion assay resulted in poor methane production due to the low content of organic material available for microorganisms and subsequent conversion into methane. This result highlights the relevance of an adequate selection of substrate/biomass ratio.

**Table 3.3.** Regression results for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

SM-VPW					
VS removal (%)			Y <sub>SM</sub> (ml CH <sub>4</sub> g VS added <sup>-1</sup> )		
	Coefficient	Prob		Coefficient	Prob
$\beta_0$	71.9	< 0.001	$\beta_0$	286.2	< 0.001
$\beta_1$	6.7	< 0.001	$\beta_1$	49.9	0.018
$\beta_2$	6.7	< 0.001	$\beta_2$	59.3	0.006
$\beta_{11}$	-5.7	< 0.001	$\beta_{11}$	-55.3	0.01
$\beta_{22}$	-0.7	0.582	$\beta_{22}$	-21.4	0.297
$\beta_{12}$	4.7	0.023	$\beta_{12}$	35.6	0.246
<i>R<sup>2</sup> = 0.86, Adj.R<sup>2</sup> = 0.81, F = 17.2, Prob&gt; F = &lt;0.0001</i>			<i>R<sup>2</sup> = 0.65, Adj.R<sup>2</sup> = 0.52, F = 5.2, Prob&gt; F = &lt;0.007</i>		
<i>Error: Sum of squares=235.9, Mean square= 16.85</i>			<i>Error: Sum of squares= 58015.6, Mean square= 4144</i>		
PL-VPW					
VS removal (%)			Y <sub>PL</sub> (ml CH <sub>4</sub> g VS added <sup>-1</sup> )		
	Coefficient	Prob		Coefficient	Prob
$\beta_0$	52.6	< 0.001	$\beta_0$	426.3	< 0.001
$\beta_1$	-15.6	< 0.001	$\beta_1$	-11.4	0.639
$\beta_2$	-3.4	< 0.001	$\beta_2$	-58.6	0.028
$\beta_{11}$	-5.8	< 0.001	$\beta_{11}$	-77.4	0.015
$\beta_{22}$	1.2	0.117	$\beta_{22}$	-132.6	< 0.001
$\beta_{12}$	-2.0	0.032	$\beta_{12}$	-121.3	0.002
<i>R<sup>2</sup> = 0.97, Adj.R<sup>2</sup> = 0.97, F = 132.7, Prob&gt; F = &lt;0.0001</i>			<i>R<sup>2</sup> = 0.73, Adj.R<sup>2</sup> = 0.62, F = 7.1, Prob&gt; F = &lt;0.0016</i>		
<i>Error: Sum of squares= 96.47, Mean square= 6.89</i>			<i>Error: Sum of squares=137003, Mean square= 9786</i>		

R<sup>2</sup>: Correlation coefficient, Adj. R<sup>2</sup>: Adjusted correlation coefficient, r: Regression coefficient, F value: Value resulted from the F-test.

Similarly to the results obtained for the response of VS removal, treatments with constant value of SC (T5, T6 and T9) exhibited an increase of  $Y_{SM}$  with increment in Veg. The improvement in methane yield seems to be related with the high biodegradability of VPW added as co-substrate. The large biodegradability of this substrate was proven when digesting 100% of VPW and resulted in the highest methane yield (T5). A similar behaviour was observed for systems T1 and T2 presenting both the same value of factor SC with T1 system being evaluated at a higher level of factor Veg. As stated in previous works, addition of vegetables as co-substrate in livestock anaerobic digestion increased biogas production (Callaghan et al., 1999; Álvarez and Liden, 2008). Based on experimental results obtained from the two responses analyzed it is observed that, although data are not adequately adjusted to the  $Y_{SM}$  model, in general an increase in both responses was observed whenever the two factors are evaluated at their maximum levels. In this sense, when considering implications related to plant scale implementation maximum levels of substrate concentration and content of VPW may be selected (within experimental region evaluated).

On the other hand, treatments with a constant value of Veg. (T7, T8 and T9) evaluated at different levels of SC factor did not follow the same tendency (Table 3.2). The highest  $Y_{SM}$  (286 mL  $\text{CH}_4$  g  $\text{VS}_{\text{added}}^{-1}$ ) corresponded to T9 (with a SC of 36.3 g  $\text{L}^{-1}$ ) while T8 with a higher SC (70 g  $\text{L}^{-1}$ ) reached 257 mL  $\text{CH}_4$  g  $\text{VS}_{\text{added}}^{-1}$ . This decrease in  $Y_{SM}$  may be rationalized by an inadequate substrate/ biomass ratio causing organic overloading which hindered methanogenic activity.

Likewise the reason for the data not to fit the model was the TVFA accumulation registered for some treatments. T3 and T5 presented an organic overloading that resulted in TVFA accumulation as shown in Fig. 3.2A. Nevertheless when TVFA were steady consumed, methane production started. Similar behaviour was reported by González-Fernández and García-Encina (2009) when digesting swine manure. This research explained that increasing substrate-microorganisms ratio resulted in high acetic acid formation and methanogenic bacteria partial inhibition. Additionally, in the case of T3, partial ammonia inhibition could also have hinder methane production at the initial stage (Table 3.4). Treatments 3, 4, 6 and 8

reached free ammonia concentrations far above the inhibition threshold of 150 mg N-NH<sub>3</sub> L<sup>-1</sup> (Angelidaki and Ahring, 1993). The lower yield obtained from T6 may be explained by the lack of vegetable waste rather than ammonia inhibition.

### 3.3.2. Poultry litter co-digestion

Experimental responses for poultry litter co-digestion are presented in Table 3.2. Response surface graphs for PL and VPW codigestion are presented in Fig. 3.3. Regression analyses resulted in second order polynomial Eqs. (3.6 and 3.7).

$$VS\ Removal = 52.7 - 15.6*SC - 3.4*Veg - 5.8*SC^2 + 1.2*Veg^2 - 2.0*SC*Veg \quad (3.6)$$

$$Y_{PL} = 426.3 - 11.4*SC - 57.6*Veg - 77.4*SC^2 - 132.6*Veg^2 - 121.3*SC*Veg \quad (3.7)$$

Regarding VS removal during the digestion process the value of the R<sup>2</sup> coefficient obtained 0.98 for Eq. (3.6) indicated that the majority of data obtained are explained by the model. Both the evaluated factors, the interaction factor and the quadratic factor for SC presented a significant effect over the response. The calculated eigenvalues for the surface ( $\lambda_1 = -5.94$  and  $\lambda_2 = 1.34$ ) showed that the surface evaluated contained a saddle point.

VS removals in the range of 50-60% were registered for all of treatments with a SC up to 80 g VS L<sup>-1</sup>. When SC value was above that threshold, a decrease on VS removal was observed with increasing SC. More specifically, when comparing treatments with constant Veg. (T7, T8 and T9 of Table 3.2) it was found that an increase in SC from 80 g VS L<sup>-1</sup> (T9) to 150 g VS L<sup>-1</sup> (T8) implied a VS removal decreased drastically from 53% to 19%. Similar pattern was obtained for pairs T1–T3 and T2–T4. At the same level of Veg., the increase of SC resulted in a sharp diminishment of VS removal. This behaviour could be related with an ammonia-mediated inhibition, as discussed below.

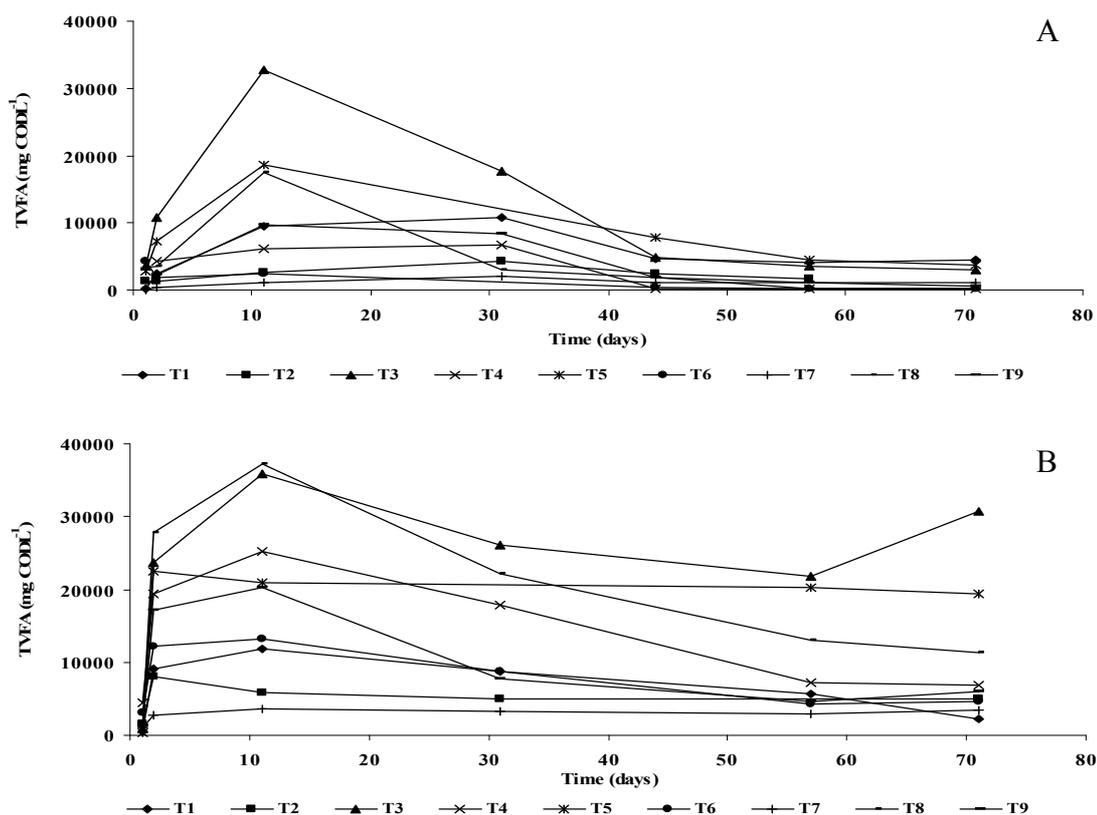
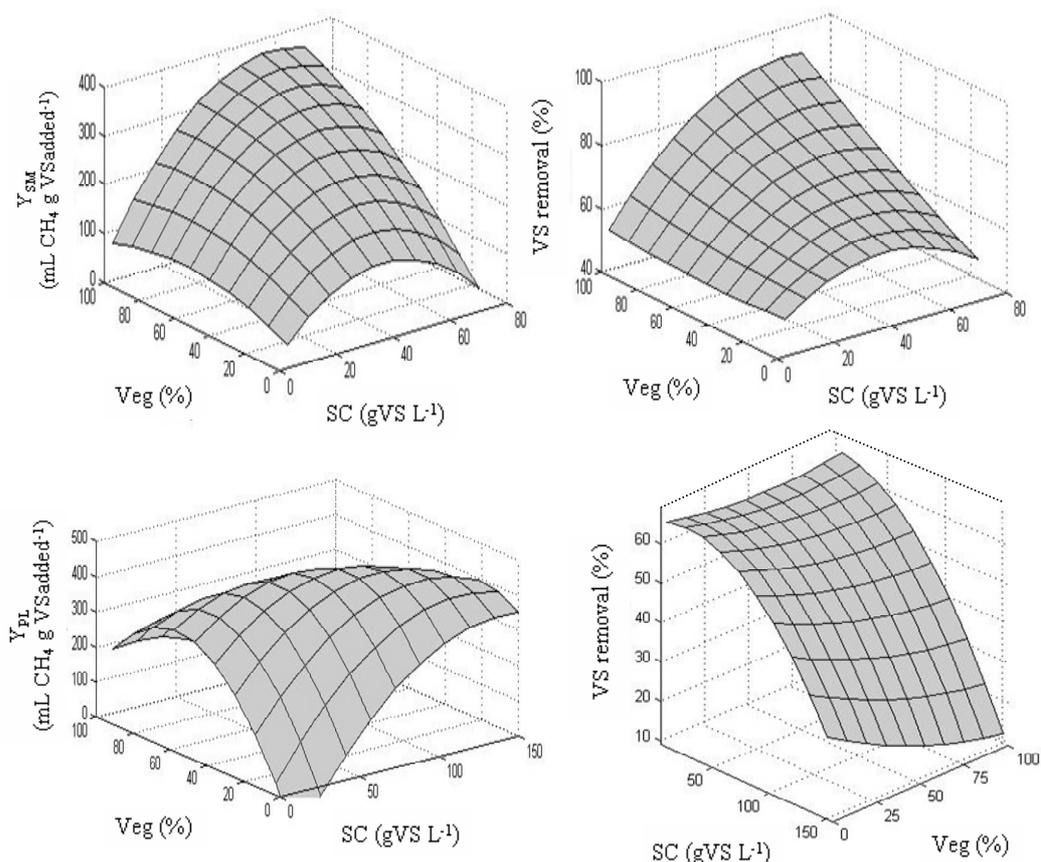


Figure 3.2. VFA concentrations during the time for: A) SM-VPW, B) PL-VPW.

Regarding treatments with constant SC (T5, T6 and T9), VS removals were in the range of 50-60% for all those treatments. Likewise, pair of treatments with the same SC (T1-T2 and T3-T4) but varying Veg. exhibited similar VS removal. Therefore, SC factor mainly determined VS removal and Veg. factor did not show a strong effect over the response (Table 3.2).

With regard to the second response evaluated, Eq. (3.7) was obtained. As shown by the determined  $R^2$  coefficient, the model explained 73% of the variability data (Table 3.3).  $P$ -values for the entire model terms were lower than 0.05, except for the linear term associated with Veg.  $\lambda_1 = -171.60$  and  $\lambda_2 = -38.34$  were the eigenvalues calculated and indicated the

presence of a maximum point. In this manner, the optimal value calculated from the model for factors SC and Veg. was 56.7 g VS L<sup>-1</sup> and 50%, respectively (Fig. 3.3). From the set of points evaluated, T9 presented the closest values to optimal value calculated mathematically, which indeed produced the highest Y<sub>PL</sub> (426 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>, Table 3.2).



**Figure 3.3.** Response surfaces plots for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

As it can be seen in Fig. 3.3, response surface plot for Y<sub>PL</sub> showed a concomitant increase of Y<sub>PL</sub> with increasing SC. However when a SC threshold was reached (approximately at 80 g VS L<sup>-1</sup>), Y<sub>PL</sub> slightly decreased. Regarding treatments with a constant value of Veg. (T7, T8

and T9) and varying SC, the highest  $Y_{PL}$  was achieved with a SC of 80 g VS L<sup>-1</sup>. An increase in SC (T8) resulted in a decrease in  $Y_{PL}$  (from 426 to 329 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>). Previous study (Webb and Hawkes, 1985) demonstrated that when digesting continuously PL alone, TS concentration above 60 g TS L<sup>-1</sup> resulted in a decrease in methane yields due to the high ammonium levels reached. In the case we studied, the threshold was higher (80 g VS L<sup>-1</sup>), probably due to the positive effect of VPW addition. On the other hand, treatments with a constant value of SC (T5, T6 and T9) and varying Veg. showed the same trend as treatments with a constant value of Veg. and varying SC. T5 with the highest Veg. presented the lowest  $Y_{PL}$ . The high Veg. in T5 resulted in a high content of TVFA in the reactor (Fig. 3.2B) resulting in a pH reduction of the system. Similar tendency was also found for T3 (100% Veg.). The buffer capacity characteristic of livestock wastes was not enough for avoiding acidification in T5 and T3, resulting in an inhibition of the digestion process (Álvarez and Lidén, 2008).

**Table 3.4.** Liquid matrix analyses for swine manure co-digestion (SM-VPW) and for poultry litter co-digestion (PL-VPW).

SM-VPW					
Treatments	Initial	Final	Initial		Final
	pH	pH	[N-NH <sub>4</sub> <sup>+</sup> + N-NH <sub>3</sub> ] (mg L <sup>-1</sup> )	[N-NH <sub>4</sub> <sup>+</sup> + N-NH <sub>3</sub> ] (mg L <sup>-1</sup> )	Free [N-NH <sub>3</sub> ] (mg L <sup>-1</sup> )
T1	7.6	8.4	326	658	146
T2	7.5	8.3	513	646	129
T3	7.5	8.3	590	1120	215
T4	7.8	8.2	1659	1960	299
T5	7.7	8.3	339	722	126
T6	7.9	8.2	1420	1470	224
T7	7.9	8.4	269	391	90
T8	7.7	8.2	1060	1570	249
T9	7.6	8.0	796	952	110

PL-VPW					
Treatments	Initial	Final	Initial		Final
	pH	pH	[N-NH <sub>4</sub> <sup>+</sup> + N-NH <sub>3</sub> ] (mg L <sup>-1</sup> )	[N-NH <sub>4</sub> <sup>+</sup> + N-NH <sub>3</sub> ] (mg L <sup>-1</sup> )	Free [N-NH <sub>3</sub> ] (mg L <sup>-1</sup> )
T1	6.6	8.5	577	870	232
T2	6.7	8.5	708	1070	283
T3	6.1	6.1	670	2270	3
T4	6.3	8.4	925	3240	687
T5	5.9	6.0	351	1110	1
T6	6.7	8.5	1025	2150	540
T7	6.9	8.5	358	580	156
T8	6.1	8.2	900	3420	574
T9	6.3	8.2	840	1950	327

Cumulative methane values for all of the treatments are shown in Fig. 3.1B, a lag phase of around 4 days was detected in all the treatments. Such lag phase was probably caused by the low pH registered at the initial stage of the assay. After such a lag phase, pH increased reaching levels which permitted methanogenic bacteria growth.

Except for T3 and T5,  $\text{N-NH}_4^+$  concentration increased during anaerobic digestion process in all the treatments (Table 3.4). Free ammonia concentration calculated for T4, T6 and T8 treatments accounted for 687, 540 and 574 mg  $\text{N-NH}_3 \text{ L}^{-1}$ , respectively. Those values were above inhibitory threshold levels. Therefore, this may be the main reason for partial inhibition of the process which in turn caused low methane production in those treatments (Fig. 3.1B).

### **3.4. CONCLUSIONS**

The co-digestion process of livestock and vegetable processing wastes was studied by a factorial design of experiments. Factors such as substrate concentration and vegetable content were strongly influencing methane yield and volatile solids removal. The addition of vegetable as co-substrate in swine manure digestion resulted in an increase in volatile solids removal. However, if the substrate/biomass ratio was not adequate, buffer capacity of swine manure was not able to avoid inhibitory effects associated with TVFA accumulation. Regarding co-digestion with poultry litter it was found that substrate concentration determined VS removal, above 80 g VS  $\text{L}^{-1}$ ,  $\text{N-NH}_3$  inhibition was detected. Methane yield was strongly affected by both factors obtaining a threshold value where TVFA or  $\text{N-NH}_3$ -mediated inhibitions were overcome.

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## *Chapter 4*

### **Anaerobic co-digestion of livestock and vegetable processing wastes: Fibre degradation and digestates stability**

**Molinuevo-Salces, B., Gómez, X., Morán, A., García-González, M. C.**



**Abstract.** Anaerobic digestion of livestock wastes (Swine manure (SM) and poultry litter (PL)) and vegetable processing wastes (VPW) mixtures was evaluated in terms of methane yield, volatile solids removal and lignocellulosic material degradation. Batch experiments were performed at 2% VS (volatile solids) ensuring TVFA (total volatile fatty acids) complete conversion and avoiding ammonia inhibition. VPW addition to livestock wastes anaerobic digestion resulted in improved methane yields and VS reductions. In the case of SM-VPW co-digestion, an increase from 111 to 244 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup> and from 50 to 86 %VS removed were achieved. Regarding PL-VPW co-digestion an increase from 158 to 223 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup> and from 70 to 92 %VS removed were obtained. Hemicelluloses and more than 50% of cellulose were degraded during anaerobic digestion. By means of thermal analyses it was demonstrated the stabilization of the wastes during anaerobic digestion process.

**Resumen.** En este estudio se evaluó la digestión anaerobia de residuos ganaderos (purín de cerdo (SM) y gallinaza (PL)) con residuos del procesado de vegetales (VPW). Además de estudiar la eficiencia del proceso en términos de eliminación de sólidos volátiles (SV) y producción metanogénica se evaluó la degradación del material lignocelulósico y el grado de estabilización de los digestatos durante el proceso. Los experimentos fueron llevados a cabo en discontinuo con un porcentaje inicial de SV del 2%, asegurando así la completa conversión de ácidos grasos volátiles y evitando inhibiciones por amonio. La adición de vegetal a la digestión anaerobia de residuos ganaderos resultó en una mejora en términos de producción de metano y reducción de SV. De la digestión de purín y vegetal (SM-VPW) se obtuvo un incremento de 111 a 244 mL CH<sub>4</sub> g SV<sub>añadidos</sub><sup>-1</sup> y un incremento de 50 a 86% SV eliminados. En el caso de la co-digestión de vegetal y gallinaza (PL-VPW), los incrementos fueron de 158 a 223 mL CH<sub>4</sub> g SV<sub>añadidos</sub><sup>-1</sup> y de 70 a 92% SV eliminados. Durante el proceso anaerobio se alcanzó una degradación del 100% de las hemicelulosas y más del 50% de la

celulosa. El análisis térmico nos permitió comprobar la estabilización del digestato conseguida durante la digestión anaerobia.

