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METHANE PRODUCTION THROUGH ANAEROBIC DIGESTION OF DEDICATED ENERGY CROPS

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List of abbreviations

AD: Anaerobic Digestion
AFEX: Ammonia Fibre Explosion
AIL: Acid Insoluble Lignin
AIR: Acid Insoluble Residue
ANOVA: Analysis of Variance
ARP: Ammonia Recycle Percolation
BD: Biodegradability
$B_{0;\,th}$: Theoretical Methane Potential
CED: Cumulative Energy Demand
COD: Chemical Oxygen Demand
DBY: Dry Biomass Yield
EE: Energy Efficient
FBY: Fresh Biomass Yield
FID: Flame Ionization Detector
FTIR: Fourier Transform Infrared Spectroscopy
GC: Gas Chromatographer
GHG: Greenhouse Gas
GE: Gross Energy yield
HMF: 5-HydroxyMethyl Furfural
HPLC: High Performance Liquid Chromatography
LHW: Liquid Hot Water
LOI: Lateral Order Index
LSD: Least Significant Difference
N: Normality
NE: Net Energy yield
PCA: Principal Component Analysis
RES: Renewable Energy Sources
RID: Refractive Index Detector
SAA: Soaking Aqueous Ammonia
SEM: Scanning Electron Microscopy
SNK: Student – Newman-Keuls test
SRB: Sulphate Reducing Bacteria
SRS: Sugar Recovery Standard
STP: Standard Temperature and Pressure
T_{80}: Technical digestion time
TCD: Thermal Conductivity Detector
TCI: Total Crystallinity Index
TKN: Total Kjeldahl Nitrogen
TOC: Total Organic Carbon
TS: Total Solids
VFA: Volatile Fatty Acid(s)
VS: Volatile Solids

Abstract

The rapid development of the biogas sector has fostered a growing use of energy crops (i.e., starch crops as cereals), raising competition for available land with food crops. To overcome this drawback, ligno-cellulosic substrates, such as dedicated non-food energy crops and agricultural residues, can be used. However, anaerobic digestion (AD) of ligno-cellulosic substrates may be limited by their composition and structural features. Hence, biomass chemical and physical-chemical pre-treatments are envisaged to overcome this constraint. In this light, this thesis aimed at: *i*) assessing biomass and methane yield, comparing alternative biomass crops with maize; *ii*) evaluating the effects of hydrothermal pre-treatments on methane yield of Arundo (a multi-annual biomass species); *iii*) investigating the effects of mild NaOH pre-treatments on chemical composition, physical structure and methane yield of two dedicated energy crops and one agricultural residue; *iv*) investigating the effects of organic acid (i.e., maleic acid) and combined inorganic and organic acid (i.e., sulphuric + maleic acid) pre-treatments on chemical composition, physical structure and methane yield of the same biomass crops and agricultural residue as in the previous case.

Three multi-annual species (Arundo, Switchgrass and Sorghum Silk), three sorghum hybrids (Trudan Headless, B 133 and S 506) and a maize hybrid, as reference for AD, were studied in the frame of point *i*). Biomass yield per hectare was assessed and samples were subjected to chemical analysis to determine their properties prior to AD batch assay. Results exhibit the remarkable variation in biomass yield, chemical characteristics and potential methane yield. The six species alternative to maize deserve attention in view of a low need of external inputs but necessitate improvements in biodegradability (i.e., harvest stage and biomass pre-treatments) to bridge the gap in net energy yield with maize.

In the frame of point *ii*), Arundo was subjected to twelve hydrothermal pre-treatments combining variations in temperature, time and acid catalyst (no catalyst; H_2SO_4 at 2% w/w immediately before steam cooking or in 24-hour pre-soaking) plus untreated control, before AD. Pre-treatments determined a variable effect on methane yield: four pre-treatments without acid catalyst achieved up to +23% CH_4 output, while pre-treatments with H_2SO_4 catalyst incurred a methanogenic inhibition in association with high SO_4^{2-} concentration in the hydrolysate, known to enhance sulphate reducing bacteria.

In the frame of point *iii*), two biomass crops (B133 sorghum and Arundo) and an agricultural residue (Barley straw) were combined with alkaline pre-treatments (increasing NaOH levels:

0.05, 0.10 and 0.15 N at 25 °C for 24 h), plus untreated controls, before AD. Pre-treatments determined an increase of methane yield and a change of chemical and physical structure, also proved by Fourier transform infrared spectroscopy and scanning electron microscopy: the benefits obtained were directly proportional to substrate recalcitrance to AD.

Lastly, as concerns point *iv*), the effects of acid pre-treatments (maleic acid at 0.3 and 0.6 M and two combinations of sulphuric and maleic acid) were tested on the same three substrates as in point *iii*), plus untreated controls, before AD. Pre-treatments significantly interacted with substrates in CH_4 yield, leading to top CH_4 increase (+62%) in Arundo pre-treated with maleic acid at 0.6 M. Pre-treatments also determined remarkable changes in chemical and physical structure of the three ligno-cellulosic substrates.

It is thereby demonstrated that pre-treatments can actually enhance biodegradability and subsequent CH_4 output of ligno-cellulosic substrates, although pre-treatment viability needs to be evaluated at the level of full scale biogas plants in a perspective of profitable implementation.

Keywords: *Anaerobic digestion; Arundo; Biomass crops; Biomass pre-treatments; Biodegradability; FTIR; Ligno-cellulosic substrates; Methane kinetic; Methane yield; Technical digestion time.*

General introduction

Since approximately 1850, the demand for energy has been satisfied through the use of fossil fuel (coal, oil and gas), leading to a rapid growth in greenhouse gas (GHG) emission, in particular carbon dioxide (CO_2). Recent data at a global level confirm that the consumption of fossil fuels accounts for the majority of anthropogenic GHG emissions; emissions continue to grow and CO_2 concentrations had increased to over 39% above preindustrial levels by the end of 2010 (IPCC, 2011), resulting in climate change. The need to reduce GHG emission, associated to unstable oil prices has strengthened the interest in renewable energy. Renewable energy sources (RES) play a role in energy supply in a sustainable manner, mitigating the climate changes at the same time. In this context, the EU directive 2009/28/EC requires EU member states to produce 20% of their energy needs from RES, in order to reduce the GHG emission by 20% and increase the energy efficiency by 20% within 2020. Agricultural sources (e.g., dedicated energy crops and agricultural by-products) may play a crucial role in order to achieve the renewable energy targets (Krasuska et al., 2010). According to the European Biomass Association (AEBIOM), bioenergy remains the major source among renewables in Europe, accounting for almost 62% of their total. In the EU-27, 8.4% of total energy consumption was covered by biomass in 2011 (ca. 92.6 Mtoe; AEBIOM, 2013).

Renewable energy from agricultural sources can be obtained in different ways (biodiesel, bioethanol, direct combustion, etc.). Among them, biogas production through anaerobic digestion (AD) is growing worldwide and is considered best suited under many viewpoints because of its economic and environmental benefits (Chandra et al., 2012b). A significant advantage of biogas compared to other sectors of renewable energy in agricultural (e.g. bioethanol and biodiesel) is the possibility to use a broad variety of organic feedstocks. Biogas can be used for different applications such as generation of heat and power from burned biogas, and, if upgraded to almost pure methane and compressed, it can be used as automotive fuel and distributed by natural gas grids. At the same time, nutrients contained in the remaining digestate can be used for crop production and play a remarkable role in promoting sustainable biomass production systems (Krishania et al., 2013a).