## **4.1. INTRODUCTION**

One of the common problems associated to the treatment of wastes by anaerobic digestion is related to inhibitions of the process caused by intermediaries or accumulation of products. As it is usually reported when digesting manures, nutrient imbalances may cause high N-NH<sub>3</sub> concentrations during anaerobic digestion of these wastes leading to process failures (Angelidaki and Ahring, 1993). The optimum carbon-nitrogen (C/N) ratio for anaerobic digestion is in the range of 20-25 (Yen and Brune, 2007). Livestock wastes (swine manure (SM) and poultry litter (PL)) present high nitrogen content resulting in low C/N ratios. On the contrary, Vegetable processing waste (VPW) presents high C/N ratios. The high organic matter content presented in VPW is rapidly hydrolyzed to total volatile fatty acids (TVFA), which may limit anaerobic degradation. The large production of TVFA during acidogenesis stage may lead to an acidification which could inhibit methanogenic activity of microorganisms (Bouallagui et al., 2005; Molinuevo et al., 2008). Co-digestion of livestock wastes with VPW has been studied leading to more balanced C/N ratios (Callaghan et al., 2002; Habiba et al., 2009). Livestock residues provide the nitrogen necessary for cell growth and carbon degradation (Fricke et al., 2007). Moreover, their high buffer capacity (González-Fernández et al., 2008) avoids pH drops. VPW supplies organic matter balancing C/N ratio and thus avoiding toxic N-NH<sub>3</sub> concentrations, improving wastes biodegradability and resulting in more stable digestion process.

The biodegradability of wastes depends among others on lignocellulosic complex structure. Lignocellulosic materials are composed by hemicelluloses, cellulose and lignin. Hemicelluloses and cellulose are complex polysaccharides presented in cell walls. Hemicelluloses are easily hydrolysable due to their amorphous structure, which is more vulnerable to enzymatic attack than cellulose or lignin structures (Ghosh and Henry, 1985). Cellulose has a simple structure and therefore few enzymes are necessary to digest it. Cellulose solubilization is dependent of inoculum source, biomass concentration and cellulose availability (Jensen et al., 2009). Anaerobic digestion of cellulose may be hindered

by cell wall components as lignin. Lignin molecules reduce cellulose bioavailability by reducing the surface area available to enzymatic penetration and activity (Haug, 1993). Lignin is a recalcitrant compound and its degradation results in a limit step (Robbins et al., 1979, Pavlostathis and Giraldo-Gomez, 1991). In addition, lignin degradation products such as phenolic acids or aldehydes have been reported as toxic substances for methanogenic microorganisms (Chen et al., 2008).

Stabilisation of waste by a proper anaerobic digestion process results in an odour-free product with reduced putrefaction potential. Thermal analysis (TA) has been demonstrated as a useful tool in order to evaluate stability of biological products, as anaerobic digestates (Gómez et al., 2007) or compost (Dell'Abate et al., 1998). Thermal analysis studies changes in the properties of materials with temperature. By means of these techniques it is possible to determine the combustible organic matter and therefore the possible energy potential of the evaluated substrate. Several studies have been carried out with regard to fibre degradation during anaerobic digestion of vegetal substrates (Tong et al., 1990; Chanakya et al., 2008). However there is a lack of information regarding fibre degradation during anaerobic digestion of livestock wastes, which indeed often contain recalcitrant organic fibre (lignin) including variable quantities of straw bedding material.

The objective of this study was to evaluate lignocellulosic complex degradation during the anaerobic co-digestion of different mixtures of livestock and vegetable wastes. As it is known that those substrates are not easily anaerobically degraded, stability of digestates was studied in order to examine process efficiencies. For this proposal, different batch experiments were carried out at an initial volatile solids concentration of 2%.

## **4.2. METHODS**

### **4.2.1. Substrates and sludge characteristics**

Three different substrates were used: SM, PL and VPW. SM was obtained from a pig farm located in Avila (Spain), with 2600 finishing pigs with an annual manure production of

8750 m<sup>3</sup>. PL was collected from a poultry farm located in Palencia (Spain) with 86000 laying hens and an annual manure production of 5850 m<sup>3</sup>. VPW were collected in a vegetable processing factory located in Segovia (Spain) and they were composed by green peas, maize, carrots and leeks. They were ground to particles of about 1 mm in size in a mill fruit. The anaerobic sludge (AS) used as inoculum was taken from an anaerobic digester in the municipal wastewater treatment plant (WWTP) of Valladolid, Spain. All of the substrates were homogenized previously to use and stored at 4°C. Table 4.1 presents chemical composition of substrates and inoculum.

**Table 4.1.** Chemical characterization of substrates and anaerobic sludge.

Parameters	VPW	SM	PL	AS
pH	4.4	7.7	9.2	7.4
VS (g L <sup>-1</sup> )	134.7 (1.6)	17.1 (0.0)	201.3 (0.8)	5.6 (0.1)
TS (g L <sup>-1</sup> )	143.6 (1.9)	25.2 (0.2)	305.2 (13.5)	12.3 (0.1)
CODs (g L <sup>-1</sup> )	70.9 (1.7)	5.1 (0.1)	12.4 (0.9)	11.7 (3.5)
CODt (g L <sup>-1</sup> )	224.1 (49.4)	29.8 (0.3)	n.d.	17.5 (0.6)
TKN (g L <sup>-1</sup> )	3.5 (0.1)	4.1 (0.1)	13.8 (1.4)	1.0 (0.0)
N-NH <sub>4</sub> <sup>+</sup> (g L <sup>-1</sup> )	0.9 (0.2)	2.8 (0.0)	12.8 (0.7)	0.6 (0.0)
C <sup>a</sup> :N	38	4	15	6
Hemicellulose (%TS)	7.8	2	4.4	n.d.
Cellulose (%TS)	17.4	22	19	n.d.
Lignin (%TS)	4.4	9.8	4.2	n.d.

<sup>a</sup> Corrected values taking into account TVFA lost (Vedrenne et al., 2008). n.d.: not determined

#### 4.2.2. Experimental set-up

Batch assays were prepared with 2% initial volatile solid (VS) concentration of substrate. An initial concentration of 2% VS was selected in order to avoid total volatile fatty acids (TVFA) and N-NH<sub>3</sub>-mediated inhibitions since the main objective of this research was to evaluate fibre degradation. The VS concentration was chosen according to Molinuevo-

Salces et al. (2010), who studied the effect of initial substrate concentration (VS) over methane yield and VS reduction co-digesting VPW-SM and VPW-PL. Different livestock and VPW wastes mixtures were prepared, denoted as C1 to C9, in order to conduct the assays at different proportions of wastes. These mixtures are shown in Table 4.2: C1-C4 stand for SM-VPW co-digestion tests, C6-C9 stand for PL-VPW co-digestion tests and C5 stand for VPW digestion.

**Table 4.2.** Proportion of the different mixtures (C1-C9) used in batch digestion tests. % expressed in dry weight (DW).

	%VPW(DW)	%SM(DW)	%PL(DW)
<b>C1</b>	0	100	0
<b>C2</b>	25	75	0
<b>C3</b>	50	50	0
<b>C4</b>	75	25	0
<b>C5</b>	100	0	0
<b>C6</b>	0	0	100
<b>C7</b>	25	0	75
<b>C8</b>	50	0	50
<b>C9</b>	75	0	25

The anaerobic assays were conducted in 500 mL bottles and filled with 200 mL of the liquid mixtures (100 mL of inoculum and 100 mL of the correspondent substrate). Blanks containing 100 mL of inoculum and 100 mL of distilled water were also run to determine the endogenous methane production of the anaerobic sludge. A solution with a concentration of 14 g  $\text{KHCO}_3 \cdot \text{L}^{-1}$  was used to adjust the pH (to 7.5) of each bottle at the beginning of the experiment (Cirne et al., 2007). The bottles were closed with a septum and flushed with  $\text{N}_2$  in order to remove the oxygen. Finally, they were incubated in a thermostatic shaker at 100 rpm and  $35 \pm 2$  °C for up to 80 days. Production of biogas was tracked by measuring the overpressure in the bottles headspace with a time frequency depending on biogas production (Colleran et al., 1992).

### **4.2.3. Analyses**

Analyses consisted of total solids (TS), VS, total and soluble chemical oxygen demand (COD<sub>t</sub> and COD<sub>s</sub>), total Kjeldahl nitrogen (TKN) and ammonium nitrogen (N-NH<sub>4</sub><sup>+</sup>). All the analyses were done according with Standard Methods (APHA, 2005). Free ammonia concentrations at the end of the different tests were calculated according to Hansen et al. (1998). Samples were analysed for lignin, acid and neutral detergent fibres (ADL, ADF, and NDF) by the method of Van Soest and Wine (1967) using a fibre analyzer (ANKOM 2000I). Cellulose and hemicelluloses content were determined by subtraction, (ADF-ADL) and (NDF-ADF), respectively. Total carbon was calculated by the addition of VS and TVFA values at the beginning of the assay. These calculations were done taking into account the TVFA volatilization during drying for VS determination (Vedrenne et al., 2008).

Biogas composition was analysed using a gas chromatograph (Varian CP 3800 GC) with a thermal conductivity detector, provided by a column CP-Molvsieve5A (15m × 0.53mm × 15µm) followed by a column CP-Porabond Q (25m × 0.53mm × 10µm). Hydrogen (13.6 mL min<sup>-1</sup>) was used as the carrier gas. The injection port temperature was set at 150 °C and the detector temperature was 175 °C. Total volatile fatty acids (TVFA) were analysed using a gas chromatograph (Varian CP 3800 GC) equipped with a Nukol capillary column (30m × 0.25mm × 0.25µm) and a flame ionization detector. The carrier gas was helium and the temperature of the injector was 250 °C. The temperature of the oven was set at 150 °C for 3 min and thereafter increased to 180 °C.

Fresh and digested dried samples for C3, C5 and C8 were taken in order to examine fibre degradation by means of scanning electronic microscopy (SEM). Dried samples were sputter-coated with gold in high vacuum (0.05-0.07 mbar) with a coater Blazers SCD 004. The samples were examined using a JOEL JSM-5600, scanning electron microscope (SEM JEOL JSM 6840 LV).

Thermal analysis (TA) was performed with a TA Instruments SDT2960 apparatus registering thermogravimetric (TG), differential thermogravimetric (DTG) and differential scanning calorimetry (DSC) measurements simultaneously. The heating rate applied to the dry samples was 10 °C min<sup>-1</sup> up to 700 °C with a flow-rate of 100 mL min<sup>-1</sup> of synthetic air (composition 21±1% O<sub>2</sub> and 79±1% N<sub>2</sub>; purity ≥99.9994%). The manometric pressure was maintained at 101 kPa. Lignin organic solvent and humic acid sodium salt standard from Sigma Aldrich were analyzed by TA. A mass spectrometry apparatus was used in line with the thermal analysis equipment to monitor the gas emissions obtained from the combustion process, connected through a capillary filament maintained at 200 °C. The mass spectrometry apparatus was a Quadrupole MS (Balzers), Thermostar GSD 300 T (Pfeiffer Vacuum, D-35614 Asslar) equipped with an electron ionization source, a Faraday cup and an SEM (channeltron<sup>TM</sup>) detector. The mass range was 0-200 amu.

### **4.3. RESULTS AND DISCUSSION**

The mixture of SM-VPW for co-digestion allowed the increase of C/N ratios to the values of 12, 20 and 29 for the tests at 25, 50 and 75% of VPW (C2, C3 and C4, respectively) when comparing with SM alone. Under similar conditions, the preparation of mixtures for PL-VPW co-digestion resulted in an increase of C/N ratios to 17, 20 and 25, in this case comparing with PL (C7, C8 and C9, respectively). Despite the addition of VPW to livestock residues resulted in an increase of C/N ratios, only those mixtures with a proportion of VPW at 50 and 75% reached optimal ratios for anaerobic digestion (Yen and Brune, 2007).

Lignocellulosic content in the substrates was in a range of 30% TS. With regard to livestock composition, hemicelluloses represented a lower fraction than in VPW. Lignin represented 10% TS in the case of SM being this value higher of that obtained for VPW (Table 4.1). VPW addition to livestock wastes resulted in mixtures with similar content of total fibre but higher values of hemicelluloses and lower values of lignin. As hemicelluloses have been proved as easily biodegradable compounds (Ghosh and Henry, 1985), VPW addition was

expected to increase the biodegradability of the mixed substrates and therefore increase the methane potential.

### **4.3.1. Anaerobic digestion process: Methane production and VS removal**

Both mixtures of wastes (SM and PL) followed the same tendency. As more vegetable was added, higher biogas and methane yields were achieved. In the case of SM, an addition of 50% (C3) and 75% (C4) VPW resulted in an increase of 39 and 120% when compared with the methane yield obtained for SM alone (C1). However, no significant increment was obtained for the mixture at 25%. Results presented in this study were lower when considering those obtained by Alvarez and Lidèn (2008) who reported a 52% increase, from 210 to 320 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>, when digesting VPW with SM and cattle manure in a ratio 50:50 (DW). The difference in results may be probably due to the composition of the substrates. It has been reported that the ultimate methane yield in manure is affected by factors as species, breed and growth stage of the animals, feed, amount and type of bedding material and degradation processes during pre-storage (Möller et al., 2004) as well as water content. SM used in the present study presented high non-biodegradable content (cellulose and lignin), which resulted in low methane production. Furthermore, VPW (green peas, carrots, maize and leeks) may contain higher fibre amount than those utilised by Alvarez and Lidèn (2008), who worked with different fruits.

Similar results were obtained for PL digestion systems. In this case addition of 50% (C8) and 75% (C9) VPW improved methane yield in 13% and 41%, respectively. Thus, methane yields were increased when digesting with VPW, while no significant benefits were observed for those treatments with C/N ration below optimum conditions. Webb and Hawkes (1985) reported methane yields for continuous PL anaerobic digestion in a range of 210-240 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>. In the case presented here, methane yields for PL alone were slightly lower. Diluting the substrate to 2% initial VS allowed the total consumption at the

end of the experiment of TVFA generated for all conditions tested. Additionally, N-NH<sub>3</sub> concentrations were close to the low limit of inhibition range (Table 4.3); therefore TVFA and ammonia-mediated inhibitions can be discarded. In this sense, the low methane yields obtained in the present study may keep relation with the lignocellulosic concentration of substrates.

**Table 4.3.** Biogas and methane yields, methane content, VS Removal, N-NH<sub>4</sub><sup>+</sup> concentrations, N-NH<sub>3</sub> concentrations and pH for C1 to C9. i and f stands for initial and final data, respectively.

	ml Biogas g VS <sub>added</sub> <sup>-1</sup>	%CH <sub>4</sub>	ml CH <sub>4</sub> g VS <sub>added</sub> <sup>-1</sup>	VS Removal(%)	
<b>C1</b>	143 (13)	66 (6)	111 (13)	50.1	
<b>C2</b>	158 (28)	65 (8)	117 (28)	62.5	
<b>C3</b>	230 (13)	66 (4)	154 (13)	81.4	
<b>C4</b>	311 (58)	59 (22)	244 (58)	85.5	
<b>C5</b>	232 (36)	55 (31)	181 (36)	97.6	
<b>C6</b>	203 (1)	51 (16)	158 (1)	70.3	
<b>C7</b>	216 (66)	61 (6)	168 (66)	67.2	
<b>C8</b>	230 (59)	65 (10)	179 (59)	78.1	
<b>C9</b>	287 (14)	64 (18)	223 (14)	91.7	

	N-NH <sub>4</sub> <sup>+</sup> i(mg/L)	N-NH <sub>4</sub> <sup>+</sup> f(mg/L)	N-NH <sub>3</sub> f(mg/L)	pHi	pHf
<b>C1</b>	1850 (71)	1913 (262)	315 (43)	7.8	8.2
<b>C2</b>	1450 (71)	1556 (199)	293 (38)	7.7	8.3
<b>C3</b>	980 (42)	1380 (163)	255 (30)	7.7	8.3
<b>C4</b>	655 (30)	923 (21)	184 (4)	7.5	8.3
<b>C5</b>	312 (7)	788 (166)	154 (32)	7.7	8.3
<b>C6</b>	1065 (49)	1531 (111)	216 (16)	7.6	8.1
<b>C7</b>	825 (73)	1255 (35)	180 (5)	7.6	8.2
<b>C8</b>	678 (6)	965 (103)	183 (19)	7.8	8.3
<b>C9</b>	410 (24)	884 (9)	162 (2)	7.5	8.3

Values obtained for VS removal were in the range 50-85% and 70-90% for SM and PL, respectively (Table 4.3). VS removal was higher when more VPW was added to the mixture being in accordance with the statement that the addition of VPW increase biodegradability, thus hydrolysis and subsequently the conversion into methane was favoured. The lowest value of VS removal obtained from SM batch test (C1) may be rationalized by the fact the

SM sample taken from the storage tank may have been partially degraded, therefore the organic matter presented in this fresh sample was probably characterized by low biodegradability. On the other hand, treatment C5 (100% VPW) presented a 97.6% VS reduction but low methane yields were achieved ( $181 \text{ mL CH}_4 \text{ g VS}_{\text{added}}^{-1}$ ). Chanakya et al. (2008) reported a similar behaviour when digesting orange peel or cabbage. They obtained VS removal in a range of 75-95% but low methane yields concluding that overload by TVFA reduced methanogenic bacteria activity. In the case presented here, two factors, namely TVFA accumulation and low nitrogen concentration, could have hindered methanogenic bacteria activity. High TVFA production ( $10000 \text{ mg COD L}^{-1}$ ) was observed when digesting VPW alone (Fig. 4.1A, 4.1B). As the system had been previously buffered and diluted to 2% initial VS, it was able to overcome such TVFA inhibition producing methane after a lag period of 20 days (Fig. 4.1C, 4.1D). On the other hand, nitrogen concentration in the range of  $50\text{-}200 \text{ mg L}^{-1}$  has been reported as necessary for anaerobic bacteria growth (McCarty, 1964). Since nitrogen concentration in C5 was in the range of  $300 \text{ mg L}^{-1}$ , this factor was also discarded. Therefore, partial inhibition of methanogenic activity due to high TVFA concentration could explain the low methane yield achieved for C5.

### **4.3.2. Fibre degradation**

NDF, ADF, ADL, cellulose (CE) and hemicelluloses (HC) concentrations for fresh (F) and digested (D) samples are presented in Table 4.4. Treatments selected for these analyses were those of digestion batch test for SM and PL at 50% VPW (C3 and C8, respectively) and samples from the system of individual digestion of VPW (C5).

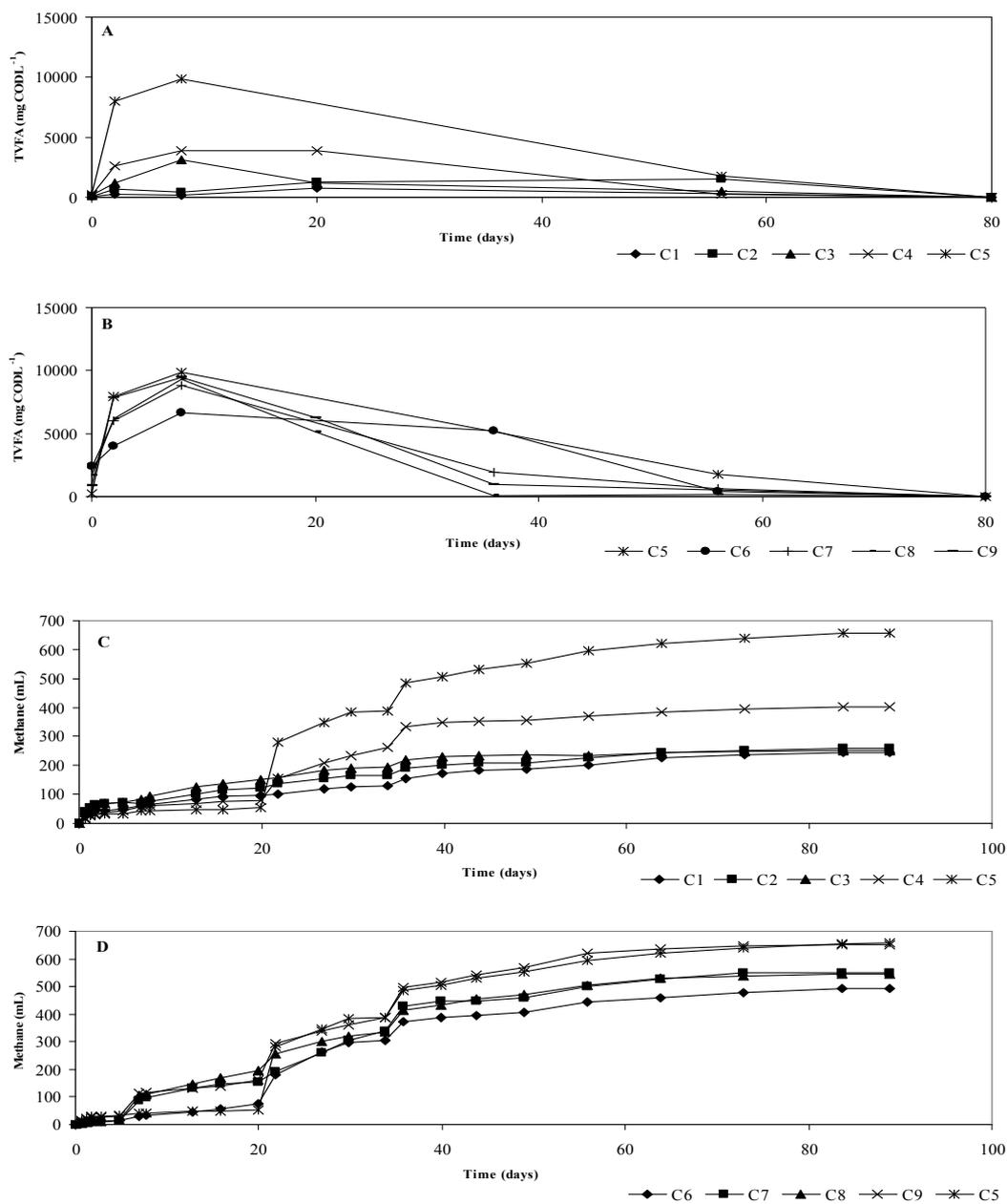
As it was expected, hemicelluloses were completely depleted since they are easily degradable (Ghosh and Henry, 1985). In the case of cellulose, it can be refractory when incorporated in a lignocellulosic complex; lignin forms a complex structure with cellulose and hemicelluloses. This structure may diminish bioavailability of cellulose and hemicellulose for enzymatic degradation (Haug, 1993), explaining that cellulose was

degraded in a range of 50-65%. Lignin content was higher for fresh sample of test C3 (50% VPW for SM co-digestion) due to the higher lignocellulosic content of pig manure. Panichnumsin et al. (2010) reported contents of 54, 108 and 83 g. kg<sup>-1</sup> VS for hemicelluloses, celluloses and lignin respectively analyzing pig manure composition. However, C3 presented the highest lignin removal (up to 80%) after anaerobic digestion whereas in C5 and C8, 50% and 35% of lignin were removed respectively (Table 4.4). It has been widely proven that lignin is recalcitrant under anaerobic digestion and mayor lignin content implies more resistance to anaerobic digestion. (Robbins et al., 1979; Pavlostathis and Grialdo-Gómez, 1991). Lignin should have not been removed since bacteria are not able to degrade it, fibre could have escaped from the bags for fibre analyses and subsequently, sub-estimated concentrations were determined after fibre analyses. Indeed, Lindberg and Knutsson (1981) studied particulate matter losses, assuming that lignin losses was a indirect determination of particulate losses from the bag, during ADF and lignin determination according with Van Soest et al. (1967).

**Table 4.4.** NDF, ADF, ADL, CE and HC measurements for initial and final stages of C3, C5 and C8.

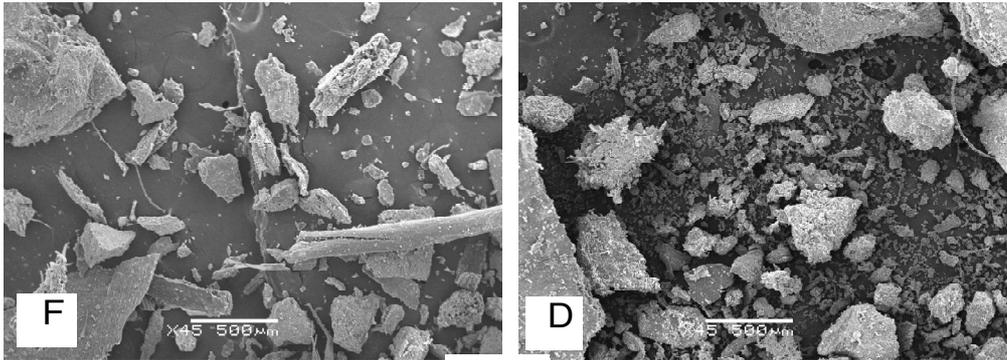
	<b>NDF (% TS)</b>	<b>ADF (%TS)</b>	<b>ADL (%TS)</b>	<b>CE (% TS)</b>	<b>HC (%TS)</b>
<b>C3F</b>	31.8 (0.2)	26.8 (0.5)	7.1 (0.2)	19.7 (0.4)	4.9 (1.3)
<b>C3D</b>	6.7 (0.6)	9.4 (0.5)	1.5 (0.0)	7.9 (0.5)	≤0.5
<b>C5F</b>	29.7 (0.2)	21.9 (2.0)	4.4(0.5)	17.4 (1.6)	7.8 (2.3)
<b>C5D</b>	10.1 (0.4)	10.7 (0.9)	1.9 (0.5)	8.8 (0.4)	≤0.5
<b>C8F</b>	28.5 (1.0)	22.4 (1.2)	4.3 (0.3)	18.2 (1.5)	6.1 (0.2)
<b>C8D</b>	9.9 (1.2)	11.4 (0.1)	2.8 (0.3)	8.6 (0.4)	≤0.5

Fig. 4.2 presents SEM pictures for fresh and digested samples of C3, C5 and C8 batch tests. By comparing fresh samples (F) with digested samples (D) it can be seen that organic matter was degraded but some particles were still present. If comparing with SEM pictures reported by Yang et al. (2009), we can conclude that those non-degraded particles could be parenchyma tissues, which are mainly composed by lignin.

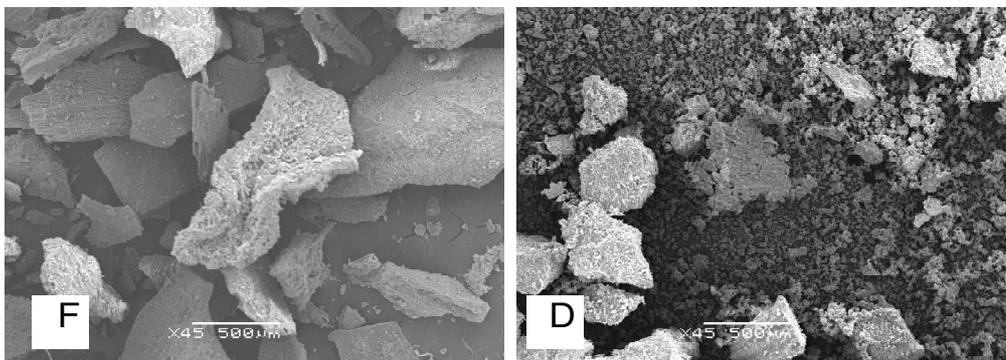


**Figure 4.1.** Results from co-digestion of batch tests: TVFA (mg COD. L<sup>-1</sup>) for SM-VPW system (A) and PL-VPW system (B). Accumulated methane production for SM-VPW system (C) and PL-VPW system (D).

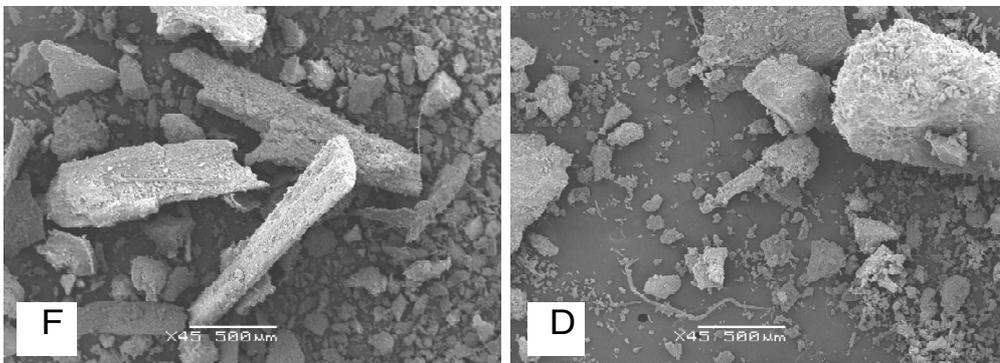
### C3



### C5



### C8



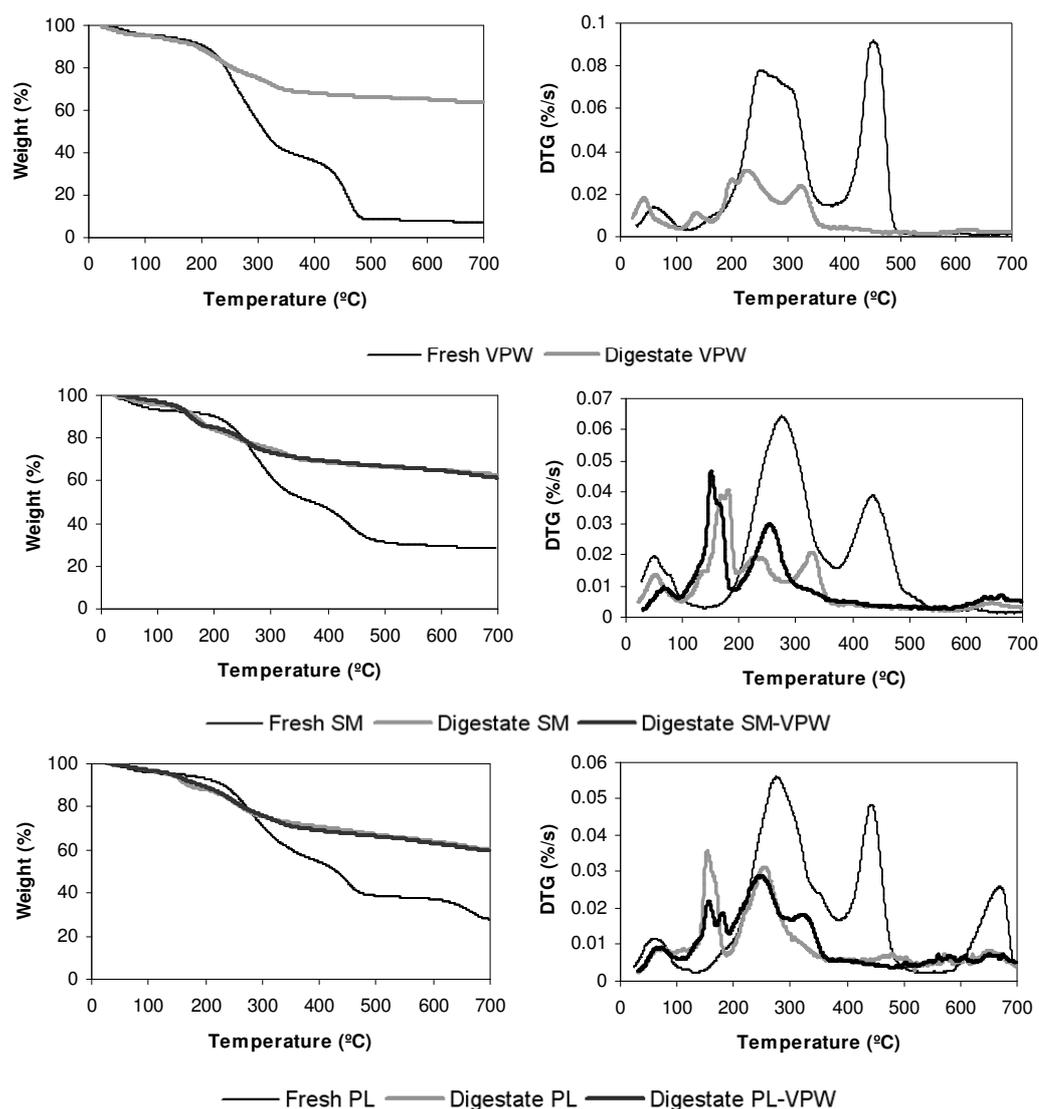
**Figure 4.2.** SEM images for fresh (F) and digested (D) samples from batch C3 ( SM-VPW), C5 (VPW) and C8(PL-VPW). All the pictures were taken with a magnification of 45x.

### **4.3.3. Evaluation of digestates stability**

In Fig. 4.3 TG and DTG profiles for fresh and digested samples are represented. TG profiles represent weight loss experienced by the sample with increasing temperature. It can be observed that a 90% loss of the weight was registered when regarding fresh VPW sample whilst close to 40% was observed for the digested VPW sample. With regard to livestock wastes, this behaviour was repeated although fresh samples registering a slightly lower weight loss at the end of the heating process when compared to fresh VPW sample. No great differences were observed from TG profiles of co-digestion samples, (SM-VPW and PL-VPW) being this not the case from DTG profiles.

DTG profiles from fresh samples were characterized by three main peaks, being the first one located at temperatures lower than 100 °C related to dehydration of the sample, the second peak was located in the range of 200-300 °C corresponded to the degradation of the easily biodegradable fraction (carbohydrates, dehydration of aliphatic structures and decarbolixation) whilst the third one was located around 400-500 °C corresponding to the aromatic structures from organic polymers and lignin type components (Dell'Abate et al., 1998; Xu et al., 2006).

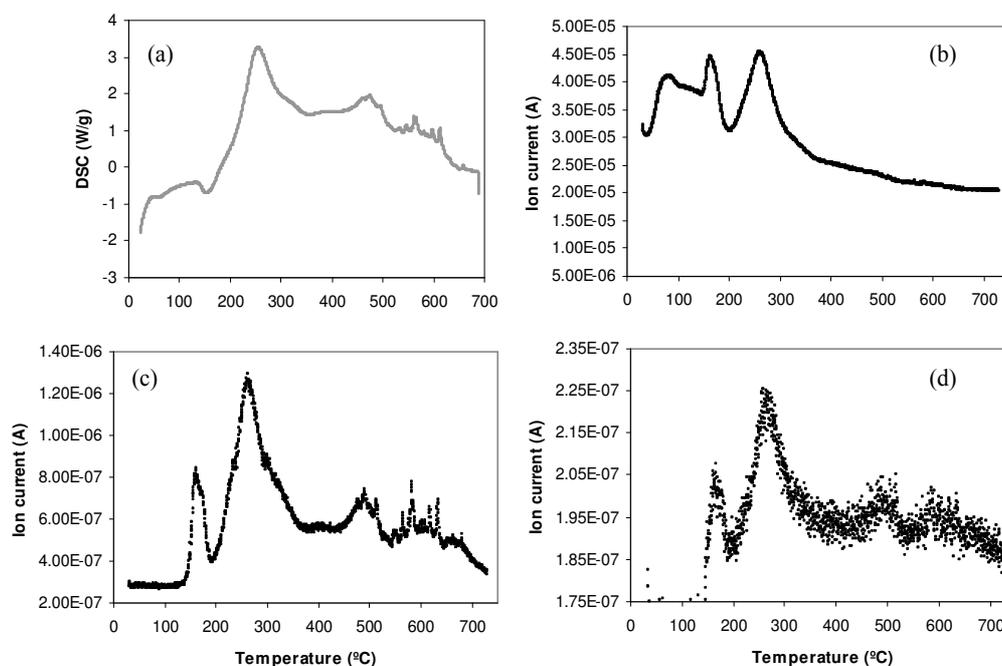
DTG profiles from digested samples presented peaks with lower intensity associated the lesser amount of material suffering oxidation. An endothermic peak at around 150 °C (similar to the DSC profile, Fig 4.4) can be observed from all digested samples, being scarcely noticeable in VPW sample but with a high intensity in those where manure was used as substrate. This peak may be the result of pyrolysis type reaction of long chain aliphatic components which experienced thermal degradation at low temperature. DSC profile and signals regarding H<sub>2</sub>O, CO<sub>2</sub> and NO<sub>2</sub> components obtained from emissions of gases evolved from TA analysis are presented in Fig. 4.4 for digested PL sample. From this figure, it is clearly observed the endothermic peak registered in digested samples which thermal degradation resulted in CO<sub>2</sub> and H<sub>2</sub>O evolution. Oxidation of nitrogen components was also registered at this low temperature.



**Figure 4.3.** TG and DTG profiles of VPW, SM and PL co-digestion systems.

Results from TA of lignin standard are presented in Fig. 4.5a and 4.5b. Lignin profile is characterized by two important mass losses starting at around 300 °C and 475 °C, corresponding to easily biodegradable and aromatic fractions of lignin, respectively. With regard to the peak related to the aromatic fraction of lignin components, the total

degradation of this type of material was observed in all digested samples (Fig. 4.3). On the other hand, humic acid sodium salt standard presented a peak around 150 °C (Fig. 4.5c, 4.5d), which could correspond to the endothermic peak above mentioned from digestate sample. The late thermal degradation observed is related to the thermal decomposition of the salt. In this sense, the hypothesis of lower methane yields due to lignin content of samples should be corrected due to the fact that the digestion was maintained by an extended period allowing degradation of complex components. Digestion for an extended period may have result in a preferential degradation of readily oxidized organic matter and enrichment in the aliphatic fraction of digestates. However, no increase in biogas yield was obtained from this late degradation process. Thus, the apparent degradation of lignin in TA could be due to an effect of lignin dilution, from the high proportion of inoculum used with regard of the mass of substrate initially added.



**Figure 4 4.** (a) DSC profile mass signals (b) H<sub>2</sub>O, (c) CO<sub>2</sub> and (d) NO<sub>2</sub> from digested PL sample.

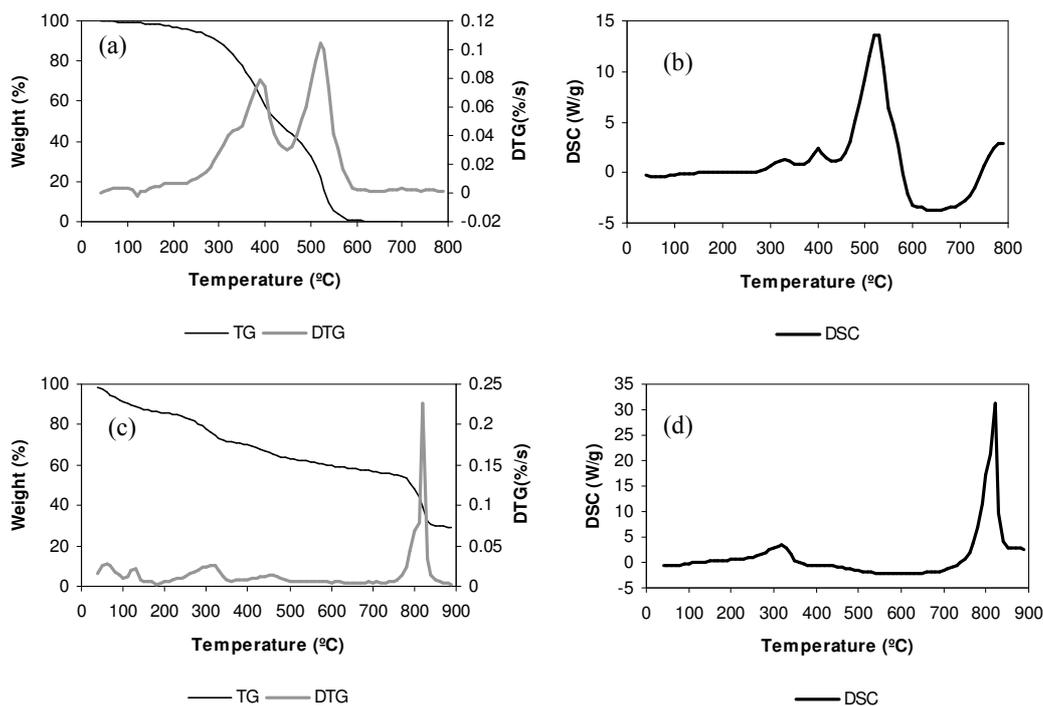


Figure 4. 5. TG, DTG and DSC profiles from (a,b) lignin and (c,d) humic acid sodium salt standards

#### 4.4. CONCLUSIONS

Vegetable processing waste (VPW) addition to livestock residues resulted in improved C/N ratios, avoiding ammonia inhibition and improving wastes biodegradability. Methane yields were increased when adding VPW but anaerobic degradation was hindered by the high amount of lignocellulosic material present in the substrates. The increase in VPW resulted in higher VS removals. After 85 days of anaerobic degradation, hemicelluloses were depleted and cellulose was removed in a range of 50%. SEM pictures helped us to conclude that lignin was not completely degraded after up to 85 days of experiment. Stability of digestates evaluated by thermogravimetric analyses showed that digested samples presented a great reduction in carbohydrate material.