In the EU-27, the biogas sector was significantly stimulated in the recent past, so that biogas production increased six-fold from 1990s to 2005 (Murphy et al., 2011) and reached 11 Mtoe with approximately 11800 operating plants in 2011; among them, 8260 plants were operating in agriculture sector, reaching 5.7 Mtoe of energy production (AEBIOM, 2013). Germany, for

example, opted to develop agricultural biogas plants by encouraging the cultivation of energy crops, reaching 7215 agricultural biogas plant in 2011 (ABIOM, 2013). As a result of this strategy, Germany is the leading European biogas producer (IEA, 2009). Meanwhile in Italy, the government passed the law no. 99/2009, establishing to pay 0.28 € kWh^{-1} of power generated by agricultural feedstocks from farm-scale biogas plants (i.e., <1 MWe). At the light of these incentives, the number of biogas plant in Italy has soared from 154 in 2007 to 994 in 2012 with a total installed power capacity of 756 MWe (Fabbri et al., 2013). However, after this increase maize is the predominant feedstock for AD, determining competition between forage and energy end uses.

Dedicated energy crops

Biofuels from dedicated energy crops as well as from other sources can be divided in two groups, according to the origin of the biomasses used: *first-generation biofuels* from the easily edible part of the plant (e.g., grains, seeds or soluble sugars) and *second-generation biofuels* from ligno-cellulosic, i.e. non-edible plant portions (Dragone et al., 2010). Dedicated energy crops for first-generation biofuels, such as maize for bioethanol or biogas and rapeseed for biodiesel, may originate competition for land with food crops (Pimentel and Patzek, 2005), leading to food price increase. The concerns for the increase in land competition are driving the development of second-generation biofuels from dedicated non-food energy crops and crop residues (Krasuska et al., 2010). In general, the characteristics of the ideal dedicated non-food energy crops are (McKendry, 2002): i) high yield; ii) low need of energy input; iii) low cost; iv) low nutrient requirement. The production of dedicated energy crops may bring several advantages: to support regional economic structures, to provide alternative economic sources in rural areas, to promote the use of marginal lands and reduce CO_2 levels (Zegada-Lizarazu et al., 2010).

Various ligno-cellulosic crops have been proposed or are being tested for energy transformation, and can be divided in two groups: annual crops such as several sorghum hybrids (fibre, sweet and forage type) and multi-annual crops such as *Arundo donax* (Arundo, also known as Giant reed), *Panicum virgatum* (Switchgrass), *Miscanthus* × *giganteus* (Miscanthus), etc. Ligno-cellulosic energy crops are mainly composed of cellulose, hemicellulose and lignin in a proportion depending on plant species and cropping factors (e.g., harvest stage). Cellulose is the main component of ligno-cellulosic substrates; it is a

linear polymer of glucose linked by β-1,4-glycosidc bonds, forming cellobiose molecules connected in long chains. The long chains are linked together by intra- and inter-molecular hydrogen bonds and van der Waals forces, constituting the micro-fibrils. Cellulose in biomass crops is present in both crystalline and amorphous forms. Hemicellulose in biomass energy crops is mainly composed of xylose and arabinose, and in a small amount of mannose, glucose, galactose and uronic acids. Sugars are linked together by β-1,4- and occasionally β-1,3-glycosidc bonds (Pérez et al., 2002). Hemicellulose serves as a connection between the lignin and cellulose microfibrils and gives the whole cellulose-hemicellulose-lignin network more rigidity (Laureano-Pérez et al., 2005). Lignin is a complex heteropolymer consisting of three different phenyl-propane units (p-coumaryl, coniferyl and synapyl alcohol) that are linked together by different types of linkages (Hendriks and Zeeman, 2009), imparting a three-dimensional structure. Lignin provide structural support, impermeability and resistance against microbial attacks.

Anaerobic digestion and substrates

AD or biomethanation is a complex biological process where the organic substrate is converted to biogas in the absence of oxygen by a microbial consortium. Biogas is composed mainly by methane (55-75%) and carbon dioxide (25-45%), and trace of hydrogen sulphide (0-5000 ppm), ammonia (0-500 ppm), nitrogen (0-5%) and water vapour (1-5%; Braun, 2007). Only a small fraction of energy content of the organic substrate is used by the microorganisms (about 14%), while the rest (86%) is stored in the end product, methane (Zehnder and Stumm, 1988; Schink, 1997). AD can be divided in four steps, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Gerardi, 2003), as reported in Figure 1. The individual steps are carried out by different microorganism groups acting in a partial syntrophic interrelation (Deublein and Steinhauser, 2008). During hydrolysis, complex organic compounds such as carbohydrates, proteins and lipids are hydrolyzed into monomers such as sugars, amino acids and fatty acids through extracellular enzymes produced by hydrolytic bacteria (Parawira et al., 2005). Monomers from hydrolysis are degraded during the acidogenic step into short-chain organic acids (e.g., butyric acid, propionic acid, acetic acid), alcohols, hydrogen and carbon dioxide, by fermentative bacteria (Chandra et al., 2012b). Intermediate compounds formed during acidogenesis are converted into acetate by proton-reducing acetogenic bacteria (Zinder et al., 1984). Acetate serves as a substrate for

methanogenic *Archaea* during methanogenesis, the last phase of AD. In this step, acetate, hydrogen and carbon dioxide are converted into methane and carbon dioxide. As general, the 70% of total methane derives from the conversion of acetate (acetoclastic methanogenesis), while the remaining 30% originates from hydrogen and carbon dioxide (hydrogenotrophic methanogenesis; Klass, 1984; Zinder et al., 1984).

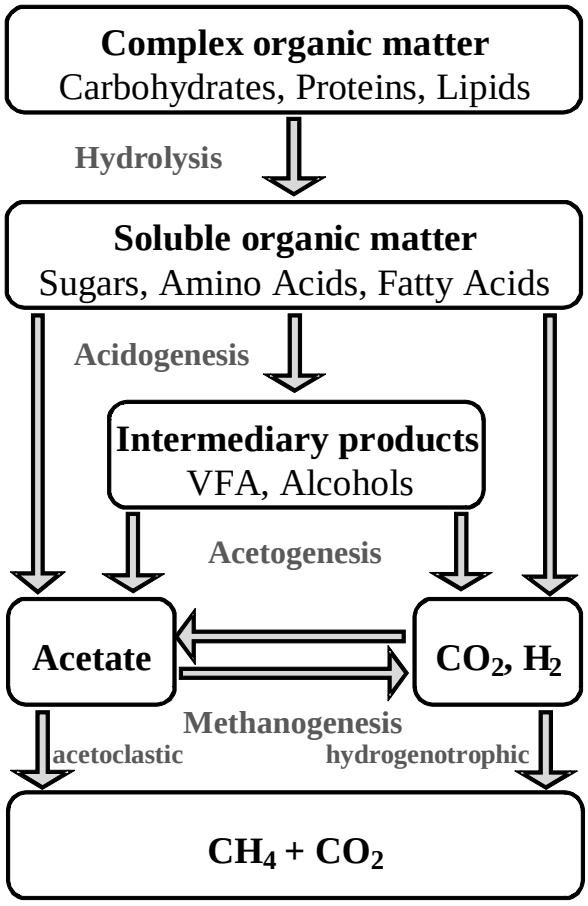

Figure 1. Pathway of anaerobic digestion (adapted from Angelidaki et al., 2002; Demirel and Scherer, 2008)