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## *Chapter 5*

# **Vegetable processing wastes addition to improve swine manure anaerobic digestion: Evaluation in terms on methane yield and SEM characterization**

**Molinuevo-Salces, B., García-González, M. C., González-Fernández, C.,  
Gómez, X., Morán, A. .**



**Abstract.** The effect of adding vegetable waste as a co-substrate for the anaerobic digestion of swine manure was investigated. The study was carried out at laboratory scale using semi-continuous fed stirred tank reactors working at 37 °C. Organic loading rates (OLRs) of 0.4 and 0.6 g VS L<sup>-1</sup>d<sup>-1</sup> were evaluated, corresponding to hydraulic retention time (HRT) of 25 and 15 days respectively. 50% vegetable addition resulted in 3 and 1.4 times increased methane yields for HRTs of 25 and 15 d, respectively. Additionally, scanning electron microscopy was employed to study changes on microbial morphotypes of samples previous and posterior to the anaerobic digestion, along with the degree of sludge degradation at the end of the experimental time.

**Resumen.** Se investigó el efecto del vegetal añadido como co-substrato en la digestión anaerobia de purín de cerdo. El estudio se llevo a cabo a escala laboratorio utilizando para ello un reactor semi-continuo con agitación y temperatura (37 °C) constantes. Se evaluaron dos cargas orgánicas, 0.4 y 0.6 g SV L<sup>-1</sup>d<sup>-1</sup>, correspondiendo a tiempos de retención de 25 y 15 días, respectivamente. Además, mediante microscopía electrónica de barrido, se estudiaron los cambios en la morfología bacteriana y en el grado de degradación del fango al final del proceso anaerobio.



## **5.1. INTRODUCTION**

Swine manure (SM) treatment is receiving increasing attention in Europe due to the rising tendency to intensive and concentrated farming activities located in small areas (Flotats et al., 2009). The wastewaters produced are characterized by high organic and nutrient concentrations, which results in pollution of the environment when not properly treated. Anaerobic digestion is considered as an efficient and cost-effective treatment capable of reducing organic content of the waste while producing energy from methane valorisation. However, digestion of SM often results in low methane yields, due to its high content of fibre, water and ammonium (Angelidaki and Ahring, 1993).

Co-digestion offers several ecological, technological and economical advantages. As Holm-Nielsen et al. (2009) pointed out; co-digestion advantages involve the reduction of greenhouse gases emissions, easier management of mixed wastes, increased biogas production and improved fertiliser value of the digestate. However, it is not clear whether some by-products might have adverse effects when added to a stable digester or used in conjunction with another type of residues (Fountoulakis et al., 2008). Successful anaerobic co-digestion of swine manure has been studied with easily degradable co-substrates, as potato by-products (Kaparaju and Rintala, 2005) or cassava pulp (Panichnumsin et al., 2010).

Since over  $7.10^6$  m<sup>3</sup> of SM are produced annually in Castilla y León (MARM, 2010) and up to 233.000 t of vegetable processing wastes (VPW) are annually produced in this same area, the co-digestion of these wastes is presented as a suitable option. SM contributes providing nutrients necessary for microorganisms growth, as well as buffer capacity overcoming potential low pHs caused by total volatile fatty acids (TVFA) accumulation of other substrates. As co-substrate, VPW enhances carbon/nitrogen (C/N) ratio thus avoiding possible ammonia mediated inhibition problems.

Recently, scanning electron microscopy (SEM) technique has been used to further study anaerobic microorganisms and their degradative effect. SEM permits the observation of spatial distribution and morphology of bacteria. For instance, external surface of anaerobic biofilms (Najafpour et al., 2008) and internal structure of anaerobic granular sludge (Alphenaar et al., 1994) have been previously studied with this technique. However, to the best of our knowledge no information is available regarding SEM examination in CSTR anaerobic sludge.

The goal of the present work was to investigate the effect of VPW addition to a semi-continuous fed anaerobic digester treating SM. Additionally; the effect of two different HRT was also addressed. Potential inhibitors of the anaerobic process were evaluated. The different populations of microorganisms were studied by means of SEM.

## **5.2. METHODS**

### **5.2.1. Raw materials**

SM was obtained from a pig farm located in Avila (Spain). VPW were collected in a vegetable processing factory located in Segovia (Spain) being composed by green peas, maize, carrots and leeks (25:25:25:25, dry weight (dw)). This residue was minced into particles of about 1 mm size in a mill fruit. Mesophilic anaerobic sludge (AS), collected in the wastewater treatment plant of Valladolid (Spain), was used as inoculum. Table 5.1 shows SM, VPW and AS chemical composition.

### **5.2.2. Experimental set-up**

Two continuous stirred tank reactors (CSTR) with total volume of 7 L and liquid phase volume of 5 L were used. One reactor was used for anaerobic digestion of SM (R1) and the other for co-digestion of VPW and SM (R2) (50:50 (dw)). The reactors were provided with agitation at 100 rpm by a mechanical stirrer. The temperature was maintained at  $37 \pm 2$  °C

with a water bath. Biogas production (quantified by water displacement) and pH were measured every weekday.

**Table 5.1.** Composition of the primary substrates (SM, VPW), inoculum (AS) and R1 and R2 inlet concentration for the different HRT studied.

Parameters	SM	VPW	AS
pH	8.3 (n.d)	4.3 (n.d)	7.5 (n.d.)
TS (g L <sup>-1</sup> )	12.5 (0.0)	124.1 (4.0)	22.2 (0.0)
VS (g L <sup>-1</sup> )	8.0 (0.0)	114.9 (4.1)	12.4 (0.0)
CODt (g L <sup>-1</sup> )	12.2 (2.6)	224.1 (49.4)	10.9 (1.2)
CODs (g L <sup>-1</sup> )	2.9 (0.5)	n.d.	4.1 (1.4)
TKN (g L <sup>-1</sup> )	3.8 (0.5)	3.3 (0.1)	1.3 (0.1)
N-NH <sub>4</sub> <sup>+</sup> (g L <sup>-1</sup> )	2.1 (0.0)	0.5 (0.0)	0.7 (0.0)
N-NH <sub>3</sub> <sup>+</sup> (g L <sup>-1</sup> )	0.4	<0.01	<0.05

Parameters	HRT= 25 d		HRT= 15 d	
	Feed R1	Feed R2	Feed R1	Feed R2
pH	7.8 (0.2)	6.9 (0.3)	7.3 (0.2)	6.5 (0.4)
TS (g L <sup>-1</sup> )	11.9 (1.4)	11.1 (2.5)	15.6 (3.8)	12.3 (2.2)
VS (g L <sup>-1</sup> )	6.2 (0.9)	7.3 (2.1)	9.4 (2.2)	8.8 (1.7)
CODt (g L <sup>-1</sup> )	9.3 (1.9)	11.1 (2.1)	14.8 (2.8)	15.0 (2.4)
CODs (g L <sup>-1</sup> )	4.6 (0.9)	6.1 (1.6)	8.8 (0.6)	9.0 (1.8)
TKN (g L <sup>-1</sup> )	1.4 (0.2)	1.0 (0.1)	1.7 (0.0)	1.0 (0.1)
N-NH <sub>4</sub> <sup>+</sup> (g L <sup>-1</sup> )	1.0 (0.2)	0.6 (0.2)	1.0 (0.2)	0.6 (0.1)
N-NH <sub>3</sub> <sup>+</sup> (g L <sup>-1</sup> )	0.1	<0.01	<0.05	<0.01

\*Data are means of three replicates, exception made for pH data and total VFAs measurements. Standard Deviation is shown in brackets.

The reactors were initially filled with mesophilic AS. After two days, reactors were fed manually once every weekday. In order to avoid imbalances or inhibitions, the first HRT was set at 25 days and the process was evaluated during three consecutive HRT. Afterwards, HRT was decreased to 15 days and the process was also evaluated for a period equivalent to three HRT. Effluent and influent samples were taken twice a week. Substrates for R1 and R2 were prepared once a week by diluting the correspondent substrate in distilled water in

order to keep constant and equal in both reactors influent total chemical oxygen demand (CODt). Table 5.1 presents chemical composition of R1 and R2 feed for both HRT.

### 5.2.3. Analytical Techniques

Total solids (TS), volatile solids (VS), CODt and soluble chemical oxygen demand (CODs), total Kjeldahl nitrogen (TKN), ammonium nitrogen ( $\text{N-NH}_4^+$ ) and alkalinity were measured twice a week according to Standard Methods (APHA, 2005). Free ammonia concentrations were calculated according to Hansen et al. (1998).

Biogas composition was analysed using a gas chromatograph (Varian CP 3800 GC) with a thermal conductivity detector, provided by a column CP-Molvsieve5A ( $15\text{m} \times 0.53\text{mm} \times 15\mu\text{m}$ ) followed by a column CP-Porabond Q ( $25\text{m} \times 0.53\text{mm} \times 10\mu\text{m}$ ). Hydrogen ( $13.6\text{ mL min}^{-1}$ ) was used as the carrier gas. The injection port temperature was set at  $150\text{ }^\circ\text{C}$  and the detector temperature was  $175\text{ }^\circ\text{C}$ . Total volatile fatty acids (TVFA) were analysed using a gas chromatograph (Varian CP 3800 GC) equipped with a Nukol capillary column ( $30\text{m} \times 0.25\text{mm} \times 0.25\mu\text{m}$ ) and a flame ionization detector. The carrier gas was helium and the temperature of the injector was  $250\text{ }^\circ\text{C}$ . The temperature of the oven was set at  $150\text{ }^\circ\text{C}$  for 3 min and thereafter increased to  $180\text{ }^\circ\text{C}$ .

Three samples were collected for SEM analyses, namely an initial mesophilic AS sample and sludges obtained from R1 and R2 at the end of the experimental time. Microbial cells in the sludge samples were fixed using 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2-7.4 for 90 min. Then, samples were washed three times for 20 min with phosphate buffered saline (PBS) at  $4\text{ }^\circ\text{C}$  and were dehydrated in ethanol graded series (30, 70, 90, 100% v/v). Dehydrated samples were dried and sputter-coated with gold in high vacuum (0.05-0.07 mbar) with a coater Blazers SCD 004. The samples were examined using a JOEL JSM 6840 LV scanning electron microscope.

## 5.3. RESULTS AND DISCUSSION

### 5.3.1. Effect of VPW addition

Operational parameters for R1 and R2 are presented in Table 5.2. Anaerobic co-digestion of SM and VPW (R2) achieved higher methane yields than SM anaerobic digestion (R1) during all the experimental time. Methane yield values were improved 3 and 1.4 times when comparing R1 and R2 for HRTs of 25 and 15 days, respectively.

Previous co-digestion studies working with similar reactor configuration and HRT also reported an enhancement in methane yield when studying VPW as co-substrate for different livestock residues. In this context, Alvarez and Lidèn (2008) improved methane yield in 1.5-folds when adding 47% VPW to cattle and pig manures mixture and Callaghan et al. (2002) achieved 1.6 times higher methane yields when adding 43% of VPW to cattle slurry. The high easily biodegradable content of VPW, 75% of the total organic fraction was reported as easy biodegradable material by Bouallagui et al., (2005), supplied carbon to the medium enhancing C/N ratio and thus improving methane yields.

**Table 5.2.** OLR, time of operation, methane yield and methane content for R1 and R2 during the different HRT applied.

	<b>OLR</b>	<b>HRT</b>	<b>Operational time</b>	<b>Methane yield</b>	<b>Methane content</b>
	g VS L <sup>-1</sup> d <sup>-1</sup>	days	days	mL CH <sub>4</sub> g VS <sub>added</sub> <sup>-1</sup>	%
<b>R1</b>	0.41 (0.06)	25	77	90 (55)	49 (4)
	0.63 (0.14)	15	45	201 (77)	69 (3)
<b>R2</b>	0.48 (0.13)	25	77	277 (49)	56 (4)
	0.59 (0.11)	15	45	285 (78)	55 (0)

Standard Deviation is shown in brackets.

### 5.3.2. Effect of HRT decrease

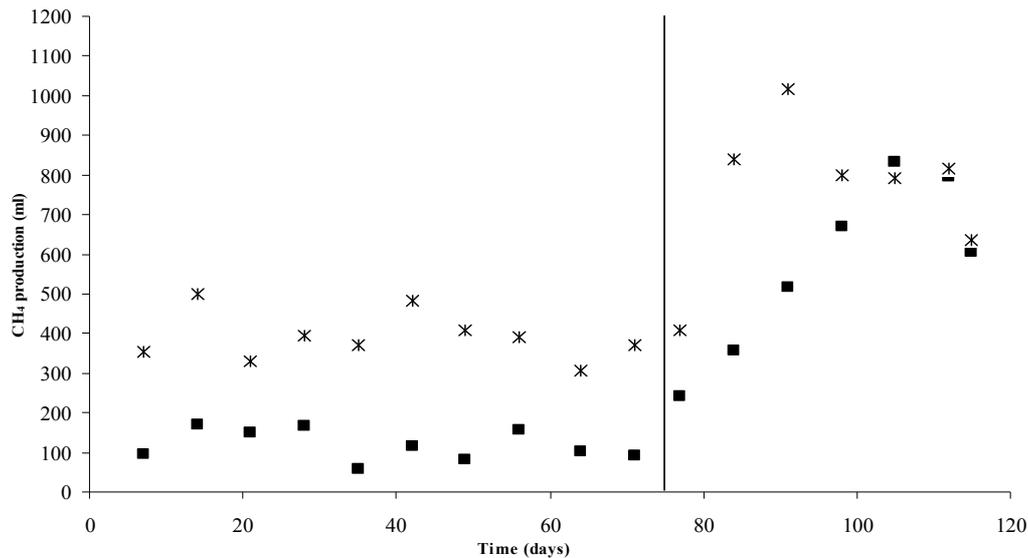
During the first period (HRT of 25 d) a low organic loading rate (OLR) was applied to both reactors in order to avoid possible inhibitions. In the case of R1, a decrease in HRT (from 25

d to 15 d) resulted in a 2.3 fold improvement in methane yield while in R2 the same decrease in HRT did not show any effect. The methane yields obtained here (for R1 at HRT of 25 d) resulted in lower values than the ones obtained in the study carried out by Kaparaju and Rintala (2005), who reported values for the anaerobic digestion of hog manure in the range of 130-150 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>. Moreover, methane content (49%) registered in the biogas was low if compared with that obtained by Kaparaju and Rintala (2005), who reported a 63% of methane in biogas. The results obtained in the present study may be rationalized by the low content of available organic material. SM used as substrate for R1 during HRT of 25 d presented low biodegradability as demonstrated by the CODs/CODt ratio (Table 5.1). This fact was probably due to previous organic matter degradation in the storage tank. When R1 was evaluated at HRT of 15 d (OLR increase from 0.4 to 0.6 g VS L<sup>-1</sup> d<sup>-1</sup>), methane yield was improved obtaining an average value of 201 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>. Data obtained in this study are then in accordance to Panichnumsin et al. (2010), who studied semi-continuously anaerobic digestion of pig manure with the same HRT, obtaining a value of 217 mL CH<sub>4</sub> g VS<sub>added</sub><sup>-1</sup>.

With regard to R2, a decrease in HRT had no effect on methane yield. The volumetric methane production (Figure 5.1) increased with HRT decrease (OLR increase), but the methane yield, which is measured in ml methane g VS added<sup>-1</sup>, remained constant.

### **5.3.3. Potential inhibitions of the anaerobic process**

It has been demonstrated that low pH (Ten-Hong et al., 1996), free ammonia concentration (Hansen et al., 1998) and TVFA accumulation (Vedrenne et al., 2008) are the main parameters responsible of methanogenic processes inhibition. Due to the acidity of VPW (Table 5.1), pH in R2 feed was lower than in R1 feed. However, SM buffered the system providing average pH values in the reactors of 8.1 and 7.8 for R1 and R2, respectively, throughout the experimental time (Table 5.3).



**Figure 5.1.** Performance of CSTRs R1 (SM) and R2 (SM and VPW). CH<sub>4</sub> production (ml). Weekly averaged accumulated methane production values are represented. Continuous vertical lines represent the change in HRT.

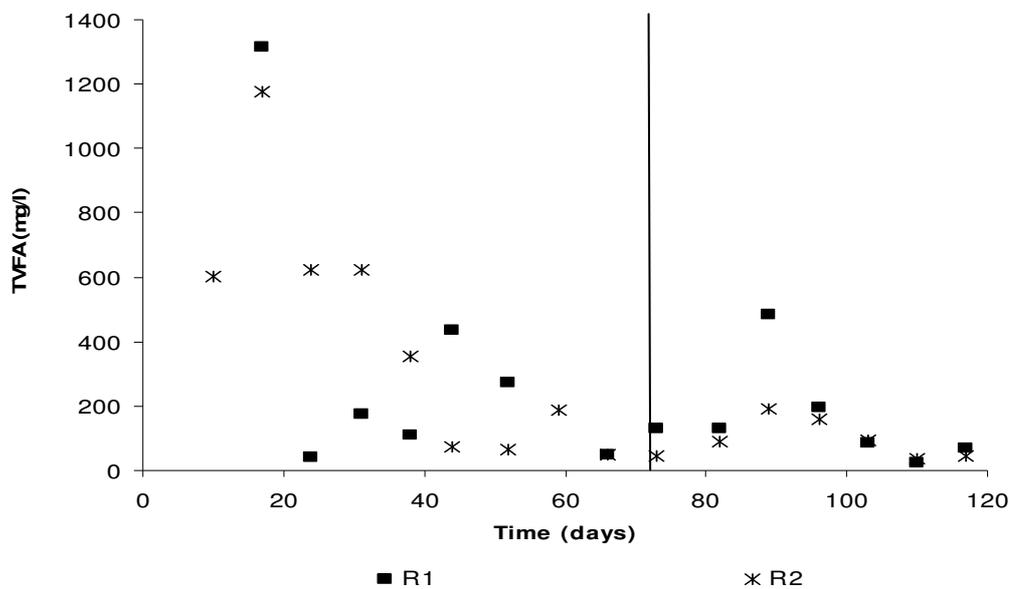
As expected, N-NH<sub>4</sub><sup>+</sup> concentration during anaerobic digestion process was dependent on the content of SM in the feeding mixture due to the high nitrogen content of this substrate (Table 5.1). Free ammonia concentrations at the end of the experiments were calculated for all treatments in accordance with Hansen et al. (1998). Even though, the ammonia concentration threshold inhibition varies widely, some authors set the concentration in the range of 100–150 mg N-NH<sub>3</sub> L<sup>-1</sup> (De Baere et al., 1984, Gallert and Winter, 1997), some others increased it up to 1100 mg N-NH L<sup>-1</sup> in batch culture at pH 8.0 with proper sludge adaptation (Hansen et al., 1998). Since ammonia levels were in the lower inhibition limit for R1 (130 and 122 mg N-NH<sub>3</sub> L<sup>-1</sup> for HRT of 25 and HRT of 15 d, respectively), no ammonia-mediated inhibition was expected (Table 5.3).

TVFA concentration inside the reactors is presented in Fig 5.2. At the beginning of the experiment, an accumulation of TVFA was detected for R2. Callaghan et al. (2002) found that co-digestion with 30% (wet weight) or more of fruit and vegetables wastes resulted in high concentrations of TVFA and the reactor performance became instable. In the present

study, after a period of adaptation of around 30 days, TVFA were almost completely consumed by methanogenic microorganisms (Fig. 5.2) indicating that no inhibition was taking place.

**Table 5.3.** pH, ammonium and ammonia for R1 and R2 during the different HRT applied.

	HRT (25 d)			HRT(15 d)		
	pH	N-NH <sub>4</sub> <sup>+</sup> (mg.L <sup>-1</sup> )	N-NH <sub>3</sub> (mg.L <sup>-1</sup> )	pH	N-NH <sub>4</sub> <sup>+</sup> (mg.L <sup>-1</sup> )	N-NH <sub>3</sub> (mg.L <sup>-1</sup> )
R1	8.1 (0.1)	1036 (120)	130	7.8 (0.2)	1082 (83)	122
R2	8.0 (0.2)	780 (120)	58	7.8 (0.2)	637 (63)	47



**Figure 5.2.** Performance of CSTRs R1 (SM) and R2 (SM and VPW). TVFA (mg/L). Continuous vertical lines represent the change in HRT.