Different organic substrates that could be used to AD can be classified into three categories as reported by Rao and Baral (2011): *i)* solid (e.g., energy crops, agricultural residues, weeds, urban solid waste, etc.); *ii)* semi-solid (e.g., manure and animal meat residues, poultry wastes, etc.); *iii)* liquid (e.g., wastes of dairy plants, pulp and paper industries, etc.). AD is sensitive to the type of substrates and their composition; in general, physico-chemical characteristics of ligno-cellulosic biomass may influence methane yield.

During AD of ligno-cellulosic biomass, hydrolysis is considered a rate-limiting step (Pavlostathis and Grialdo-Gomez, 1991), by several factors: cellulose crystallinity, degree of polymerization, surface area for enzymatic attack and, especially, lignin content (Chang and Holtzapple, 2000). Lignin is the most recalcitrant component to anaerobic biodegradation (Taherzadeh and Karimi, 2008) and shields cellulose and hemicellulose (Frigon and Guiot, 2010), reducing the available surface area for enzymatic attack and hampering the degradation of structural carbohydrates. Hence, biomass recalcitrance is directly related to the properties of substrate (Agbor, et al., 2011). Therefore, in order to improve methane production from ligno-cellulosic substrates, a pre-treatments step is often necessary (Chang and Holtzapple, 2000; Taherzadeh and Karimi, 2008).

Pre-treatments of ligno-cellulosic biomass

Pre-treatments of ligno-cellulosic substrates prior to AD could accelerate the hydrolytic step and improve final biogas production through various activities, depending of the type pre-treatment: hydrolysis of hemicellulose and cellulose, reduction of cellulose crystallinity, breakage of the impermeable/resistant layer of lignin, solubilization or redistribution of lignin, increasing area and porosity of the substrate, making carbohydrates more accessible for enzymatic attack. However, efficient pre-treatments must avoid degradation or loss of carbohydrates while minimizing or avoiding the formation of by-products that are rate limiting (e.g., furfurals) and determine a slower kinetics in methane production due to methanogens needing a period of adaptation (Benjamin et al., 1984). Moreover, pre-treatments necessitate to be cost effective as prerequisite for a large scale use. A vast literature focuses on pre-treatment effects to enhance bioethanol production, but up to now only few studies have been published on pre-treatment impacts to enhance the methane yield of ligno-cellulosic substrates. Pre-treatments may be grouped into physical, chemical and biological treatments, depending on the physico-chemical agent involved. Each pre-treatment has a specific effects on the cellulose-hemicellulose-lignin network (Hendriks and Zeeman, 2009), therefore, single pre-treatments may also be combined to improve their global effect on biodegradability (Talebnia et al., 2010).

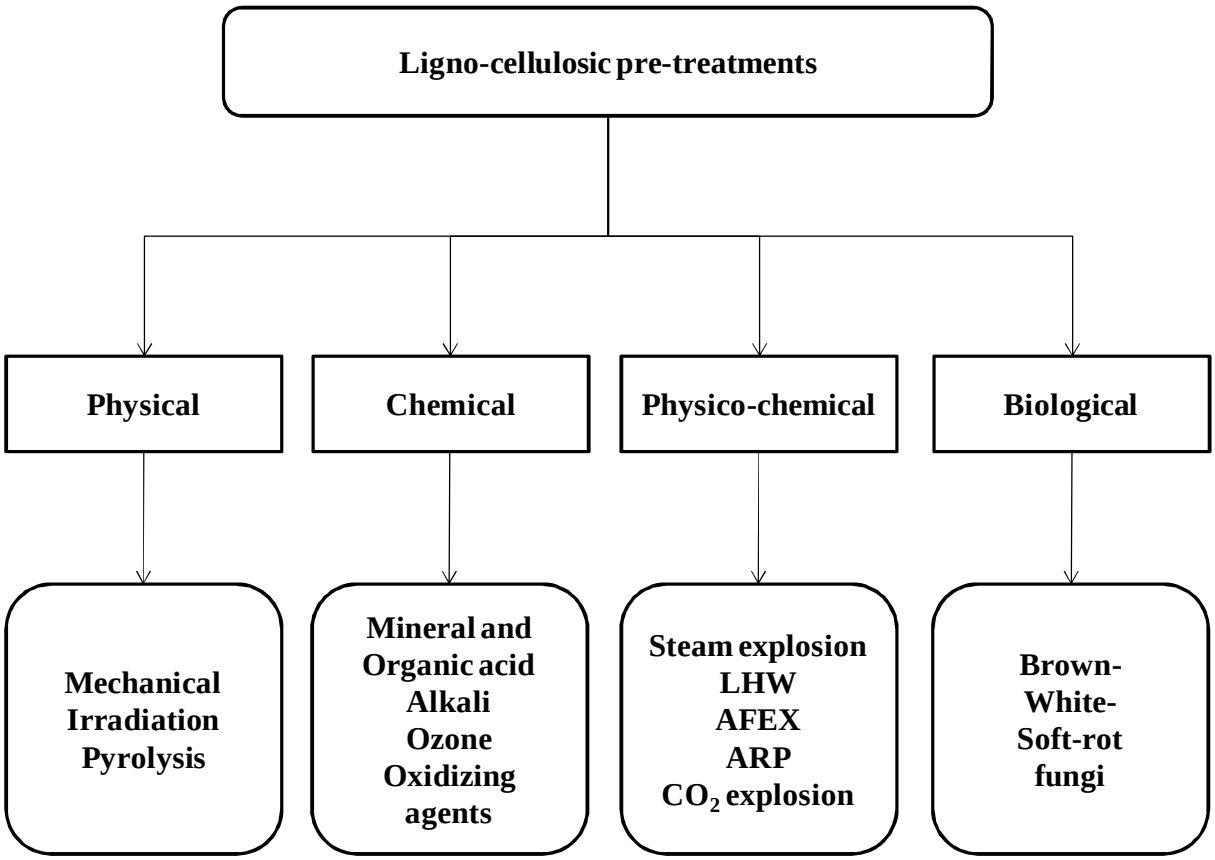

Figure 2. Pre-treatment processes of ligno-cellulosic materials. LHW, liquid hot water; AFEX, ammonia fibre explosion; ARP, ammonia recycle percolation (adapted from Talebnia et al., 2010).