### 5.3.4. Microorganisms populations studied by SEM

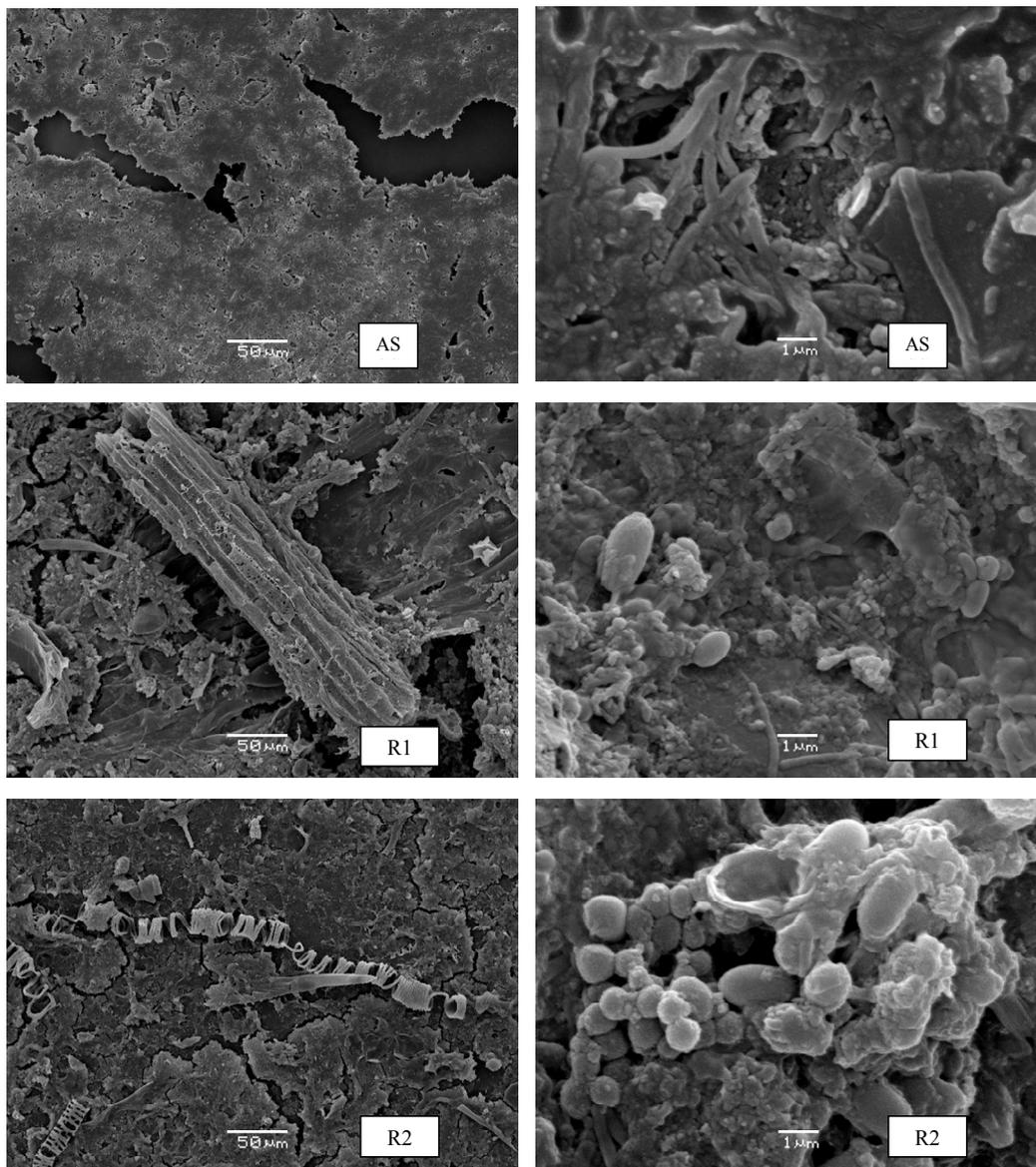
Fig. 5.3 shows SEM pictures of the initial mesophilic anaerobic sludge (AS) used as inoculum and from R1 and R2 sludge at the end of the experimental time. 300x pictures gave us a general overview of the different sludges developed in the reactors. Initial AS sludge presented a homogeneous appearance. This matrix, obtained from the second anaerobic digester of a WWTP, had been previously anaerobically digested before being used for the experiments. Sludge with similar appearance was reported by Baharuddin et al. (2010) analysing by SEM a palm oil mill effluent anaerobic sludge. On the contrary, anaerobically digested matrixes for R1 and R2 presented a heterogeneous structure with different particles not been degraded during anaerobic digestion. Those particles could correspond to lignin and cellulose structures. For instance, R2 picture (300x) presented a trachea, which are xylem cells with lignified walls typically encountered in vegetable structure (Kaviani et al., 2008). It is worth mentioning that VPW substrate used for the present study was around 20% TS composed by cellulose and lignin, which indeed is non-degradable by through anaerobic digestion (Robbins et al., 1979). As previous studies demonstrated, anaerobic bacteria are not able to degrade lignin (Robbins et al., 1979), which can even reduce cellulose bioavailability by reducing the surface area available for enzymatic penetration (Haug, 1993).

When the magnification was increased up to 10000x, differences between AS and R1-R2 pictures were clearly appreciated. Long rod-shaped bacteria were the dominant morphotypes in AS-picture and they appeared embedded inside a matrix of mudding aspect. A different fact was elucidated in pictures corresponding to R1 and R2, where rods and cocci were the predominant bacteria morphotypes and they were loosely distributed in the surface.

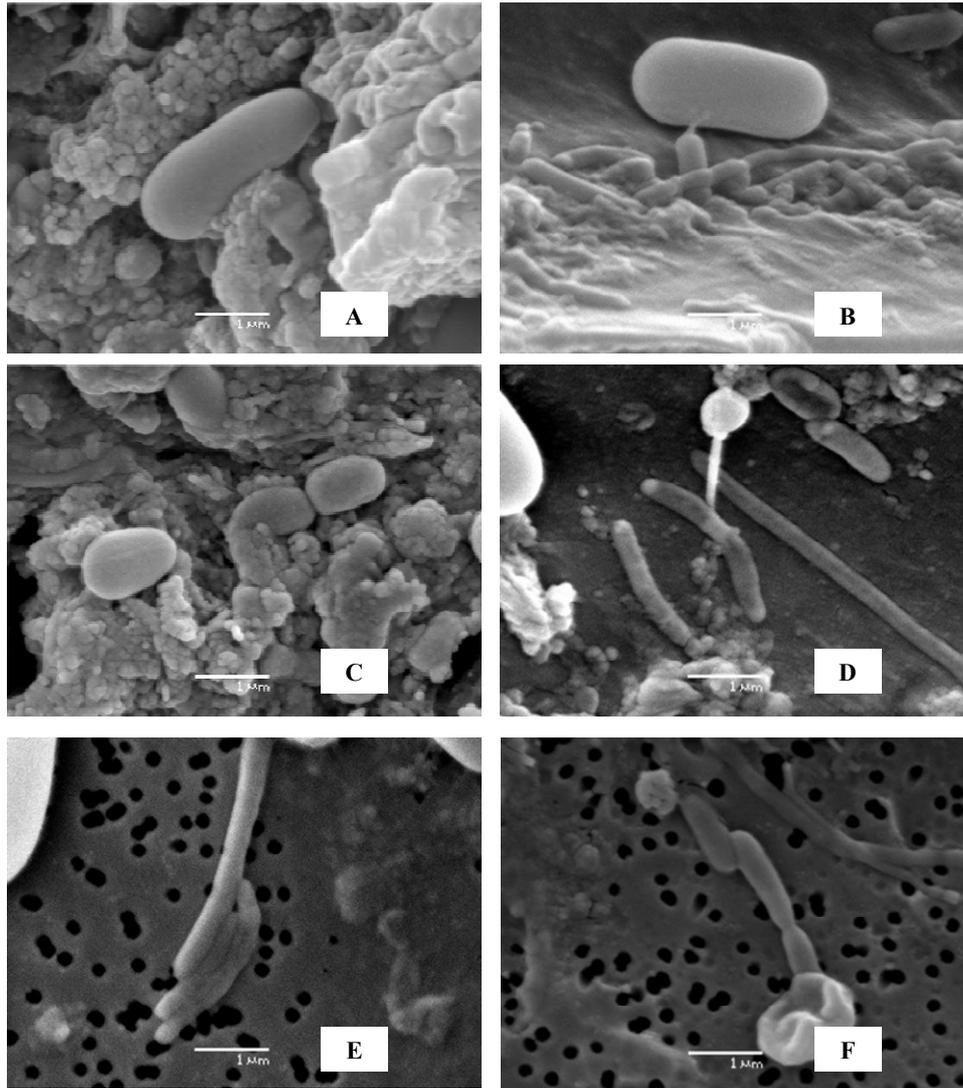
Even more detailed SEM pictures (mag. 20000x) are shown in Fig. 5.4. Pictures A, B and C present bacilli-shape bacteria and, in accordance to Sanz et al. (2003), pictures D, E and F show long rod-shaped microorganisms similar to *Methanosaeta sp.*

(mag. x 300)

(mag. x 10000)



**Figure 5.3.** SEM images for initial anaerobic sludge (AS) and final sludges for R1 and R2. The left column presents a magnification of 300x and the right one presents a magnification of 10000x. White arrows indicate bacterial morphotypes as cocci, rods or long rod-shaped bacteria.



**Figure 5.4.** Detailed SEM images of anaerobic sludge (mag. x 20000). Bacillus morphotype (A,B,C) and long-rod shaped microorganisms (D,E,F).

### **3.4. CONCLUSIONS**

The present study demonstrated the positive effect on methane production of vegetable addition in semi-continuous operated anaerobic digester treating swine manure. The high buffer capacity of swine manure coupled with the high C/N ratio of vegetable wastes

improved process performance where no inhibitions were detected. As shown by SEM pictures, lignin and cellulose were not completely degraded at the end of the experimental time and microbial composition was found to change to cocci and rods morphotypes after 120 days of anaerobic digestion.

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## *Chapter 6*

# **Anammox for ammonia removal from pig manure effluents: Effect of organic matter content on process performance**

**Molinuevo, B., García, M.C., Karakashev, D., Angelidaki, I.**

*Bioresource Technology 100 (2009) 2171–2175*



**Abstract.** The anammox process, under different organic loading rates (COD), was evaluated using a semi-continuous UASB reactor at 37°C. Three different substrates were used: initially, synthetic wastewater, and later, two different pig manure effluents (after UASB-post-digestion and after partial oxidation) diluted with synthetic wastewater. High ammonium removal was achieved, up to  $92.1 \pm 4.9\%$  for diluted UASB-post-digested effluent ( $95 \text{ mg COD L}^{-1}$ ) and up to  $98.5 \pm 0.8\%$  for diluted partially oxidized effluent ( $121 \text{ mg COD L}^{-1}$ ). Mass balance clearly showed that an increase in organic loading (from  $95 \text{ mg COD L}^{-1}$  to  $237 \text{ mg COD L}^{-1}$  and from  $121 \text{ mg COD L}^{-1}$  to  $290 \text{ mg COD L}^{-1}$  for the UASB-post-digested effluent and the partially oxidized effluent, respectively) negatively affected the anammox process and facilitated heterotrophic denitrification. Partial oxidation as a pre-treatment method improved ammonium removal at high organic matter concentration. Up to threshold organic load concentration of  $142 \text{ mg COD L}^{-1}$  of UASB-post-digested effluent and  $242 \text{ mg COD L}^{-1}$  of partially oxidized effluent, no effect of organic loading on ammonia removal was registered (ammonium removal was above 80%). However, COD concentrations above  $237 \text{ mg L}^{-1}$  (loading rate of  $112 \text{ mg COD L}^{-1} \text{ day}^{-1}$ ) for post-digested effluent and above  $290 \text{ mg L}^{-1}$  (loading rate of  $136 \text{ mg COD L}^{-1} \text{ day}^{-1}$ ) for partially oxidized effluent resulted in complete cease of ammonium removal. Results obtained showed that, denitrification and anammox process were simultaneously occurring in the reactor. Denitrification became the dominant ammonium removal process when the COD loading was increased.

**Resumen.** Se evaluó el proceso anammox con diferentes cargas orgánicas utilizando para ello un reactor UASB operando en modo semi-continuo a 37° C. Se utilizaron tres sustratos diferentes, agua residual sintética y dos efluentes resultantes de la digestión anaeróbica de purines de cerdo (un primer efluente obtenido después de la digestión en un reactor UASB y un segundo efluente tomado después de una oxidación parcial). Se obtuvo una eliminación de amonio de un  $92.1 \pm 4.9\%$  en el caso del primer efluente ( $95 \text{ mg DQO L}^{-1}$ ) y un  $98.5 \pm 0.8\%$  en el caso del segundo ( $121 \text{ mg DQO L}^{-1}$ ). El balance de materia demostró que un incremento

en la carga orgánica (de 95 mg DQO L<sup>-1</sup> a 237 mg DQO L<sup>-1</sup> y de 121 mg DQO L<sup>-1</sup> a 290 mg DQO L<sup>-1</sup> para el primer y segundo efluente, respectivamente) afectó negativamente al proceso anammox y favoreció el proceso de desnitrificación heterotrófica. Se observó que la oxidación parcial utilizada como pre-tratamiento mejoró los porcentajes de eliminación de amonio a altas concentraciones de materia orgánica. Hasta concentraciones de 142 mg DQO L<sup>-1</sup> en el caso del primer efluente y 242 mg COD L<sup>-1</sup> en el caso del segundo no se registraron efectos negativos de la carga orgánica en la eliminación de amonio (las eliminaciones de amonio fueron superiores al 80% en ambos casos). Sin embargo, por encima de 237 mg DQO L<sup>-1</sup> (112 mg DQO L<sup>-1</sup> day<sup>-1</sup>) para el primer efluente, y por encima de 290 mg DQO L<sup>-1</sup> (136 mg DQO L<sup>-1</sup> day<sup>-1</sup>) para el segundo, se observó un cese en la eliminación de amonio. Los resultados indicaron que ambos procesos biológicos, anammox y desnitrificación, estaban ocurriendo simultáneamente en el reactor y que la desnitrificación se convertía en el proceso dominante cuando la carga orgánica aplicada al reactor se incrementaba.

## **6.1. INTRODUCTION**

Pig farming is a major European Union (EU) agricultural industry. Nowadays, farmers in the EU are confronted with an increasing number of environmental regulations, concerning the application of the manure produced, as direct fertilizer on agricultural land. Phosphorus and nitrogen pollute potable water and cause eutrophication. As the main part of nitrogen in manure, ammonium is readily oxidized to nitrate, which is poorly absorbed by soil colloids, thus facilitating its transfer to surface waters. Manure also contributes to increased greenhouse gas emissions (GHG) (Thorman et al., 2007). Special attention needs to be given to N<sub>2</sub>O gas emissions, which can be minimized by developing a sustainable manure management strategy. Thus, sustainable solutions for pig manure treatment regarding nitrogen removal need to be implemented with respect to environmental and agricultural benefits.

Anaerobic ammonium oxidation (anammox) has received special attention since its discovery, because it is an efficient biological alternative to conventional nitrogen removal from wastewaters. Under anaerobic conditions, ammonium is oxidized to nitrogen gas with nitrite as the electron acceptor and carbon dioxide is used for growth of the anammox microorganisms involved. In comparison to traditional nitrification–denitrification process, this autotrophic process consumes 100% less biodegradable organic carbon and at least 50% less oxygen (Tal et al., 2006) and has, therefore, a lower operating cost.

The anammox process is suitable for wastewater with low carbon: nitrogen (C:N) ratios. At C:N ratios above 1, the anammox bacteria are no longer able to compete with heterotrophic denitrifying bacteria (Güven et al., 2005).

The organic loading rate was found to affect the anammox process performance, but the exact inhibitory levels still remain unclear (Sabumon, 2007; Wang and Kang, 2005). An organic matter concentration above 300 mg chemical oxygen demand (COD) L<sup>-1</sup> was

previously found to inactivate anammox communities in a UASB reactor fed with fat milk as organic matter source (Chamchoi et al., 2008). Concentrations of 50 mM of acetate resulted in 70% inhibition in the anammox process (Dapena-Mora et al., 2007). Therefore it is necessary to clearly establish the COD levels inhibiting the anammox process.

Ammonia removal via anammox has been developed for the treatment of many different wastes with low organic matter content (below 1700 mg COD L<sup>-1</sup>), such as water from the secondary clarifier of municipal wastewater treatment plants in a down flow biofilter (Li et al., 2005), nitrous organic wastewater in ASBR reactors (Jing-Ping et al., 2006) and landfill leachate in a continuous reactor (Liang and Liu, 2008). Only a few studies have investigated the possibility of using the anammox process for ammonia removal from animal waste treatment water, which is indeed a residue with high organic matter and nitrogen content (Waki et al., 2007). However, there is still a big gap regarding effect of different pre-treatments (reducing organic and ammonia loads) of the waste streams on anammox process performance.

The aim of the present study is to investigate the performance of the anammox process under different organic loadings in a semi-continuous UASB reactor fed with two pretreated pig manure effluents (UASB-post-digested effluent and partially oxidized effluent) as organic matter source.

## **6.2. METHODS**

### **6.2.1. Substrate characteristics**

Three different substrates were used in this study:

#### **Synthetic wastewater (SWW)**

Synthetic wastewater was used for the start-up of the lab-scale UASB reactor.

The synthetic wastewater composition used throughout this study was (g L<sup>-1</sup>): NaHCO<sub>3</sub>, 2.6; K<sub>2</sub>HPO<sub>4</sub>, 0.025; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.3; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.00625; EDTA, 0.00625; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.19; NaNO<sub>2</sub>, 0.26; NaNO<sub>3</sub>, 1.22 dissolved in distilled water. Trace element solutions I and II (1.25 ml per litre medium) were also added as previously described (Chamchoi and Nitisoavut, 2007).

#### **Pig manure effluent after UASB-post-digestion (UASB-postdigested effluent)**

This effluent was collected after three treatment steps: fullscale anaerobic digestion (AD), decanter separation and postdigestion in a lab-scale UASB reactor for reduction of the residual organic matter (Karakashev et al., 2008).

Full-scale anaerobic digestion was performed continuously in a thermophilic (55 °C) biogas plant (Hegndal biogas plant, Denmark) with hydraulic retention time (HRT) of 15 days and organic loading rate of 4.6 kg COD m<sup>-3</sup> day<sup>-1</sup>. After full-scale AD, the effluent was continuously separated in a decanter centrifuge (Alfa Laval, NX 309B-31, Rodovre, Denmark) operated at 5000g. After centrifugation, digested manure was separated into a solid organic fibre fraction (10-15% wet weight) and a liquid fraction (85-90% wet weight). The liquid fraction was post-digested in UASB to reduce the residual COD. The reactor operational parameters were: temperature 55 °C, total volume 334 ml, liquid volume 255 ml, HRT 4 days. The reactor was fed 12 times per day with a feeding rate of 2.63 ml min<sup>-1</sup> for 2 min. The reactor was inoculated with 0.05 L of anaerobic granular sludge obtained from a potato factory (Kruiningen, The Netherlands). The average organic loading rate of the reactor was 3.8 g total COD L<sup>-1</sup> day<sup>-1</sup>. Chemical characteristics after this treatment are shown in Table 6.1.

#### **Pig manure effluent after partial oxidation (Partially oxidized effluent)**

A partial oxidation (nitrification) of the UASB-post-digested effluent was carried out to create more favourable conditions for the anammox process through partial removal of COD

and partial conversion of ammonium to nitrite. A mixture of nitrifying sludge (20%) (Lundtofte WWTP, Denmark) and 80% of UASB-post-digested effluent was aerated for 30 h with an aeration rate of 1500 ml air min<sup>-1</sup>.

The chemical characteristics of the partially oxidized effluent are presented in Table 6.1.

**Table 6.1.** Chemical characteristics of the pig manure effluents.

Parameters	Unit	Average Value ± SD*	Average Value ± SD
		Effluent after UASB	Effluent after partial oxidation
TS	g L <sup>-1</sup>	nd**	nd
VS	g L <sup>-1</sup>	nd	nd
COD	g L <sup>-1</sup>	4.74 ± 1.05	2.42 ± 0.29
N-NH <sub>4</sub> <sup>+</sup>	g L <sup>-1</sup>	3.78 ± 0.46	0.67 ± 0.16
N-NO <sub>2</sub>	g L <sup>-1</sup>	1.7 ± 0.20	0.7 ± 0.24
N-NO <sub>3</sub>	g L <sup>-1</sup>	4.01 ± 0.40	1.65 ± 0.58

\* SD represents standard deviation from triplicate sampling experiments

\*\* Not determined

## 6.2.2. Experimental setup

### Lab-scale UASB reactor for the anammox process

A lab-scale UASB reactor operated in semi-continuous mode was used. The UASB was inoculated with 40 ml granules from anaerobic granular sludge (potato factory, Kruiningen, The Netherlands) and 40 ml anammox seed sludge (provided by the Laboratory of Microbial Ecology, Ghent University, Belgium).

The reactor was operated at 37 °C with a total volume of 334 ml; the liquid volume was 255 ml. The flow rate was set up at 120 ml day<sup>-1</sup> and the HRT was 2.1 d. The UASB reactor was initially fed with synthetic wastewater. The effect of organic matter concentration was tested with two pig manure effluents, after UASB and after partial oxidation. Addition of the effluent to the artificial wastewater was done gradually in increments, 2%, 3% and 5% for

UASB-post-digested effluent and 5%, 7%, 10% and 12% for partially oxidized effluent. When no ammonia removal was detected, the experiment was terminated.