Physical pre-treatments include mechanical treatment, irradiation and pyrolysis (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Kumar et al., 2009a). Milling, chipping, grinding, are mechanical pre-treatments that comminute the ligno-cellulosic substrates. The objective of these pre-treatments is a reduction of particle size and crystallinity, increasing the available specific surface area (Palmowski and Müller, 2000). Irradiation pre-treatments can be used to increase biodegradability of ligno-cellulosic substrates by gamma rays, electron beam and microwaves (Taherzadeh and Karimi, 2008). Microwave irradiation was effectively shown to increase surface area, decrease polymerization and crystallynity of cellulose, solubilise hemicellulose and partial depolymerization of lignin (Odhner et a., 2012; Sapci, 2013). The major drawback of microwave pre-treatment can be the formation of rate-limiting AD compounds as furfurals. However, after pre-treatment total biogas production can be

improved, also decreasing the initial lag-phase of AD (Beszédes et al., 2001; Jackowiak et al., 2011).

Chemical pre-treatments may be carried out with different chemical agents such as acids, alkalis, oxidizes and ozone. Acid pre-treatments can be classified into strong or dilute-acid hydrolysis, based on the dose; moreover, organic acids can be used. Among acid reagents, sulphuric, hydrochloric and nitric acid are those most frequently applied (Taherzadeh and Karimi, 2007). During strong acid pre-treatment, the substrate is treated with high acid concentration at ambient temperature. Conversely, under dilute-acid pre-treatment, the acid concentration is around 4 g 100 $g^{-1}_{substrate}$. Dilute-acid pre-treatment is usually performed in a short time (e.g., 5-20 min) at high temperature (160-260 °C) and pressure (0.7-4.8 MPa; Kumar et al., 2009a), resulting in a physico-chemical pre-treatment as reported below. The main reaction that occurs during acid pre-treatment is the hydrolysis of hemicellulose; also the solubilisation of lignin that will quickly condensate and precipitate in acid environment may happen (Liu and Wyman, 2003; Hendriks and Zemman, 2009), improving the access to cellulose. However, acid agents, especially sulphuric acid, are corrosive, hazardous and toxic. In addition, during acid pre-treatments and especially at high temperature, the rate-limiting compounds as furfurals may originate from hemicellulose degradation, influencing methane production kinetics. Moreover, during the AD of substrates pre-treated with sulphuric acid, the H_2S production may be enhanced, lowering biogas quality. Organic or aqueous organic solvents mixed with mineral acid (HCl or H_2SO_4) can be used during organosolv pre-treatments, causing a break in the internal lignin and hemicellulose bonds. Recently, the use acids dicarboxylic has also been introduced, as described below (Chapter # 4). Alkali pre-treatments may be carried out at lower temperature and pressure but for a longer time (hours or days), than other pre-treatments (Moiser et al., 2005); different alkali agents can be used, such as sodium, potassium, calcium and ammonium hydroxides. Among them, sodium hydroxide has been most widely used, leading to a definition of this method as soaking aqueous ammonia (SAA), involving that biomass is treated into a batch reactor at moderate temperature (25-60 °C; Kim and Lee, 2005). Alkali pre-treatment causes the solvation, saponification, swelling, partial solubilisation of hemicellulose and disruption or redistribution of lignin (Taherzadeh and Karimi, 2008; Hendriks and Zemman, 2009). Swelling, after alkaline pre-treatment, causes a decrease of polymerization, increase of internal surface, separation of structural linkages between lignin and structural carbohydrates, and disruption of the lignin structure (Fan et al., 1987). Furthermore, during AD the residual

alkali agent in the pre-treated substrate prevents a drop of pH during the acidogenic phase, increasing the efficiency of methanogenesis (Palvostathis and Gossett, 1985). During oxidative pre-treatments, hydrogen peroxide or peracetic acid can be used, causing the removal of hemicellulose and lignin (Hendriks and Zemman, 2009). Ozonolysis pre-treatment, using ozone, is usually carried out at room temperature, and can degrade lignin and part of hemicellulose, although it is quite expensive (Vidal and Molinier, 1988; Taherzadeh and Karimi, 2008).

Microorganisms such as brown-, white- and soft-rot fungi, can be used in *biological pre-treatments* to degrade hemicellulose and lignin (Sun and Cheng, 2002). Lignin is degraded by specific degradation enzymes such as laccase and peroxidase (Okano et al., 2005; Lee et al., 2007). Biological pre-treatments are safe, environmentally friendly and less energy intensive compared to other pre-treatment methods; however, the rate of hydrolysis is very low (Talebnia et al., 2010).

Physico-chemical pre-treatments include steam explosion, liquid hot water, ammonia fibre explosion and ammonia recycle percolation. Steam explosion combines chemical and physical techniques. During pre-treatment, the biomass is treated with saturated steam at high pressure (0.7-4.8 MPa) and temperature (160-260 °C), in combination with acid (e.g., dilute-sulphuric acid) for several seconds or minutes (Sun and Cheng 2002; Kumar et al., 2009a). Thereafter, the system is rapidly depressurized, disrupting the ligno-cellulosic structure (Brodeur et al., 2011), causing hemicellulose degradation and lignin transformation (Sun and Cheng 2002; Kumar et al., 2009a; Brodeur et al., 2011). Conversely, if pressure is slowly released, the process is defined steam cooking.

Liquid hot water (LHW) is similar to steam cooking, but uses water in the liquid phase at high temperature (90-170 °C) for a few minutes (Agbor et al., 2011; Brodeur et al., 2011); a catalyst (e.g. an acid) can be added. During pre-treatment, water penetrates into biomass, hydrate cellulose, and solubilyzate hemicellulose completely and part of lignin. Two fractions can be obtained: a liquid fraction rich of hydrolyzed hemicellulose, and a solid fraction rich of cellulose more susceptible to enzymatic attack (Broduer et al., 2011). The lower temperature used minimizes the formation of rate-limiting compounds, but requires more energy due to the large volumes of water involved (Agbor et al., 2011).

Ammonia fibre explosion (AFEX) is a physico-chemical pre-treatment similar to steam explosion. Biomass is exposed to liquid ammonia at high temperature (60-100 °C) and pressure (3 MPa) for a few minutes (5-30 min), followed by immediate reduction of pressure

(Taherzadeh and Karimi, 2008; Kumar et al., 2009a; Agbor, et al., 2011; Broduer et al., 2011). Ammonia recycle percolation (ARP) is similar to AFEX, but the aqueous ammonia (5-15 % wt) passes through biomass at a flow of about 5 ml min^{-1} at high temperature (140-210 °C) for 10-15 min (Brodeur et al., 2011); after pre-treatment, the ammonia is recovered and recycled. AFEX and ARP cause biomass swelling, disruption in the lignin-carbohydrates linkage, alteration and removal of lignin, hemicellulose and partial decrystallization of cellulose (Taherzadeh and Karimi, 2008; Kumar et al., 2009a; Agbor, et al., 2011; Broduer et al., 2011).