### 6.2.3. Analytical methods

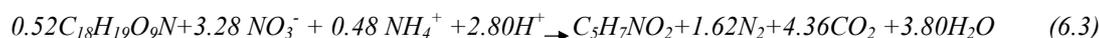
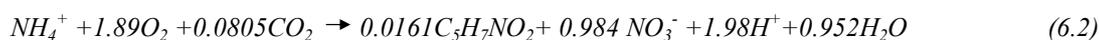
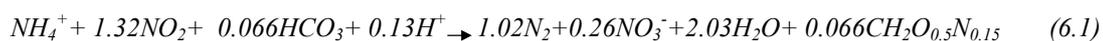
COD,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^- \text{-N}$ , and  $\text{NO}_3^- \text{-N}$ , were measured according to APHA (Standard Methods for the Examination of Water and Wastewater, 2001).

### 6.2.4. Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) was used to study the anammox community dynamics. An Amx368 probe targeting all known anammox bacteria was used. Hybridization procedures were followed as given by Hugenholtz et al. (2001). FISH images were analyzed with an epifluorescence microscope and digital image analyzer (Image-Pro Plus 5.1 software).

### 6.2.5. Mass balance calculations

Based on nitrogen mass balance over the entire system, taking into consideration the different nitrogen conversion processes possible, the removal of ammonium was established. The nitrogen conversion processes considered were: anammox process (Eq.(6.1)), autotrophic nitrification (Eq. (6.2)) and heterotrophic denitrification process (Eq. (6.3)).



It was assumed that the oxygen concentration was  $8.7 \text{ mg L}^{-1}$ , which is the saturated dissolved oxygen concentration in fresh water at 1 atm, 22 °C.

## 6.3. RESULTS AND DISCUSSION

### 6.3.1. Ammonium removal from UASB-post-digested effluent

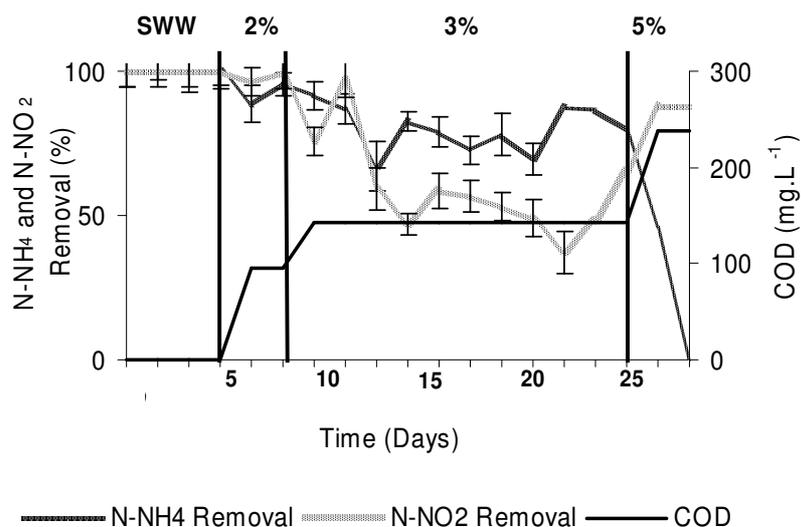
An UASB reactor was chosen in our experiments due to the high stability of this type of reactor for anammox process performance (Jin et al., 2008). The granulation process of anammox sludge was generally evaluated by the particle size distribution. Granules of smaller size (0.3-0.6 mm) dominated the upper portion of UASB sludge bed while the granules of larger size (0.8-2.00 mm) occupied the lower portion of reactor sludge bed. Compared to HRT of different anammox reactor configurations ranged from 3 h for anaerobic biological filtrated reactor, ABF (Isaka et al., 2006), through 12-16 h for UASB and upflow stationary fixed film reactor, USFF (Jin et al., 2008), up to 1 d for sequencing batch reactor, SBR (Third et al., 2005), HRT of our UASB reactor system was chosen to be a bit higher (2.1 d) with respect to treat high strength organic residue. After a starting up period, with synthetic wastewater as feed, UASB-post-digested effluent ( $4.7 \text{ g COD L}^{-1}$ ) was gradually introduced to the feed of the anammox UASB reactor. An addition of 2% (v/v) and 3% (v/v) of UASB-post-digested effluent (organic load of  $95 \text{ mg COD L}^{-1}$  and  $142 \text{ mg COD L}^{-1}$ , respectively) resulted in very high ammonium removal ( $92 \pm 4.9\%$  for 2% (v/v) and  $80 \pm 7.8\%$  for 3% (v/v) effluent addition, respectively). Ammonium removal fell sharply to 0% when 5% (v/v) UASB-post-digested effluent was added (organic load of  $237 \text{ mg COD L}^{-1}$ , corresponding to loading rate of  $112 \text{ mg COD L}^{-1}\text{day}^{-1}$ ) (Fig. 6.1).

This addition resulted in decrease of the  $\text{NO}_2^- \text{-N} : \text{NH}_4^+ \text{-N}$  ratio, from 1:0.67 to 1:0.33 for addition of 2% and 5% post-digested effluent, respectively. Results obtained indicated that the chemical composition of the UASB-post-digested effluent was not suitable for optimal performance of the anammox process, as the  $\text{NO}_2^- \text{-N} : \text{NH}_4^+ \text{-N}$  ratio for the best anammox process performance obtained by Strous et al. (1999), was 1:1.32. UASB-post-digested

effluent had relatively high ammonium levels (Table 6.3) resulting in an  $\text{NO}_2^- \text{-N} : \text{NH}_4^+ \text{-N}$  ratio much lower than the reported optimum for anammox. As anammox bacteria are often inhibited by sulphide concentrations above 1 mM (Dapena-Mora et al., 2007), another reason for process inhibition could be sulphide formation due to some activity of sulphate reducers naturally presented in pig manure. In respect to organic load, several studies have reported that presence of organic matter combined with high concentration of nitrite, negatively affected anammox bacteria due to the existing competition between anammox bacteria and heterotrophic denitrifiers (Ahn et al., 2004; Chamchoi et al., 2008; Dong and Tollner, 2003; Jianlong and Jing, 2005; Tal et al., 2006). In mixed culture environment anaerobic ammonia oxidizers are always in competition with heterotrophic denitrifiers for nitrite. When enough organic matter is available, anammox bacteria are not longer able to outcompete denitrifiers due to difference in the growth rates for both groups of microorganisms. In our study, mass balance for nitrogen showed that  $19.9 \pm 0.24\%$  and  $11.3 \pm 1.3\%$  of  $\text{NH}_4^+ \text{-N}$  were removed by anammox process when organic loads of UASB post-digested effluent  $95 \text{ mg COD L}^{-1}$  and  $142 \text{ mg COD L}^{-1}$ , respectively were applied (Table 6.2). Other process, responsible for removal of the major part of the ammonium, such as heterotrophic denitrification, was also involved (Table 6.2).  $7 \pm 0.24\%$ ,  $6 \pm 0.96\%$  and  $10 \pm 0\%$  of  $\text{NH}_4^+ \text{-N}$ , was removed via heterotrophic denitrification for organic loads of  $95 \text{ mg COD L}^{-1}$ ,  $142 \text{ mg COD L}^{-1}$  and  $237 \text{ mg COD L}^{-1}$ , respectively.

Anammox bacteria have a very slow growth rate compared to heterotrophic denitrifiers (Strous et al., 1999). According to Kang and Wang (2006), removal of  $\text{NH}_4^+ \text{-N}$  and  $\text{NO}_2^- \text{-N}$  is controlled by the COD concentration in the reactor. Results obtained clearly showed that anammox activity decreased and heterotrophic denitrification increased when the organic loading is increased. Similar findings were previously reported for nitrogen removal from animal waste treatment water (Waki et al., 2007).

Aerobic nitrification (Eq. (6.2)) was to some extent involved in ammonium removal due to air entering the reactor system during feeding or sampling (data not shown).



**Figure 6.1.**  $\text{NH}_4^+$  - N and  $\text{NO}_2^-$  - N removal during gradual implementation of the effluent after UASB post-digestion. Error bars represent standard deviation from a triplicate sampling analysis.

**Table 6.2.** Participation of different processes for ammonium removal from effluent after UASB post-digestion.

% (v/v) of UASB- post-digested effluent added to SW	Effluent COD( $\text{mg L}^{-1}$ )	% Ammonia removal $\pm$ SD*			
		Anammox	Denitrification	Nitrification	Other
0	0	48.20 $\pm$ 9.09	2.98 $\pm$ 0.02	5.56 $\pm$ 1.04	39.84 $\pm$ 11.28
2	95	19.98 $\pm$ 0.24	7.14 $\pm$ 0.21	3.18 $\pm$ 0.19	69.70 $\pm$ 0.64
3	142	11.32 $\pm$ 1.29	6.32 $\pm$ 0.96	3.36 $\pm$ 0.62	79.22 $\pm$ 2.22
5**	237	0.00	9.61	5.08	85.31

\* SD represents standard deviation from a triplicate experiment.

\*\*When no ammonia removal was detected the reactor was stopped, so standard deviation was not achieved in this case.

Physical processes, such as ammonia volatilization or ammonia stripping, could have been involved. Ammonia stripping at moderate temperature was previously observed. High ammonia removal (more than 90%  $\text{NH}_3$ -N reduction) by ammonia stripping was reported by Liao et al. (1995) in swine manure wastewaters at 20 °C.

### **6.3.2. Ammonium removal from partially oxidized effluent**

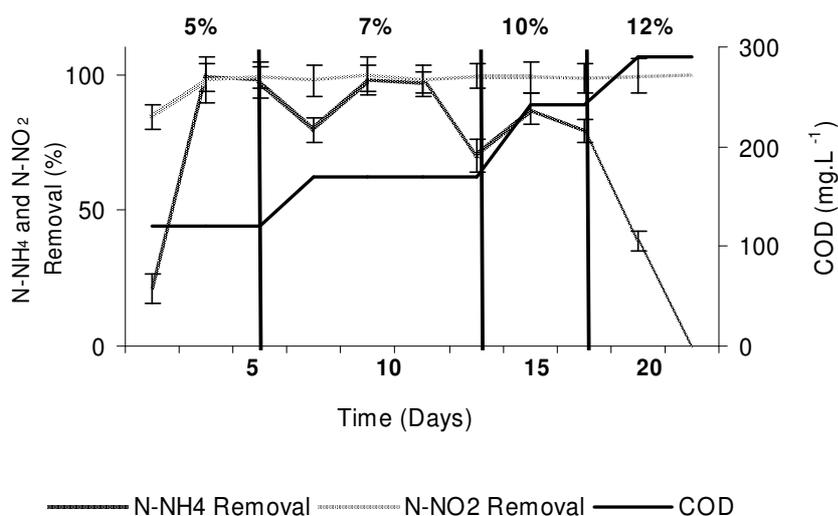
A partial oxidation of the UASB-post-digested effluent resulted in a 51% COD reduction, from 5 g COD  $\text{NH}_4^+\text{-N L}^{-1}$  to around 2.5 g COD  $\text{L}^{-1}$ , due to activity of heterotrophic aerobic microorganisms. 83%  $\text{NH}_4^+\text{-N}$  reduction, from 3.78 g  $\text{NH}_4^+\text{-N L}^{-1}$  to 0.67 g  $\text{NH}_4^+\text{-N L}^{-1}$  was also registered.

The addition of 5% (v/v) of partially oxidized effluent (organic load of 121 mg COD  $\text{L}^{-1}$ ) resulted in high ammonium removal (up to  $98.5 \pm 0.8\%$ ), compared with ammonium removal for UASB-post-digested effluent ranged between  $92 \pm 4.9\%$  and  $80 \pm 7.8\%$  for 2% and 3% (v/v) effluent addition, respectively. As high concentration of free ammonia was previously proven to be inhibitory for anammox reaction (Waki et al., 2007), partial oxidation of ammonia to nitrite in our study definitely facilitate anammox reaction.

The feeding rate was increased gradually corresponding to organic loads of 169, 242 and 290 mg COD  $\text{L}^{-1}$  (7%, 10% and 12% (v/v) of partially oxidized effluent), small steps to permit the anammox bacteria to adapt to higher organic loads. Ammonium removal ranged between 83% and 86% when 169 mg COD  $\text{L}^{-1}$  and 242 mg COD  $\text{L}^{-1}$  was added. When organic load of 290 mg COD  $\text{L}^{-1}$  (loading rate of 136 mg COD  $\text{L}^{-1} \text{ day}^{-1}$ ) was applied, the ammonium removal was totally absent (Fig. 6.2).

On average, 98.9% of nitrite removal was achieved during all the experiments (Fig. 6.2).  $\text{NO}_2^-\text{-N}:\text{NH}_4^+\text{-N}$  ratios obtained were 1:1.23, 1:1.16 and 1:1.13 when organic loads of 169 mg COD  $\text{L}^{-1}$ , 242 mg COD  $\text{L}^{-1}$  and 290 mg COD  $\text{L}^{-1}$  were applied, respectively. This ratio was very close to the theoretical  $\text{NO}_2^-\text{-N}:\text{NH}_4^+\text{-N}=1:1.32$  for anammox (Strous et al., 1999).

When organic load of 290 mg COD  $\text{L}^{-1}$  was applied, a ratio of 1:2.56 was obtained. In this case the heterotrophic denitrification was the major reaction involved in ammonium removal. 22.5% of  $\text{NH}_4^+\text{-N}$  was removed by heterotrophic denitrifiers (Table 6.3).



**Figure 6.2.**  $\text{NH}_4^+$ -N and  $\text{NO}_2^-$ -N removal during gradual implementation of the effluent after partial oxidation. Error bars represent standard deviation from a triplicate experiment.

Nitrate consumption was also found to be much higher than in the rest of the experiments (data not shown). Similar findings about dominance of heterotrophic denitrification over the anammox process were reported previously by Jetten et al. (1999). Results from the mass balance (Table 6.3) showed that the anammox process performance improved by up to 3 times.  $33 \pm 1.2\%$  and  $41.8 \pm 3.4\%$  of  $\text{NH}_4^+$ -N was removed by the anammox process for organic loads of  $121 \text{ mg COD L}^{-1}$ , and  $169 \text{ mg COD L}^{-1}$ , while  $11.3 \pm 0.3\%$  of  $\text{NH}_4^+$ -N was removed for addition of UASB-postdigested effluent with organic load of  $142 \text{ mg COD L}^{-1}$ . Partial oxidation decreased ammonia concentration, which resulted in final  $\text{NO}_2^-$ -N:  $\text{NH}_4^+$ -N ratios very close to the theoretical ratio for optimal anammox process performance.

As expected, when more partially oxidized effluent was added, the participation of the anammox process in the total ammonium removal decreased from approximately 30-0% (no anammox ammonia removal), when partially oxidized effluent with organic load of  $290 \text{ mg COD L}^{-1}$  (Table 6.3) was implemented. On the other hand, the denitrification part in total ammonium removal increased from approximately 9.3% for  $242 \text{ mg COD L}^{-1}$  added to

22.5% for 290 mg COD L<sup>-1</sup> of partially oxidized effluent added. When 290 mg COD L<sup>-1</sup> (12% of partially oxidized effluent) was added, no ammonium removal was detected, so the anammox reaction was completely inhibited. Inhibitory level of COD detected in this study was lower compared to inhibitory COD level of 300 mg COD L<sup>-1</sup> previously reported by Chamchoi et al. (2008).

**Table 6.3.** Participation of different processes for ammonium removal from effluent after partial oxidation.

% (v/v) of partially oxidized effluent added to SW	Effluent COD(mg.L <sup>-1</sup> )	% Ammonia removal ± SD			
		Anammox	Denitrification	Nitrification	Other
5	121	33.23 ± 1.23	13.42 ± 4.68	5.74 ± 0.48	47.61 ± 6.39
7	170	41.75 ± 3.35	14.84 ± 2.73	7.13 ± 0.69	36.28 ± 6.77
10	242	29.97 ± 1.07	9.31 ± 0.32	6.24 ± 0.11	54.47 ± 1.5
12 **	290	0.00	22.52	13.63	63.85

\* D represents standard deviation from triplicate experiment.

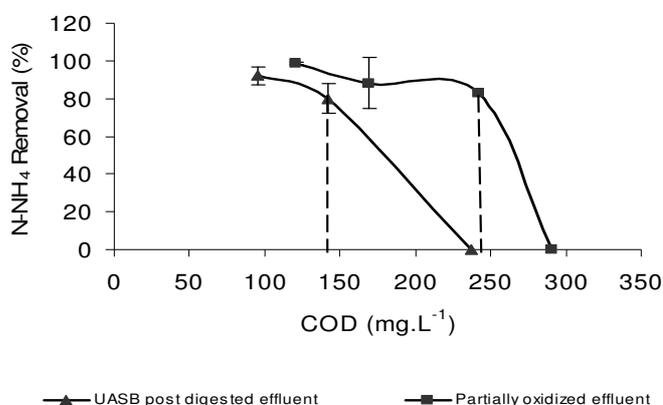
\*\* When no ammonia removal was detected the reactor was stopped, so standard deviation was not achieved in this case.

A change in the physiological characteristics of the biomass was observed. A biomass aggregation was registered when a concentration of 290 mg COD L<sup>-1</sup> was introduced to the reactor. The biomass settled and began to turn black, until the ammonium removal disappeared. This finding indicates that anammox communities decreased while denitrifiers increased, due to change in the environmental conditions (in this case the addition of organic matter). Ammonia removal by denitrification was 22.5% from total NH<sub>4</sub><sup>+</sup>-N removal, while ammonia removal via anammox was zero. This may be due to the fact that denitrifiers have a higher growth yield (yield coefficient of heterotrophic denitrifiers; Y = 0.3) compared to anammox bacteria (Y = 0.066 ± 0.01) (Strous et al., 1999). Moreover, denitrification reactions are thermodynamically more favourable than ammonia oxidation reactions (Ahn et al., 2004).

FISH analyses (data not shown) revealed that there was a reduction in the number of anammox bacteria when an effluent with organic load of 290 mg COD L<sup>-1</sup> was applied, while a large amount of anammox cells were found when an organic load of 121 mg COD L<sup>-1</sup> (5% of partially oxidized effluent) was applied.

### 6.3.3. Organic matter concentration vs. ammonium removal via anammox

Fig. 6.3 shows the effect of organic loading on ammonium removal for both effluents – UASB-post-digested and partially oxidized.



**Figure 6.3.** Effect of organic matter concentration on ammonium removal. Error bars represent standard deviation from a triplicate experiment. Dotted lines show COD threshold inhibitory levels for each effluent.

In order to quantify the effect of organic loading on ammonium removal more precisely, a COD inhibitory organic load threshold concentration was defined when ammonium removal dropped to around 80%. Results obtained showed that up to threshold concentrations of 142 mg COD L<sup>-1</sup> for UASB-post-digested effluent and 242 mg COD L<sup>-1</sup> for partially oxidized effluent, almost no effect of organic loading on ammonia removal was registered as ammonium removal was above 80% (Fig. 6.3). This finding indicates that although organic matter inhibited the anammox process performance in both cases-post-digested and partially

oxidized effluent, a partial nitrification improved the ammonium removal when a high concentration of organic matter is presented in the effluent.

## **6.4. CONCLUSIONS**

Organic loadings negatively affected anammox process performance, as confirmed by low ammonium removal rates obtained. Loading rates above 112 mg COD L<sup>-1</sup> day<sup>-1</sup> for UASB-post-digested effluent and above 136 mg COD L<sup>-1</sup> day<sup>-1</sup> for partially oxidized effluent, inhibited ammonium removal and decreased anammox bacterial numbers, due to denitrifier competition. Anammox and denitrification always occurred simultaneously showing that both processes could coexist in the same environment. So, environmental conditions (COD, nitrite, nitrate, ammonium, pH, and temperature) have to be controlled to get a good balance between anammox and denitrification communities.

In this study we demonstrated that livestock wastewaters can be successfully treated by the anammox process. However, the COD concentration in the wastewaters treated by anammox in full-scale plants determines whether anammox or denitrification would be the dominant route for ammonia removal.

## **Acknowledgements**

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## *Chapter 7*

### **Performance comparison of two photobioreactors configurations (open and closed to the atmosphere) treating anaerobically degraded swine slurry**

**Molinuevo-Salces, B., García-González, M.C., González-Fernández, C.**

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**Abstract.** The purpose of the study was comparison of two configurations of photobioreactors; an open-type photobioreactor, open to atmosphere and a tubular type photobioreactor, closed to the atmosphere. Organic matter was fairly removed under both configurations at 50-60% and biomass carbon content on dry weight basis accounted for 45%. Both configurations were able to completely exhaust ammonium, however different mechanism removals were responsible for the different influent loads applied. In terms of nitrogen recovery by biomass assimilation, the open configuration ranged 38-47% whereas the closed type presented 31%. It is worth to mention that nitrification-denitrification was taking place under both photobioreactor configurations. Approximately 80% phosphate removal was achieved regardless the configuration and biomass P content was slightly higher in the closed-type reactor. For nutrient recycling, biomass harvesting is described as the key issue of this technology. Nevertheless, the closed configuration highlighted the great potential of the biofilm formation by retaining 96% of the total biomass produced.

**Resumen.** El objetivo del estudio fue comparar dos configuraciones de fotobioreactor, uno de ellos una laguna abierta a la atmósfera y el otro un reactor tubular y cerrado a la atmósfera. Se obtuvieron eliminaciones de DQO del 50-60% registrándose un contenido en carbono (peso seco) en la biomasa obtenida del 45%. El amonio fue totalmente eliminado en ambos tipos de fotobioreactor aunque los mecanismos de eliminación fueron distintos en las distintas cargas aplicadas. En el reactor abierto se recuperó un 38-47% de nitrógeno en forma de biomasa mientras que en el cerrado solo se recuperó un 31%. Además, el proceso de nitrificación-desnitrificación tuvo lugar en ambos reactores. Se eliminó en torno al 80% de fósforo en ambos casos siendo ligeramente menor la asimilación del fósforo por parte de la biomasa en el caso del reactor cerrado. En cuanto a recuperación de nutrientes en forma de biomasa para posteriores usos, el fotobioreactor cerrado presentó una ventaja ya que el

96% del total de biomasa producida se encontró formando parte de una biopelícula adherida a las paredes del dicho reactor.

## 7.1. INTRODUCTION

Piggery effluents present serious concerns such as soil acidification or water bodies eutrophication. These problems may be reduced through a better management of this high strength wastewater. Traditional practices include both anaerobic and aerobic treatments. The first technology is well known as an energy source in the form of methane, however anaerobic effluents still present a high nutrient concentration that requires further treatment. On the other hand, the aerobic treatment is able to reduce organic matter and nutrients by supplying mechanical aeration to the system and stripping gases such as CO<sub>2</sub> and NH<sub>3</sub> or N<sub>2</sub>O to the atmosphere. In order to achieve sustainable technologies it is necessary to maximize the nutrients recovery and to strongly reduce gas emissions. Nutrient transfer into phyto-biomass such as microalgae is among the main technologies proposed by Lens et al. (2001) for nutrients recovery.

Microalgal technology offers several advantages over conventional treatments including recovery of nutrients and CO<sub>2</sub> release avoidance due to their autotrophic metabolism. Furthermore when consortia of microalgae and bacteria are employed as the degrading microorganisms a symbiotic relation takes place. In one hand, bacteria produce the CO<sub>2</sub> needed for microalgae growth whereas microalgae supplies oxygen produced photosynthetically to the bacteria. Thus, with this type of microorganisms consortia mechanical aeration may be eliminated of the process. It should be stressed that according to Oswald (1995) a mechanical aerated ponds requires 0.8-6.4 kW h kg BOD<sup>-1</sup> removed while photosynthetically oxygenated ponds consumes 0-0.57 kW h kg BOD<sup>-1</sup>.

During last decades ponds were the most usual and cost effective reactors employed regarding microalgae technology (Oswald, 1995; Nurdogan and Oswald, 1995; Garcia et al., 2000). However, three major drawbacks were observed on this type of photobioreactors. Due to the high pH achieved during photosynthesis by CO<sub>2</sub> consumption out the medium, ammonia may be stripped instead of assimilated or converted to NO<sub>x</sub>. In addition, the ponds

require a constant agitation in order to avoid gradients and provide light homogeneously to the microalgae. The third main inconvenient is related to the harvesting process of the suspended cells. New technologies are being developed to overcome those limitations. Among them, immobilisation of microalgae in different polymers has been already proven (Jiménez-Pérez et al., 2004; Travieso Córdoba et al., 1995). However, long term use is not guarantee since the polymers usually are degraded (Travieso Córdoba et al., 1995). Another immobilisation strategy is the possibility of growing the biomass in the photobioreactor's wall. In that manner a bio-film is formed due to the microalgae natural adherence ability (Craggs et al., 1997). This type of closed photobioreactor not only overcome the harvesting problem but also provides a high oxygen production to the medium (Torzillo et al., 2003).

Research has been done employing different photobioreactors with different types of wastewater. The results presented in those investigations exhibits each photobioreactor advantages but comparison of two photobioreactors types, open to the atmosphere and closed type, treating the same wastewater has not been conducted yet. Microalgal studies have consistently exhibited efficient removal of nitrogen and phosphorus; however influent concentrations were far below the ones employed herein. Furthermore a wide number of those investigations involve the bubbling of air into the medium for CO<sub>2</sub> supplementation. The present research uses the symbiotic relationship taking place between bacteria and microalgae, thus avoiding any external air supply device.

Therefore, the aim of this study was the comparison of two photobioreactor types (open and closed to the atmosphere) with regard to the effluent and biomass quality. To fulfil this goal increasing loads of swine anaerobically digested slurry (ADS) was fed to both reactors and evaluated for organic matter, nitrogen and phosphorus removal. In addition, carbon, nitrogen and phosphorus uptake was also analyzed to offer an in-depth study which allows the understanding of the different mechanisms nutrients removal depending upon the photobioreactor employed.

## **7.2. METHODS**

### 7.2.1. Microorganisms and culture conditions

Wildly grown microalgae were collected from a lagoon containing aerobically treated swine slurry located in Cuellar (Segovia).

Microalgae were identified microscopically as *Oocystis* sp. and *Scenedesmus* sp.

The aerobic bacteria were obtained from a swine slurry degrading sequencing batch reactor operated at hydraulic retention time (HRT) of approximately 9 days. Prior to inoculation, microalgae and bacteria were centrifuged at 9000 rpm for 10 min and resuspended in distilled water.

### 7.2.2. Substrate composition

Swine manure was obtained from an anaerobic digester working at HRT of 25 days. The effluents were centrifuged and the supernatants diluted to the desired loading rate. The average composition of the centrifuged ADS was  $3858 \pm 915$  mg COD L<sup>-1</sup>,  $1664 \pm 367$  mg N-NH<sub>4</sub><sup>+</sup> L<sup>-1</sup>,  $6.1 \pm 1$  mg TS L<sup>-1</sup> and  $2.5 \pm 0.5$  mg VS L<sup>-1</sup>. The slurry was fed into the photobioreactor at increasing ammonium loading rates (ALR). The inlet concentration was set in accordance to the ammonium concentration since it has been previously addressed as an inhibitory compound (González et al., 2008a).

### 7.2.3. Photobioreactors

The experimental set-up consisted of two different reactors, namely an open pond and a tubular enclosed photobioreactor. Both reactors had a total working volume of 6 L and were operated at HRT of 8.5 days. Each photobioreactor was constantly illuminated using four fluorescents lamps at 12,000 lx (Philips 50 W). The lightning of the reactor provided also heating of the cultivation medium. Thus, daily measurements of temperature resulted in 35

$\pm 7$  and  $30 \pm 2$  °C for the closed and open photobioreactors, respectively. Dissolved oxygen (DO), pH and temperature were monitored in situ.

Both photobioreactors were initially filled with tap water and inoculated with 70 and 35 mg VSS L<sup>-1</sup> of microalgae and activated sludge bacteria, respectively. Right after inoculation, the reactors were fed with diluted ADS.

A complete description of the closed photobioreactor may be found elsewhere (González et al., 2008b). The culture broth was recirculated inside the tube at 90 mL min<sup>-1</sup>. Regarding the open photobioreactor, the culture broth was gently suspended by means of magnetic stirrers. Therefore, both reactors may be considered as completely-mixed reactors. In the case of the open-type photobioreactor, the volume was daily checked and any water lost due to evaporation was corrected. From now on, sampling periods from O1 to O5 will stand for the open and C1 to C5 for the closed-type photobioreactor.

The final effluent was collected in a settler (0.75 L) for biomass sedimentation. Biomass was withdrawn periodically from the bottom of the settler (Table 7.1). This purge was analyzed for total solids (TS), volatile solids (VS), total carbon (TC), total Kjeldahl nitrogen (TKN) and total phosphorus (TP) at the different loading rates. Liquid samples of the diluted ADS influent and effluent from the top of the settler were periodically withdrawn in order to monitor the pH, total chemical oxygen demand (COD), soluble COD, TP, soluble phosphorus (P sol), TKN, ammonium (N-NH<sub>4</sub><sup>+</sup>), nitrate (N-NO<sub>3</sub><sup>-</sup>) and nitrite (N-NO<sub>2</sub><sup>-</sup>).

#### **7.2.4. Chemical analysis**

DO and temperature in the bioreactor were determined using a Multiline P4 Oxical-SL Universal Meter (WTW, Germany). A Crison micropH 2002 (Crison Instruments, Barcelona, Spain) was used for pH determination. TS, VS, TKN, P sol and COD were also analyzed according to Standard Methods (Eaton et al., 2005). TC was measured using a

Shimadzu TOC-5050A analyzer (Japan).  $\text{N-NH}_4^+$ ,  $\text{N-NO}_3^-$  and  $\text{N-NO}_2^-$  concentrations were determined using electrodes, Orion 900/200 (Thermo Electron Corporation, Beverly, USA)

### 7.3. RESULTS AND DISCUSSION

#### 7.3.1. Biomass growth under both photobioreactor configurations

Nutrients (N and P) removal is directly linked to photosynthetic activity whereby also related to biomass production. Biomass growth was measured as the dry weight (total solids) of biomass produced per day and litre of reactor. In the case of the open-type photobioreactor, the average daily biomass production increased gradually with ALR. More specifically, biomass production increased from 0.051 to 0.332 g dry weight (DW)  $\text{L}^{-1} \text{day}^{-1}$  through O1 to O4. However during O5, this parameter decreased to 0.173 g DW  $\text{L}^{-1} \text{day}^{-1}$  likely due to the high ALR applied.

**Table 7.1.** Biomass growth under both photobioreactor types

Load	g DW L <sup>-1</sup> d <sup>-1</sup>	Load	g DW L <sup>-1</sup> d <sup>-1</sup>
O1	0.051	C1	
O2	0.157	C2	0.001
O3	0.210	C3	
O4	0.332	C4	0.007
O5	0.173	C5	0.007

As mentioned before one of the major drawbacks is the cost associated with the biomass harvesting. In this context, the closed-type photobioreactor overcome this limitation as seen in Table 7.1. Through C1 to C3, almost no biomass withdrawn was required since all the biomass produced was attached to the photobioreactor's wall. A slightly higher production was achieved during C4 and C5 (0.007 g DW  $\text{L}^{-1} \text{day}^{-1}$ ) but still far below biomass produced in the open-type reactor. The biomass accumulated for biofilm formation accounted for 96% of the total biomass produced. This high biomass retention was achieved by keeping the superficial liquid velocity at 0.01  $\text{m s}^{-1}$ , hence avoiding biofilm detachment (González et al.,

2008). At the end of the experimentation time, the biofilm was scrapped out mechanically. The dried biomass production for the whole experimentation time (121 days) accounted for  $0.163 \text{ g DW L}^{-1} \text{ day}^{-1}$ .

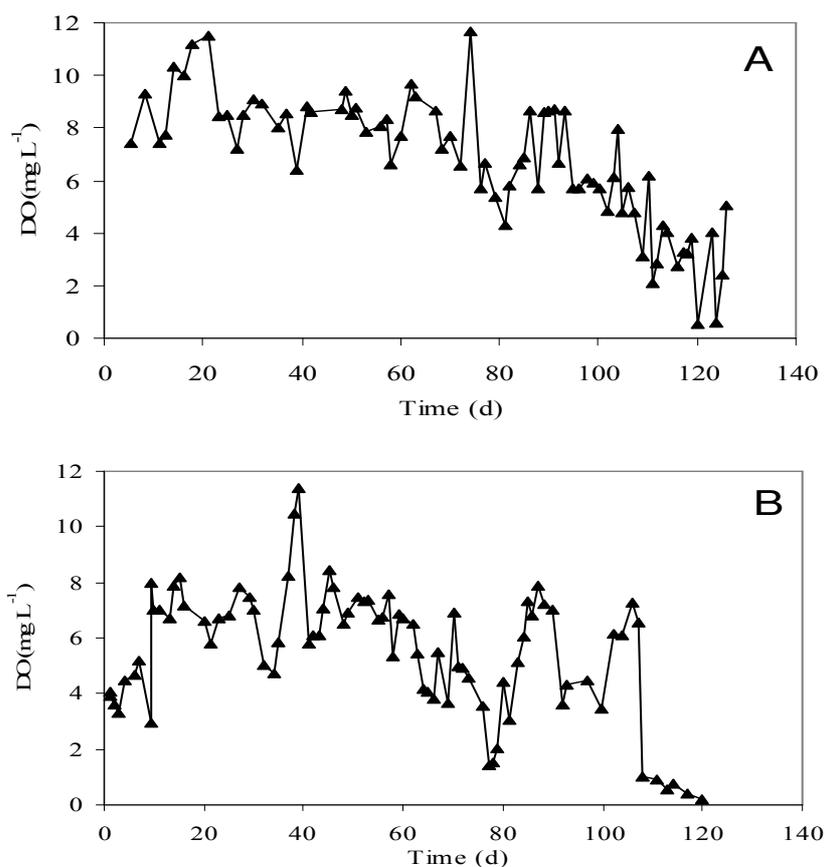
### 7.3.2. Organic matter removal under both photobioreactor configurations

COD concentrations increased concomitantly with the increasing ammonium loads (Table 7.2). The open-type reactor showed different COD removals depending mainly on two factors, namely the soluble COD accounting for total COD and the dissolved oxygen measured in the medium. During the first load O1, 37% and 42% soluble and total COD removal was achieved. These low removals correspond to the start-up of the reactor and biomass acclimatization. Moreover, during O1 soluble COD accounted for 61% of the total COD, whereas for the rest of the loads accounted for approximately 80%. Soluble COD represents the most easily fraction of organic matter to be mineralized by the consortia. Even though microalgae may remove organic matter this degradative process is mainly carried out by heterotrophic bacteria. Furthermore, total COD requires a first hydrolysis step prior to be degraded by the consortia and therefore depends strongly on DO.

**Table 7.2.** Organic matter concentration and removal efficiencies through the different loads obtained under both photobioreactor types.

Load	INFLUENT		%REMOVED IN THE EFFLUENT	
	COD tot (mg/L)	COD sol (mg/L)	COD tot (mg/L)	COD sol (mg/L)
O1	305.8 ± 63.3	186.1 ± 38.2	42.0 ± 7.5	37.0 ± 15.6
O2	484.9 ± 66.7	374.3 ± 86.6	52.9 ± 5.6	50.2 ± 6.9
O3	816.8 ± 63.7	686.4 ± 93.3	57.6 ± 6.5	60.2 ± 8.5
O4	1181.9 ± 249.0	960.1 ± 154.9	46.9 ± 8.1	51.8 ± 8.6
O5	1806.4 ± 286.0	1529.8 ± 291.8	38.7 ± 10.9	36.8 ± 12.0
C1	247.7 ± 22.7	208.2 ± 46.2	54.9 ± 7.9	45.7 ± 5.9
C2	672.3 ± 94.3	612.2 ± 56.1	47.0 ± 8.5	50.4 ± 5.6
C3	1015.5 ± 124.3	880.5 ± 83.9	51.7 ± 3.4	48.6 ± 19.4
C4	1252.6 ± 204.1	1064.8 ± 243.2	67.22 ± 5.9	53.2 ± 9.9
C5	1439.9 ± 75.5	1241.1 ± 190.2	60.5 ± 7.9	40.9 ± 19.9

The highest COD removals were achieved during O2 and O3, 52.9% and 57.6%, respectively. These results were slightly lower than the obtained (70%) in open photobioreactors treating piggy effluents (de Godos et al., 2009). COD removals decreased concomitantly with increasing loads. During O4 and O5, lower medium DO concentrations were recorded corresponding to higher ALR. More specifically, DO averaged 8.3 and 7.3 for O2 and O3, while during O4 and O5 the OD measured was 5.5 and 2.9, respectively (Fig. 7.1A).



**Figure 7.1.** Dissolved oxygen measured in situ in (A) the open type photobioreactor and (B) the closed type photobioreactor.

In the case of the closed-type photobioreactor, soluble COD accounted for 85-90% total COD over the whole experimental period. Fifty percentage of soluble COD and total COD were steady removed regardless the load during the three first loads (C1, C2 and C3). As it can be seen in Fig. 7.1B, DO decreased from 6 to 7 mg O<sub>2</sub> L<sup>-1</sup> to around four during C4 and C5, however during these lasts loads higher total COD removals (>60%) were achieved. The efficiency of this photobioreactor was underestimated during the first loads and probably biomass growth was limited by influent concentration. Slightly higher values (70% total COD removal) were shown by González et al. (2008b) when operating the same type of photobioreactor. However, it should be noted that in the present study anaerobically digested manure was treated and therefore organic matter was hardly degradable compared with the fresh manure treated by González et al. (2008b).

### **7.3.3. Ammonium removal under both photobioreactor configurations**

As previously stated, ammonium in the influent was employed to determine the different loads applied to the photobioreactors. ALR in the open-type reactor ranged from 15.4 to 80.4 mg N-NH<sub>4</sub><sup>+</sup> L<sup>-1</sup> day<sup>-1</sup>. Ammonium was almost exhausted during the tree first loads. Nevertheless, the N-NH<sub>4</sub><sup>+</sup> concentration at the effluent gradually increased together with further increase ALR. In that manner, N-NH<sub>4</sub><sup>+</sup> was removed up to 93.8% and 88.3% for loads corresponding to O4 and O5, respectively. This drop in ammonium removal was likely due to substrate/microorganisms imbalances at such high ALR. At this point, it should be stressed that during this experiment high ammonium concentration was fed to the reactors. No inhibition of the process was detected since an extension of HRT to 10 days was enough to decrease the ammonium effluent concentration (data not shown). Furthermore, ammonia inhibition was reported as inhibitory compound at pH higher than 8 (Abeliovich and Azov, 1976) which was not the case of O4 and O5.

Due to the strong photosynthetic activity, CO<sub>2</sub> of the medium was rapidly consumed and therefore pH increased markedly during O1 and O2. The high pH reached during this loads caused ammonia stripping. In order to quantify theoretically the fraction that was stripped, the free ammonia concentration of the different loads were calculated according to Hansen et al. (1998). The results showed that free ammonia volatilization was the main driven force of ammonium removal during O2 (Table 7.3). The ammonium influent concentration (72.4%) was stripped out during this stage. Further ALR increase resulted in a decrease of ammonia volatilization and nitrification became an important mechanism for ammonium removal. No nitrification was observed during the two initials loads. However, N-NO<sub>x</sub> steadily increased at increasing loading rates. In this context, ammonium was nitrified and accounted for 3.7%, 23.2% and 33.9% of the ammonium initial concentration for O3, O4 and O5, respectively. The pH of these later loads ranged from 6.6 to 7.9, indicating that more equilibrated activity was reached between bacteria and microalgae. Therefore, even though ammonia volatilization (Garcia et al., 2000) and assimilation (Wolf et al., 2007) had always been thought as the main mechanism for ammonium removal in open ponds, the present study demonstrated that denitrification also may take place when pH was close to neutrality. It should be noted that denitrification requires low DO and O3 presented medium DO of 7 mg L<sup>-1</sup>. Nevertheless, DO may be much lower inside the flocks formed during the treatment (De Kreuk et al., 2005). Additionally, even though nitrate is not the preferred nitrogen form for microalgae uptake (Guerrero and Lara, 1987; Travieso et al., 2006) this fact may also have contributed to the nitrogen removal from the medium.

A general overview of the ammonium removal in the open type reactor showed that ammonia stripping was the main driving force for ammonium removal during the first two loads. Further increases in influent loads resulted in a strong nitrification together with an increasing nitrogen content of the biomass in each load. The overall balance of nitrogen balance during O3 and O4 (which where the loads were completely removal of ammonium was achieved) showed that N assimilation on biomass accounted for 38% and 47%, whereas 52% and 29% of the ammonium was denitrified.

Likewise, in the case of the closed-type photobioreactor fairly good removals (>80%) were obtained during the three initial loads (C1, C2 and C3). Removal rates were even higher for higher ALR, namely 99.8% and 98.8% for C4 and C5, respectively. As seen in Table 7.3 during load C1, the same trend as in O1 and O2 was observed. The high photosynthetic activity resulted in a high pH which subsequently stripped ammonia out of the system (50.7%). Ammonia stripping accounted with lower values for the rest of the loads as shown in Table 7.3. The oxidation of ammonium to N-NO<sub>x</sub> was attained from the second load C2 onwards. In fact during C3 nitrification was maximum and 34.7% of the ammonium was oxidised. This strong nitrification activity resulted in a decrease of pH to 6.7. However, pH was restored to around 8.0 in the following two loads together with a nitrified fraction diminishment with increasing ALR (C4 and C5).