Objectives

At the light of the pending issues in the research on anaerobic digestion of ligno-cellulosic biomass, the overall objective of this thesis was to investigate biomass yield, methane yield and pre-treatment effects on substrate physico-chemical structure, in view of improving methane yield from ligno-cellulosic feedstocks represented by dedicated annual and multi-annual energy crops.
The specific objectives were:

i. to assess biomass yield in field plots and methane output in an anaerobic incubation assay, comparing alternative biomass crops with whole plant maize, the reference crop used as substrate for anaerobic digestion;

ii. to evaluate the effects of hydrothermal pre-treatments on biodegradability and methane yield of a promising multi-annual species in South European environments as *Arundo donax*;

iii. to investigate the effects of NaOH pre-treatments at low temperature on chemical composition, physical structure and methane yield of dedicated biomass crops and agricultural residues.

iv. to investigate the effects of organic acid (i.e. maleic acid) and combined mineral and organic acid (i.e. sulphuric + maleic acid) at low temperature on chemical composition, physical structure and methane yield of dedicated crops and agricultural residues.

CHAPTER 1

Biomass yield, methane output and energy balance in maize vs. alternative energy crops

Based on: Barbanti, L., Di Girolamo, G., Grigatti, M., Bertin, L., Ciavatta, C., 2014. **Anaerobic digestion of annual and multi-annual biomass crops.** Industrial Crops and Products 56, 137-144.

Abstract

This chapter addresses the AD of seven biomass crops: three multi-annual species, *Arundo donax* (Arundo), *Panicum virgatum* (Switchgrass) and Sorghum Silk; three sorghum hybrids (B 133, S 506 and Trudan H.); one Maize hybrid as reference crop for AD. Dry biomass yield (DBY) was assessed in a field plot experiment and biomass samples were subjected to chemical analysis (proteins and lipids, soluble sugars, starch, structural carbohydrates and lignin). Thereafter, an AD assay was carried out in batch mode with 4 g VS l^{-1} at 35 °C for 58 days, during which time potential methane yield (ml CH_4 g^{-1} VS) was determined. Gross energy yield (GE = DBY × VS × potential CH_4 yield × methane lower heating value) and cumulative energy demand (CED) led to net energy yield (NE = GE - CED) and energy efficiency (EE = GE/CED) as indicators of crop suitability for AD. Arundo, B 133 and S 506 achieved ±10% DBY compared to Maize (this latter, 27.8 Mg ha^{-1}). Conversely, Maize prevailed in terms of potential methane yield (316 ml CH_4 g^{-1} VS). Among the six alternative crops, Arundo and Switchgrass exhibited the lowest values (average, 216 ml CH_4 g^{-1} VS), associated with low kinetics of degradation. This is consistent with the two crops' characteristics: low easily degradable fractions as lipids, soluble sugars and starch; high structural carbohydrates and lignin. Maize achieved a top level also in GE (286 GJ ha^{-1}, corresponding to ca. 8400 Nm^3 CH_4 ha^{-1}) and NE (248 GJ ha^{-1}). B 133 and S 506 were undifferentiated from Maize in NE (their average, 215 GJ ha^{-1}), whereas Trudan H. and the three multi-annual species were outperformed (average NE, 149 GJ ha^{-1}). Conversely, Maize ranked worst in EE (7.4 GJ GJ^{-1}) while sorghum B 133 and Arundo attained top levels (average, 12.1 GJ GJ^{-1}), thanks to a good GE associated with a modest CED in B 133; to a very low CED in Arundo. It is concluded that alternative crops to maize deserve attention in view of a low need of external inputs but necessitate improvements in biodegradability (harvest stage and biomass pre-treatments) to bridge the gap in the amount of net energy produced.

1.1 Introduction

Policy makers all over the world are showing increasing concern for the growth in energy consumption, while promoting the conversion from a fossil fuel-based to a bio-based economy (Richardson, 2012). The agricultural sector participates in this effort, supplying biomass to be transformed into various forms of energy. Among them, AD can successfully

be used for biogas and, ultimately, methane production. Biofuels including methane represent an important strategy to reduce GHG emissions by substituting fossil fuels, thus complying with the Kyoto Protocol and subsequent legislation such as EU Directive 2009/28/EC.

Biosolids of agro-industrial origin (e.g., crop, market and transformation residues; animal manure and slurries) are valuable feedstocks for AD in view of methane production. Beside them, dedicated biomass crops are increasingly being used, resulting in potential competition for available land with food crops (Murphy et al., 2011).

In the scientific literature, several studies address AD with biomass crops. Beside maize that is the reference feedstock for AD experiments, several sorghum (*S. bicolor*) hybrids including fibre, sweet and forage genotypes have been tested in view of methane production (Jerger et al., 1987; Chynoweth et al., 1993; Bauer et al., 2010; Mahmood and Honermeier, 2012; Mahmood et al., 2013; Sambusiti et al., 2013). However, only some works combine specific CH_4 yield and crop biomass yield, assessing CH_4 yield per hectare (Kralik et al. 2008; Bauer et al., 2010; Kerckhoffs et al., 2011; Mahmood and Honermeier, 2012; Monteiro et al., 2012; Mahmood et al., 2013). In multi-annual biomass species, Switchgrass (*P. virgatum*) has recently been investigated in view of CH_4 production (Massé et al., 2011; Frigon et al., 2012). Lastly, Arundo (*A. donax*) is the subject of the most recent AD experiments (Di Girolamo et al., 2013; Ragaglini et al., 2014). Despite the abundance of studies on the topic, the substitution of maize that requires high cropping inputs and the best cropland with less demanding species is rarely echoed in the literature on biogas (Kralik et al., 2008; Bauer et al., 2010; Kerckhoffs et al., 2011; Mahmood et al., 2013).

Bio-energies which are expected to supply a significant share of future energy demand will require better integrated policies to prevent adverse impacts from land competition. In this respect, a recent report by the Netherlands Environmental Assessment Agency forecasts a drastic reduction of the energy deriving from biomass, due to the lack of surface available for sustainable biomass crops (PBL, 2012). It is generally acknowledged that energy crops should not be cultivated in previous forestland, pastures and virgin soils, because converting these lands to energy crops enhances GHG emissions, in turn accelerating climate change (Campbell et al., 2008). Moreover, the use of good agricultural lands for energy crops is held responsible of increases in food price volatility and the associated risks for food security (FAO, 2008). To overcome these drawbacks, the use of marginal land is considered a sustainable practice for the cultivation of energy crops (PBL, 2012; Campbell et al., 2008). Likewise, biomass crops necessitating low amounts of subsidiary energy (fertilizers, fuels,

etc.) may be a more sustainable source of energy in areas where surplus land is available, compared to maize. Especially multi-annual species are proposed as alternatives to maize involving much lower crop inputs (Lewandoski et al., 2003; Heaton et al., 2004; Angelini et al., 2005; Mantineo et al., 2009; Massé et al., 2011).

In this light, the computation of the energy flows involved in the cropping phase, i.e. the amount of energy produced in exchange for that of subsidiary energy consumed to obtain crop biomass, is considered an important tool to evaluate crop suitability in view of anaerobic digestion. However, the appraisal of energy flows in biomass crops for methane production is rarely echoed in the literature, in contrast to biomass crops for combined heat and power generation (Angelini et al., 2005; Mantineo et al., 2009).

Given these premises, we assessed biomass yield in field plots and specific CH_4 yield in an AD assay under batch conditions, comparing six promising biomass crops with maize. Thereafter, the appraisal of the energy flows associated with the cultivation phase allowed us to calculate net energy yield and energy efficiency, the two traits expressing ultimate crop performance in view of anaerobic digestion. The six plants potentially alternative to maize were three hybrids of biomass sorghum and three multi-annual herbaceous species. They were selected for a high potential of biomass production and low need of external inputs. The aim of this work was to assess if a more efficient production of methane could be achieved replacing maize that is the principal feedstock in the diet of many biogas plants at present.

1.2 Material and methods

1.2.1 Crop management

In the year 2010 seven biomass crops were grown at the experimental farm, University of Bologna, in Cadriano (44° 33’ N, 11° 21’ E, 32 m above sea level), Italy. The experimental farm features deep alluvial soils with a clayey-loamy texture (average sand, silt and clay, 340, 360 and 300 g kg^{-1}, respectively), under a warm-temperate climate (700 mm, 8.3 and 18.3 °C as average yearly precipitation, minimum and maximum temperature, respectively). Three of the seven crops were multi-annual species: *Arundo donax* L. (Arundo, also known as Giant reed); *Panicum virgatum* L. (Switchgrass) cv. Alamo; the inter-specific hybrid *Sorghum arundinaceum* Stapf × (*S. halepense* Pers. × *S. roxburghii* Stapf), known as Sorghum Silk (S. Silk). The four annual crops included: three sorghum [*Sorghum bicolor* (L.) Moench] genotypes, namely a fibre (Biomass 133; B 133), sweet (Sucros 506; S 506) and forage hybrid

(Trudan Headless; Trudan H.); one maize hybrid (Klips, FAO 700 maturity). Maize is the dedicated crop most widely used as feedstock for anaerobic digestion in Italy (Fabbri et al., 2011) as well as in Europe (Herrmann and Rath, 2012). Among multi-annual species, Arundo has proved a promising crop for energy uses in South European areas (Lewandoski et al., 2003); Switchgrass is especially valued in the US, having proved also adapted to the Po Valley in Italy (Monti et al., 2011); Sorghum Silk should combine the good characteristics of forage sorghum (thin stemmed "Sudan" genotypes) with the multi-annual habit, having already staged high biomass potential under Mediterranean conditions (Corleto et al., 2009).
Arundo and Switchgrass had been established in 2002 and were still in full production as of 2010; Maize was seeded on April 1; S. Silk on April 28; the three sorghum hybrids on May 18. All the crops were grown with four replicates in plots of 90 m^2 (multi-annual species) and 27 m^2 (annual species). Weed control was performed through hoeing integrated by hand weeding. In all the crops except Maize, fertilization consisted of 120 kg of N ha^{-1} as urea, incorporated during the early development stage. In Maize 250 kg of N ha^{-1} were split applied as urea, to ensure the achievement of full yield potential of this highly demanding plant. 200 kg of P_2O_5 ha^{-1} had been supplied as triple superphosphate to Arundo and Switchgrass prior to planting in 2002; 92 kg of P_2O_5 ha^{-1} to S. Silk, Trudan H., B 133, S 506 and Maize before seeding in 2010. No K fertilizer was applied, given the good soil status of this specific nutrient. All the crops except Maize were grown in rain fed conditions on a soil with a good water capacity in a year (2010) showing a normal weather pattern; Maize was irrigated with a total 168 mm in the summertime. Both nitrogen dose and irrigation volume represent normal cropping inputs for maize in Northern Italy. No chemical treatment against pests or diseases was needed in any of the seven crops. Maize was harvested as whole plant at hard dough stage on August 5; the three multi-annual species on October 5 at initial senescence; the three sorghum hybrids on October 18 at hard dough stage. Fresh biomass yield (FBY), total solids (TS; 48 h at 105°C) and dry biomass yield (DBY) per hectare were assessed. Biomass samples were oven-dried (60 °C) and ground at 2 mm for chemical analysis and the anaerobic digestion assay.

1.2.2 Chemical characteristics

On dried and ground samples of the seven biomass crops, TS (48 h at 105 °C) and VS (4 h at 550 °C) were singly determined in the four field replicates. Thereafter, on average samples

the following analyses were carried out in triplicates: total organic carbon (TOC) by the dichromate oxidation method; total Kjeldahl nitrogen (TKN), through distillation after hot digestion with 96% H_2SO_4; C/N as TOC/TKN; proteins, meaning total protein content, calculated as TKN × 6.25; lipids, meaning total lipid content, through the Soxhlet method with diethyl ether; starch by the amyloglucosidase-α-amylase method (McCleary et al., 1997). Ligno-cellulosic biomass like the seven investigated crops involves the appraisal of extractives, meaning the water- and ethanol-soluble fraction of VS: extractives were determined in triplicates on 1 g of dried and ground biomass sample by sequential 12-hour water and ethanol extraction (1:20 w/v) at room temperature. The liquid phase was separated through centrifugation at 10000 relative centrifugal force for 10 min at 4 °C. On the liquid phase after water extraction, soluble sugars were assessed by means of HPLC (Aminex XPX-87H column (300 x 7.8 mm) at 63 °C; mobile phase, 4 mM H_2SO_4; flow rate, 0.6 ml min^{-1}) provided with a refractive index detector (Shimadzu RID-10A). After the sequential water and ethanol extraction, the residual solid fraction was oven dried at 40 °C for 24 h. Thereafter structural carbohydrates (cellulose and hemicellulose) were determined by means of HPLC (same conditions as above), subtracting the amount of starch from the sulphuric acid glucan (starch + cellulose) value to obtain the actual amount of cellulose (Sluiter et al., 2010). AIL was determined in the solid residue after 24 hours at 550 °C. For both structural carbohydrates and AIL, the U.S. National Renewable Energy Laboratory (NREL) guidelines (Sluiter et al., 2011) were followed. All data were expressed on VS.

1.2.3 Anaerobic digestion

Methane yield from the six biomass crops and maize was assessed in AD under batch conditions. The inoculum was collected from a commercial AD plant (operative conditions: 55 °C, fed with maize silage and fresh vegetable residues). It was subsequently adapted to mesophilic conditions (35 °C in the dark with repeated manual stirring) until the end of biogas emission (ten days) (Angelidaki et al., 2009). The starved inoculum had the following characteristics: TS, 36 mg g^{-1}; VS, 22.6 mg g^{-1} fresh weight; TOC, 11.2 mg g^{-1} fresh weight; TKN, 3.5 mg N g^{-1} fresh weight; very low C/N ratio (3.2); total alkalinity, 28.2 g $CaCO_3$ l^{-1}. The NH^+_4-N content was fairly high (2.77 mg g^{-1}), still in the normal range for this kind of products. The AD assay was conducted at 35 °C in 100 ml serum bottles (Sigma-Aldrich) at a 4 g VS l^{-1} loading for 58 days, suspending the samples in 48 ml of inoculum and diluting by

deionized water to a final volume of 60 ml. The high inoculum rate (80% v/v), corresponding to a 4.5:1 inoculum to substrate ratio (VS/VS), was adopted to avoid potential inhibition determined by high organic load or insufficient nutrients or alkalinity (Angelidaki and Sanders, 2004; Angelidaki et al., 2009). Additional serum bottles were prepared with i) the sole inoculum at the same dilution rate (blank); ii) glucose at the same organic load and inoculum as plant samples (control). The seven plant materials, the sole inoculum and glucose were tested in 4 replicates, totalling 36 serum bottles. After filling, the bottles were flushed with N_2 for 60 seconds to ensure anaerobic conditions, capped with butyl rubber stoppers and sealed with aluminium crimps. The bottles were placed in an incubator and continuously stirred (120 rpm) during the first week, then manually stirred every other day for the rest of the incubation.