**Table 7.3.** Ammonium concentration of the influent, total removal efficiencies, ammonium removed by stripping and by nitrification through the different loads obtained under both photobioreactor types.

Load	Mean T in situ	Mean Ph in situ	N-NH <sub>4</sub> <sup>+</sup> (mg L <sup>-1</sup> )	% Removed N-NH <sub>4</sub> <sup>+</sup>	% Removed by stripping	% Removed by nitrification
O1	28.9	8.6	131.9 ± 21.1	97.8 ± 3.4	23.6	0
O2	29.9	9.5	185.6 ± 17.9	99.9 ± 0.2	72.4	0
O3	29.4	7.9	355.9 ± 48.4	99.3 ± 1.0	5.4	3.7
O4	31.2	6.6	496.6 ± 50.4	93.8 ± 1.9	0.4	23.2
O5	31.5	7.6	689.0 ± 71.5	88.3 ± 2.9	3.2	33.9
C1	33.8	9.0	95.7 ± 16.8	84.7 ± 6.2	50.7	0
C2	33.8	7.5	259.7 ± 27.7	80.1 ± 5.0	3.1	22.5
C3	34.4	6.7	392.2 ± 61.1	89.4 ± 1.8	0.5	34.7
C4	38.3	8.0	493.2 ± 69.9	99.8 ± 0.5	12.1	7.2
C5	31.0	8.0	735.8 ± 23.3	98.8 ± 2.1	7.8	3.0

Denitrification was also detected in the closed photobioreactor. Bubbles were accumulated and released periodically in order to keep a constant hydraulic volume within the tube. At the last stage of the experimentation period some gas bubbles were sampled. The gas analysis confirmed that nitrogen gas was the main gas contained in those bubbles. Once again, even though DO monitored in situ showed values over 3 mg O<sub>2</sub> L<sup>-1</sup> (Fig. 7.1B), the biofilm formation in this type of reactor resulted in a DO gradient within the tube. In that

manner close to the tube wall anoxic microzones may be attained whereas oxygen produced by microalgae diffused into the liquid bulk (Muñoz and Guieysse, 2006; Toet et al., 2003). Nitrate uptake may also account for nitrate decrease along the loads. Indeed, Lau et al. (1998) indicated that nitrate was taken up similarly by immobilized and free cells.

A general overview of the ammonium removal in the closed type reactor showed that ammonia stripping was important only during the first load. The following two loads also resulted in a high percentage of ammonium being nitrified; however the last two loads showed a decrease in N-NO<sub>x</sub> which was caused by a simultaneous nitrification–denitrification process. In the closed-type reactor, the overall nitrogen balance was not carried out by loads since biomass was getting attached to the walls, hence hindering the N uptake at each load. However, the net nitrogen balance of the system during the 121 days of experimentation showed that 10.5% was stripped out, 11.3% nitrified, 31.3% assimilated, 41% denitrified and 5% still remained in the effluent.

#### **7.3.4. Phosphate removal under both photobioreactor configurations**

Phosphate is removed from the medium mainly by biomass uptake. Microorganisms' consortia employed PO<sub>4</sub><sup>-3</sup> for metabolic activities. The biomass uptake of soluble form of phosphorus may be enhanced by sustained high DO. In fact, PO<sub>4</sub><sup>-3</sup> “luxury uptake” has been described as a phenomenon by which microorganism store within the cells more PO<sub>4</sub><sup>-3</sup> than the strictly required for their growth (Powell et al., 2008). Thus, luxury uptake was expected to occur since both reactors presented constantly DO higher than 2 mg L<sup>-1</sup> (Fig. 7.1).

In the case of the open-type photobioreactor, PO<sub>4</sub><sup>-3</sup> was removed to approximately 80% regardless the ALR applied except for the last load (Table 7.4). During O5, PO<sub>4</sub><sup>-3</sup> removal dropped to 54.3% in accordance with the decrease of ammonium removal at the same load. At this last stage of the experiment, microorganisms were not able to completely remove none of both nutrients. The high loads applied together with the relatively short HRT

employed for this type of consortia may be the reason of such behaviour. It should be noticed that high pH attained during the two first loads (O1 and O2) may involved  $\text{PO}_4^{3-}$  precipitation (Nurdogan and Oswald, 1995). Therefore, those removal percentages presented for O1 and O2 may be not only caused by biological processes but chemical precipitation as well. In addition, removals reached were high compared with 10% reported by de Godos et al. (2009) who worked with high rate ponds.

**Table 7.4.** Soluble P concentration and removal efficiencies through the different loads obtained under both photobioreactor types.

Load	INFLUENT	% REMOVED IN THE EFFLUENT
	P sol (mg/L)	P sol (mg/L)
O1	5.3 ± 3.2	79.6 ± 16.0
O2	9.7 ± 3.1	79.1 ± 8.4
O3	19.2 ± 6.4	75.9 ± 11.0
O4	25.5 ± 5.8	79.2 ± 2.1
O5	30.4 ± 2.4	54.3 ± 11.7
C1	3.8 ± 1.9	81.3 ± 5.3
C2	13.2 ± 3.2	84.1 ± 6.1
C3	15.6 ± 1.1	82.7 ± 9.9
C4	24.0 ± 4.3	74.9 ± 5.4
C5	34.3 ± 5.4	73.2 ± 8.7

The closed-type photobioreactor exhibited similar removals as the open type. The removal percentage for C1 was likely affected as well by  $\text{PO}_4^{3-}$  precipitation due to the high pH reached during this load. The only remarkable fact was the decrease during C5 that was caused by the sharp decrease of DO at the last stage of this experiment. During the last samplings of the closed photobioreactor, DO was below  $1 \text{ mg O}_2 \text{ L}^{-1}$  which caused some  $\text{PO}_4^{3-}$  release (Fig. 7.1B). Oxygen limitation in the bulk medium has been previously shown to be an important factor that may hinder  $\text{PO}_4^{3-}$  removal (González et al., 2008b).

### 7.3.5. Carbon, nitrogen and phosphorus biomass uptake under both photobioreactor configurations

Microalgae are receiving considerable attention nowadays due to their high potential for CO<sub>2</sub> mitigation. These microorganisms employ the fixed CO<sub>2</sub> to produce carbohydrates, lipids, proteins and nucleic acids (Spolaore et al., 2006). The cell carbon content depends on the microalgae strain (Hase et al., 2000) as well as on different cultivation conditions (Ruiz-Marín et al., 2010). In the case of the open-type photobioreactor the percentage of carbon (%C) on dry weight biomass ranged 43.3-48.4 (Table 7.5). The C content was maximum during O2 and O3 which were proven as the two loads where highest degradative activity was achieved by the highest N-NH<sub>4</sub><sup>+</sup> and COD removals. These values were in accordance with data reported by Hase et al. (2000) who calculated 46% C for microalgae grown in open raceway reactor.

Nitrogen is removed out of the medium by microbial assimilation and converted to mainly proteins (Mitchell and Richmon, 1988). As seen it can be seen in Table 7.5, N content accounted for 7-8% of the dry biomass obtained from the open-type photobioreactor. Those values were slightly higher than the ones (5-7%) reported for microalgae grown in open air turfs (Pizarro et al., 2002). In the case of the closed type, the percentage increased up to 10%. Nitrogen assimilation was higher in this type of photobioreactor likely due to the higher nitrogen availability in the medium in this configuration whereas in the open one an important percentage was stripped out.

Microbial assimilation of phosphorus includes the formation of phospholipids, nucleotides and nucleic acids for microorganism's growth. Factors such as temperature and light intensity (Powell et al., 2008) or substrate nature (Wilkie and Mulbry, 2002) have been described to affect P assimilation. In our particular case, light intensity was constant while temperature slightly varied (Table 7.3). The open reactor operated at approximately 30°C, while the closed one ranged 31-38°C. Even though higher temperature would result in higher metabolic activity, the opposite behaviour was observed when comparing both photobioreactors. The open-type photobioreactor exhibited an increasing P biomass uptake together with increasing loading rates (Table 7.5). In this context, P content in the dry biomass increased from 1.2% to 2.2% through O1 to O4. Nevertheless, P content decreased

concomitantly with a decrease in  $\text{PO}_4^{-3}$  removal during the last loading O5. The closed-type photobioreactor did not show any clear tendency regarding P content of the biomass. However, a lower amount of P per gram of dried biomass was obtained when compared to the open reactor. This slight difference may be attributed to how biomass was grown. In this context, Ruiz-Marín et al. (2010) reported a higher P biomass uptake by free *Scenedesmus obliquus* compared to immobilized ones. Thus, in the present study the different configuration of reactors, namely open type (suspended biomass) and closed type (biofilm attached biomass) can be inferred as the reason for such an small increase in the P uptake of the open-type photobioreactor. The thick biofilm formed on the closed photobioreactor likely triggered somehow light diffusion and hence P uptake decreased.

**Table 7.5.** Biomass uptake in terms of C, N and P through the different loads obtained under both photobioreactor types

<b>Biomass uptake (mg/g DW)</b>			
<b>Load</b>	<b>TC</b>	<b>TKN</b>	<b>TP</b>
<b>O1</b>	438.6	69.6	11.7
<b>O2</b>	484.1	76.5	17.2
<b>O3</b>	471.5	75.2	18.0
<b>O4</b>	457.1	82.1	22.3
<b>O5</b>	433.1	79.6	12.7
<b>C1</b>		.	
<b>C2</b>	439.3	100.3	12.5
<b>C3</b>		.	
<b>C4</b>	388.5	85.8	10.8
<b>C5</b>	404.2	88.1	11.0

Microbial analysis of the culture broth in the two photobioreactors revealed that the initial species *Oocystis sp.* and *Scenedesmus sp.* were outcompeted at the end of the experiment by mainly *Oocystis sp.* together with *Chlorella sp.* and *Protoderma sp.* in the case of the open pond and by *Protoderma sp.*, *Chlamydomonas sp.*, *Chlorella sp.* and a lesser amount of *Oocystis sp.* in the case of the closed tubular reactor. Therefore, the different conditions encountered under the different reactors produced changes in microalgal community and

probably in the removal mechanisms as well. Unfortunately, the identification of this fact was out of the scope of this research.

## **7.4. CONCLUSIONS**

Anaerobically digested swine slurry was fed at increasing ALR to two different photobioreactors (open to the atmosphere where biomass grown suspended and closed tubular photobioreactor involving the biofilm formation). Closed type presented an almost cell-free effluent during the first loads since all the biomass was getting attached to the reactor's walls. As expected, biofilm formation was proved as a useful tool to avoid harvesting costs.

Organic matter was similarly removed under both configurations. With regard to nitrogen, even though both reactors were able to achieve complete depletion of ammonium, different mechanism removals were elucidated depending on the photobioreactor type. Consortia demonstrated a higher efficiency in ammonium removal than in phosphate in both photobioreactors.

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## *Chapter 8*

### **Conclusions**



## CONCLUSIONS

Concentrated livestock farming in Castilla y León is leading to the production of high amounts of livestock needing stabilisation. In the present research work, anaerobic digestion was presented as a proper solution offering several advantages as energy production, organic matter reduction and stabilisation. Moreover, Anammox and microalgae-based treatments were proven to be effective methods for removing nitrogen from anaerobically degraded swine manure. The following conclusions can be drawn from the present study:

1. Co-digestion of manures with vegetable processing waste (VPW) was evaluated by a central composite design of experiments followed by response surface methodology analysis (*Chapter 3*). Two different factors; the content of VPW added to the mixture and the substrate concentration were examined for two responses, methane yield and volatile solid (VS) removal. After studying anaerobic co-digestion under batch conditions for swine manure (SM) and VPW it was concluded that the addition of VPW resulted in increments of methane yield and VS removal. C/N ratios were enhanced, thus increasing biodegradability of the wastes resulting in a higher biogas potential. However, the degradation of SM registered a lack of buffer capacity to overcome total volatile fatty acids (TVFA) accumulation in some of the evaluated mixtures. Regarding the co-digestion of poultry litter (PL) and VPW, it was observed that substrate concentration was the major factor affecting the responses. Values greater than 80 g VS L<sup>-1</sup> resulted in N-NH<sub>3</sub>-mediated inhibition, thus reducing methane yields as well as VS removal. When studying the effect of VPW addition to semi-continuous anaerobic digestion of SM, a similar tendency was found (*Chapter 5*). Hence, it was observed that biogas production and methane content were increased as compared with results obtained from the anaerobic digestion of swine manure. Once again, the increase in the easily biodegradable fraction within the substrate led to better biogas results.

2. Wastes co-digestion avoided potential inhibitors such as pH drops and TVFA accumulation due to the high buffer capacity of manures. However, partial TVFA-mediated inhibition resulted in a lag phase leading to a delay in methane production. In this context, substrate/biomass was found to play an important role. On the other hand,  $N-NH_3$ , which is considered as a potential inhibitor, was found to hinder the process in some of the tested mixtures under batch digestion.

3. The degradation of the lignocellulosic complex under anaerobic conditions was studied under batch co-digestions of SM-VPW and PL-VPW. Hemicelluloses were completely depleted, 50% of the cellulose was removed and the lignin was not degraded under anaerobic treatment. Moreover, lignin could have hindered cellulose bioavailability for exoenzyme attack resulting in the low removal percentage of cellulose. Scanning Electron Microscopy (SEM) was shown to be a helpful tool for observing the degradation of wastes, concluding that lignin was present after the anaerobic treatment (*Chapter 4*).

4. Microorganisms involved during the digestion process were studied by means of SEM. It was possible to study changes in the spatial distribution and morphology from the initial to the final stages. It was observed that after anaerobic digestion, bacteria morphology changed from a filamentous shape to cocci and bacilli (*Chapter 5*).

5. Besides the valorisation of the wastes by production of methane and the reduction of organic matter, a final product named “digestate” was also obtained from the anaerobic process. Anaerobic digestate could be used as soil amendment. Regarding this possible use, thermal analysis was used to evaluate the transformation of organic matter. Anaerobic digestion was proven to be a suitable method for achieving the stabilisation of the organic wastes. It was found that co-digestion resulted in digestate with a greater content of carbohydrate type materials (*Chapter 4*).

6. The Anammox process and microalge-based technology was shown to be a suitable alternative to conventional nitrification-denitrification processes for nitrogen removal from

digested SM. Digestates were successfully treated by both processes. Ammonium and nitrite were removed by up to 99% when treating digestates with Anammox technology (**Chapter 6**). The dominant process for ammonium removal was determined by the concentration of chemical oxygen demand (COD) in the influent. The COD concentration threshold was found to be at 112 mg COD L<sup>-1</sup> d<sup>-1</sup> for anaerobically digested swine manure. However, if partial aeration was applied as pre-treatment for enhancing Anammox activity, the inhibitory threshold increased up to 136 mg COD L<sup>-1</sup> d<sup>-1</sup>.

7. Regarding the microalgae-based process (**Chapter 7**), complete depletion of ammonium, 80% removal of phosphorus and 60% for COD was achieved. Different ammonium removal mechanisms were observed within the different reactor configurations and the different applied loads. Nutrients were assimilated by microalgae-bacteria consortium achieving a valuable product which could be easily harvested in the case of the closed biofilm reactor, since it was attached to the reactor wall.

## CONCLUSIONES

La alta carga ganadera en determinadas zonas de Castilla y León está dando lugar a un gran producción de residuos, los cuales deber ser tratados. En este trabajo de investigación se presenta el proceso de digestión anaerobia como una posible solución al problema, ya que presenta diversas ventajas como son la alta producción energética, la reducción del contenido de materia orgánica y la estabilización del residuo. Además, se estudian los procesos de anammox y tratamientos con microalgas con el objetivo de tratar los nutrientes presentes en el purín degradado anaeróbicamente. A continuación se presentan las conclusiones generales obtenidas en este trabajo de investigación:

1. Se evaluó en discontinuo la co-digestión anaerobia de dos residuos ganaderos (purín de cerdo y gallinaza de ponedora) con residuos del procesado de vegetales (VPW) mediante un diseño central compuesto y un análisis de superficie de respuesta (*Capítulo 3*). Se examinaron dos factores (contenido en VPW y concentración inicial de sustrato) sobre dos respuestas (rendimiento de producción de metano y eliminación de sólidos volátiles (SV)). De la digestión de purín y VPW se concluyó que la adicción de VPW afectaba positivamente a ambos factores debido a la obtención de mejores relaciones carbono/nitrógeno (C/N), lo cual incrementó la biodegradabilidad del material mejorando los resultados. En el caso de la digestión de gallinaza y VPW se observó que la concentración de sustrato determinó ambas respuestas evaluadas, así valores superiores a 80 g SV L<sup>-1</sup> registraron inhibiciones por amonio con la consiguiente disminución en dichas respuestas. Esta misma tendencia se encontró cuando se estudió la adicción de VPW a la digestión anaerobia de purín de cerdo operando en semi-continuo (*Capítulo 5*) observándose un aumento en la producción de biogás y en el contenido en metano.

2. Mediante la co-digestión de residuos se evitaron inhibidores potenciales del proceso como bajadas de pH o acumulación de ácidos grasos volátiles (AGV) debido a la capacidad tampón de los residuos ganaderos. Sin embargo, se observaron inhibiciones parciales por

AGV resultando en un retraso en la producción de metano concluyendo que la relación sustrato/microorganismo tiene un papel fundamental en el proceso. Por otro lado, el amonio, considerado otro inhibidor potencial en la digestión anaerobia de este tipo de residuos, fue el causante de la inhibición en algunas de las condiciones evaluadas.

**3.** Se estudió la degradación anaerobia del complejo lignocelulósico en las co-digestiones de purín-VPW y gallinaza-VPW (Capítulo 4). Las hemicelulosas y la celulosa se degradaron al 100% y 50%, respectivamente, mientras que la lignina no se degradó durante el proceso anaerobio. La biodisponibilidad de la celulosa para las exoenzimas pudo estar afectada por la lignina obteniéndose por ello un resultado tan bajo en la degradación de la celulosa. Para observar la degradación de los residuos se utilizó la microscopía electrónica de barrido (SEM) permitiéndonos concluir que la lignina se encontraba presente después del tratamiento anaerobio.

**4.** La microscopía electrónica de barrido también se utilizó para estudiar los microorganismos involucrados en el proceso anaerobio. Se estudió la distribución espacial y los cambios en la morfología de los mismos al inicio y al final del tratamiento, observando un cambio en la morfología de las bacterias, de formas filamentosas al inicio a formas cocoidales o bacilares al final (*Capítulo 5*).

**5.** Ya que el digestato tiene un alto potencial como fertilizante agrícola, se realizó un estudio de análisis térmico para evaluar la transformación de la materia orgánica, demostrando que la digestión anaerobia es un método adecuado para obtener material orgánico estable y que mediante la co-digestión se obtiene un digestato con mayor cantidad de carbohidratos (*Capítulo 4*).

**6.** Se estudiaron dos métodos alternativos a la nitrificación-desnitrificación obteniéndose buenos resultados en ambos casos. Mediante el primero de ellos, el tratamiento anammox (*Capítulo 6*), se obtuvieron eliminaciones de amonio y nitrito del 99% y se observó que el funcionamiento de dicho proceso depende de la concentración de materia orgánica en el

substrato. Para concentraciones superiores a  $112 \text{ mg DQO L}^{-1} \text{ d}^{-1}$  en purín degradado anaerobicamente se observó un descenso en la eficiencia del proceso anammox. Para mejorar dicho proceso, se aplicó aireación parcial como pre-tratamiento incrementando la concentración a  $136 \text{ mg DQO L}^{-1} \text{ d}^{-1}$ .

El segundo proceso estudiado fue un sistema de tratamiento con microalgas (**Capítulo 7**). Se obtuvieron eliminaciones de amonio, fósforo y materia orgánica del 100, 80 y 60%, respectivamente. Se observaron distintos mecanismos de m de nitrógeno dependiendo de la carga orgánica aplicada y del tipo de reactor utilizado. Por otro lado, se obtuvo una biomasa algal más fácilmente cosechable en caso del reactor biofilm cerrado. Dicha biomasa podría ser utilizada para diversas aplicaciones.

*Appendixes*

**Agradecimientos**

**Research impressions**



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## **RESEARCH IMPRESSIONS**

1. Belt scrapers where poultry litter waste was obtained.
2. Pig slurry tank.
3. Vegetable processing wastes (VPW).Vegetable processing factory.
4. VPW processed in the laboratory.
5. Removing oxygen from anaerobic Batch reactors.
6. Thermostated shaker with anaerobic Batch digestion.
7. Detailed picture of continuous stirred reactor (CSTR).
8. CSTR reactors used for anaerobic digestion.
9. Upflow anaerobic sludge reactor (UASB) for Anammox treatment.
10. Partial oxidation of digested swine manure.
11. Microalgae opened photobioreactor.
12. Microalgae biofilm photo bioreactor.