1.2.4 Biogas and CH_4 assessment

The incubation lasted until no increase above 5% of methane production was detectable (58 days), during which time the bottles remained sealed. Twelve times during the incubation (day 2, 4, 6, 9, 12, 16, 20, 26, 34, 41, 48 and 58), the biogas production accumulated in the headspace of the sealed bottles was measured with a water-displacement system constituted of a 1 l Schott bottle and a graduated cylinder (Mariotte bottle) with water. This was connected to the batch with a syringe needle only for the time needed to measure water displacement (ca. 10 s). After equilibrium and reading, the water displacement apparatus was disconnected. Then the gas in the bottle headspace was analyzed for the biogas components (H_2, O_2, CH_4 and CO_2) with a μGC-TCD, model 3000A (Agilent Technologies, Milan, Italy) under the following conditions: injector temperature, 90 °C; column temperature, 60 °C; sampling time, 20 s; injection time, 50 ms; column pressure, 25 psi; run time, 44 s; carrier gas, nitrogen. Biogas and CH_4 data are expressed at STP (Standard Temperature and Pressure; 273 K, 100 KPa). Biogas from each bottle was measured at each sampling time and cumulated as ml g^{-1} VS. CH_4 production was calculated based on volume displacement and percent methane content at each current reading and its previous reading (Lou et al., 2012):

$$CH_4(\text{ml}) = \left[\frac{(A + B) \times \%\,CH_{4t}}{100}\right] - \left[\frac{B \times \%\,CH_{4(t-1)}}{100}\right] \qquad \text{[eq. 1]}$$

where A is displaced gas volume; B is headspace gas volume; t is current sampling time; t-1 is previous sampling time.

Specific methane yield was cumulated over time by summing the amount of methane produced at each date at the net of the inoculum, expressed as ml CH_4 g^{-1} VS. At each sampling, the calculation was:

$$\text{Specific } CH_4 \text{ (ml g}^{-1}\text{ VS)} = \frac{(V_{\text{biogas sample}} \times CH_{4\ \text{sample}}) - (V_{\text{biogas inoculum}} \times CH_{4\ \text{inoculum}})}{VS} \quad \text{[eq. 2]}$$

where $V_{\text{biogas sample}}$ and $V_{\text{biogas inoculum}}$ are respective biogas volume (ml) in sample and inoculum; $CH_{4\ \text{sample}}$ and $CH_{4\ \text{inoculum}}$ are percent methane contents in sample and inoculum, respectively.

Methane production kinetics was fitted by means of a mathematical model (Gompertz function) having the following equation:

$$CH_4 = CH_{4;0} \times \exp^{-\exp\left(\frac{x - x_0}{b}\right)} \quad \text{[eq. 3]}$$

where CH_4 represents specific methane yield (ml g^{-1} VS) at time t (d), $CH_{4;0}$ is potential methane yield, i.e. the function asymptote, x is substrate degradation rate (d) and x_0 is the point of inflection (d). To this aim, the Sigma Plot 10 software (Systat Software Inc., Chicago, Illinois, USA) was used. Thereafter, daily methane yield (ml CH_4 g^{-1} VS d^{-1}) was estimated as the first derivative of fitted function.

1.2.5 Energy Assessments

Gross energy yield per unit crop surface (GE; GJ ha^{-1}) was calculated as:

$$GE\ (GJ\ ha^{-1}) = DBY \times VS \times CH_{4;0} \times 0.03402 \quad \text{[eq. 4]}$$

where 0.03402 GJ Nm^{-3} is methane lower heating value (CTI, 2009).

In parallel to this, the amount of subsidiary energy consumed per unit crop surface was assessed through the Cumulative Energy Demand (CED) method in a life cycle based analysis

(Frischknecht and Jungbluth, 2003), focusing the analysis “from cradle to farm gate” (VDI, 2012). Briefly, the method accounts the amount of energy of products entirely spent in the agricultural process (e.g., fertilizers, fuels, etc.) or the small fraction attributable to the process (farm facilities and equipment). Therefore the CED of sub-processes implied in cropping (e.g., seed and agricultural machinery production) was also accounted in this indicator. Conversely, the CED required for the anaerobic digestion process was not calculated, as this falls beyond the scope of the present work which is to assess the suitability of crops for bio-methane production. In the three multi-annual species the CED of the establishment year was spread over an expected life span of 10 years, in order to calculate an annual equivalent CED to be compared with annual crops. Likewise, normal weed and pest control practices for each specific crop were accounted in CED, although they were not carried out in the experimental plots.

Based on this, two different indicators were adopted for energy assessments in the seven crops: i) net energy yield (NE), the difference between produced and consumed energy (GE - CED); ii) energy efficiency (EE), their ratio (GE/CED).

1.2.6 Statistical analysis

For each trait, normal distribution and equal variance of the data were controlled through the Kolmogorov-Smirnov and Bartlett test, respectively. The dataset was then submitted to one way analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, CA, USA). The Student - Newman-Keuls (SNK) test at $P \leq 0.05$ was adopted to separate means of statistically significant traits.

1.3 Results and discussion

1.3.1 Biomass yield and characteristics

The seven biomass crops exhibited a large variation in fresh and dry biomass yield (Table 1.1). Fresh biomass yield ranged between 46 (S. Silk*)* and 96 Mg ha^{-1} (Maize), the four annual crops consistently passing the three multi-annual species (averagely 89 and 53 Mg ha^{-1} in the two respective groups). Compared to this, dry biomass yield staged only a 60% variation between 18 (S. Silk) and 29 Mg ha^{-1} (B 133), due to a lower moisture at harvest in the multi-annual vs. the annual species (average TS were 422 and 294 mg g^{-1} in the two respective groups).

Volatile solids varied in a tight range between 926 mg g^{-1} TS (Arundo) and 961 mg g^{-1} TS (Maize) (Table 1.1); no clear distinction is detectable between the two aforementioned plant groups. The C/N ratio reflected TOC and TKN relative variation (data not shown), ranging from about 50 (annual crops and Arundo) to above 100 (Switchgrass and S. Silk) (Table 1.1).

Remarkable differences were evidenced in the compositional analysis of the seven biomass crops (Table 1.2). These plants, belonging to the *Poaceae* family, are intrinsically poor in proteins and lipids. However proteins outlined a ca. 1:2 ratio between the group composed by Switchgrass and S. Silk, and that of the other five crops (Table 1.2). Lipids showed a wider range between 9.5 mg g^{-1} VS (multi-annual species) and 33.6 mg g^{-1} VS (Maize) (Table 1.2).

Also soluble sugars exhibited notable differences among crops (Table 1.2): sorghum hybrids B 133 and S 506 prevailed over Trudan H. soon followed by Maize, whereas the three multi-annual species exhibited the lowest values, averaging about 3-fold less than B 133 and S 506.

Starch, the main carbohydrate in storage organs, was very low in the three sorghum hybrids and the multi-annual species which produce little or no grain (Table 1.2). Conversely, starch was much higher in Maize thanks to a relevant share of the grain component in plant biomass (data not shown). Comparing soluble sugars and starch content (Table 1.2), it is worth noting that annual sorghum hybrids, namely B 133 and S 506, were quite richer in the former than in the latter component, in contrast to Maize. Starch slightly prevailed over soluble sugars in the three multi-annual species, although these species were very poor in both non-structural carbohydrates.

Cellulose and hemicellulose, the two structural carbohydrates, outlined three different plant groups (Table 1.2): Maize showed the lowest levels of both (130 and 123 mg g^{-1} VS, respectively); the three sorghum hybrids staged intermediate values (average, 233 and 183 mg g^{-1} VS in the two respective carbohydrates); the three multi-annual species attained top levels (average, 301 and 233 mg g^{-1} VS in cellulose and hemicellulose, respectively).

Acid insoluble lignin described a similar pattern as structural carbohydrates, apart from a certain spread of the data in multi-annual species (Table 1.2).

Table 1.1 Biomass yield and main characteristics of the seven biomass crops.

Crop	FBY	DBY	TS	VS	C/N
	Mg ha^{-1}		mg g^{-1}	mg g^{-1} TS	
Arundo	61.7 c	26.8 ab	438 a	926 c	56.9 b
Switchgrass	52.6 cd	22.4 abc	431 a	957 a	118.9 a
S. Silk	45.6 d	18.2 c	397 a	943 b	107.9 a
Trudan H.	75.1 b	20.8 bc	275 b	929 c	48.7 b
B 133	89.4 a	29.2 a	325 b	949 ab	54.3 b
S 506	94.9 a	27.0 ab	284 b	940 b	54.2 b
Maize	95.7 a	27.8 a	290 b	961 a	39.5 b

FBY, fresh biomass yield; DBY, dry biomass yield; TS, total solids; VS, volatile solids. ANOVA always significant at $P \leq 0.01$. In each trait, different letters indicate statistically different means (SNK test; $P \leq 0.05$).

Table 1.2 Compositional analysis of the seven biomass crops.

Crop	Proteins	Lipids	Soluble sugars	Starch	Cellulose	Hemicellulose	AIL
	mg g^{-1} VS						
Arundo	51.2 a	9.5 d	24.0 e	39 b	315 a	237 a	193 a
Switchgrass	23.8 b	9.6 d	39.9 d	54 b	283 a	235 a	177 b
S. Silk	26.9 b	9.5 d	42.1 d	48 b	306 a	227 a	161 c
Trudan H.	60.4 a	18.2 b	76.4 b	39 b	238 b	190 b	154 c
B 133	52.0 a	16.0 c	130.0 a	53 b	232 b	181 b	149 c
S 506	52.1 a	15.9 c	134.4 a	48 b	229 b	179 b	148 c
Maize	64.7 a	33.6 a	63.7 c	319 a	130 c	123 c	77 d

AIL, acid insoluble lignin. ANOVA always significant at $P \leq 0.01$. In each trait, different letters indicate statistically different means (SNK test; $P \leq 0.05$).

Differences in biomass yield among species belonging to the same family but featuring a different morphology and habit (annual vs. multi-annual) are often echoed in the literature. In this work, alternative crops at limited need of external inputs were compared with maize grown with the normal husbandry adopted in Northern Italy (Giardini and Vecchiettini, 2000). Hence the good biomass yield of maize must be considered at the light of the higher inputs supplied: 250 vs. 120 kg of N ha^{-1} in the six alternative crops and 168 mm of irrigation vs. none. The two factors combined involve a significant increase in financial and energy costs, necessitating a higher biomass yield to counterbalance them. Moreover, the supply of a nutrient at high environmental impact (N) and the use of a resource at limited availability (water) represent two flaws the cultivation of energy crops should seek to avoid (Fernando et al., 2010).

The six crops alternative to maize expressed a varying degree of competitiveness in terms of biomass yield per hectare. Various types of sorghum (*S. bicolor*) are candidate to replace maize in view of biogas production. The assessment of biomass and methane yield in the two crops combined was carried out in specific works (Kralik et al., 2008; Bauer et al., 2010; Kerckhoffs et al., 2011; Mahmood et al., 2013), whereas other sources addressed sorghum alone (Mahmood and Honermeier, 2012; Monteiro et al., 2012).

Within multi-annual species, Switchgrass is rated among top biomass producers (Lewandowski et al., 2003), especially under limited water supply (Lewandowski et al., 2003; Heaton et al., 2004). In the Southern U.S. the same Switchgrass variety (Alamo) harvested at the same time of the year (autumn) as in this experiment outlined a dry biomass yield ranging between 12.2 and 26 Mg ha^{-1} (Lewandowski et al., 2003). My data (22.4 Mg ha^{-1}) lies in the upper half of this range, indicating favourable growth conditions in this experiment. Conversely, limiting growth conditions are evinced from the low biomass yield (< 10 Mg ha^{-1}) in the two works where Switchgrass was aimed for biogas production in Canada (Massé et al., 2011; Frigon et al., 2012). Arundo, too, is regarded as a perennial species with good biomass potential (Lewandowski et al., 2003), especially in the Mediterranean environment (Angelini et al., 2005). This is supported by the dry biomass yield recorded in this study (26.8 Mg ha^{-1}), quite close to the upper limit for experiments carried out in South European countries (range, 7.6 to 30.2 Mg ha^{-1}) (Lewandowski et al., 2003). At last, Sorghum Silk is still scarcely investigated: up to now this species attained a good yield in a multi-location experiment in Southern Italy (Corleto et al., 2009), in contrast to the modest performance evidenced in this study.

1.3.2 Biogas and methane yield

To avoid inhibition determined by high organic load, insufficient nutrients or alkalinity (Angelidaki et al., 2009; Angelidaki and Sanders,2004), this experiment was run at 4 g VS l^{-1} with inoculum at the rate of 80% (v/v). This fostered a rapid onset of biogas production in all treatments (Fig. 1.1.a), and a rapid settling of methane content at a plateau level of 60-65% in all crops (data not shown). Specific CH_4 yield of the seven biomass crops cumulated during the 58 days of incubation followed three different patterns (Fig. 1.1.a): Maize featured enhanced methanation, achieving a top level of potential methane yield (316 ml g^{-1} VS; Table 1.3). The three sorghum hybrids and S. Silk showed an intermediate behaviour, attaining an average 262 ml CH_4 g^{-1} VS (Table 1.3). Lastly, Arundo and Switchgrass performed the lowest potential yield, averaging 216 ml CH_4 g^{-1} VS (Table 1.3). As general, the Gompertz function (eq. 3) provided a very good fitting ($R^2_{adj.}$ always at 0.99**) and a prudential estimate of potential CH_4 yield vs. cumulative CH_4 yield at the end of the incubation (average, -4%). A first order kinetics (i.e., exponential rise to max equation) was also fitted to methane data, leading to a slight over-estimation of cumulative CH_4 yield at the end of the incubation (average, +7%). It was therefore dismissed (data not shown).

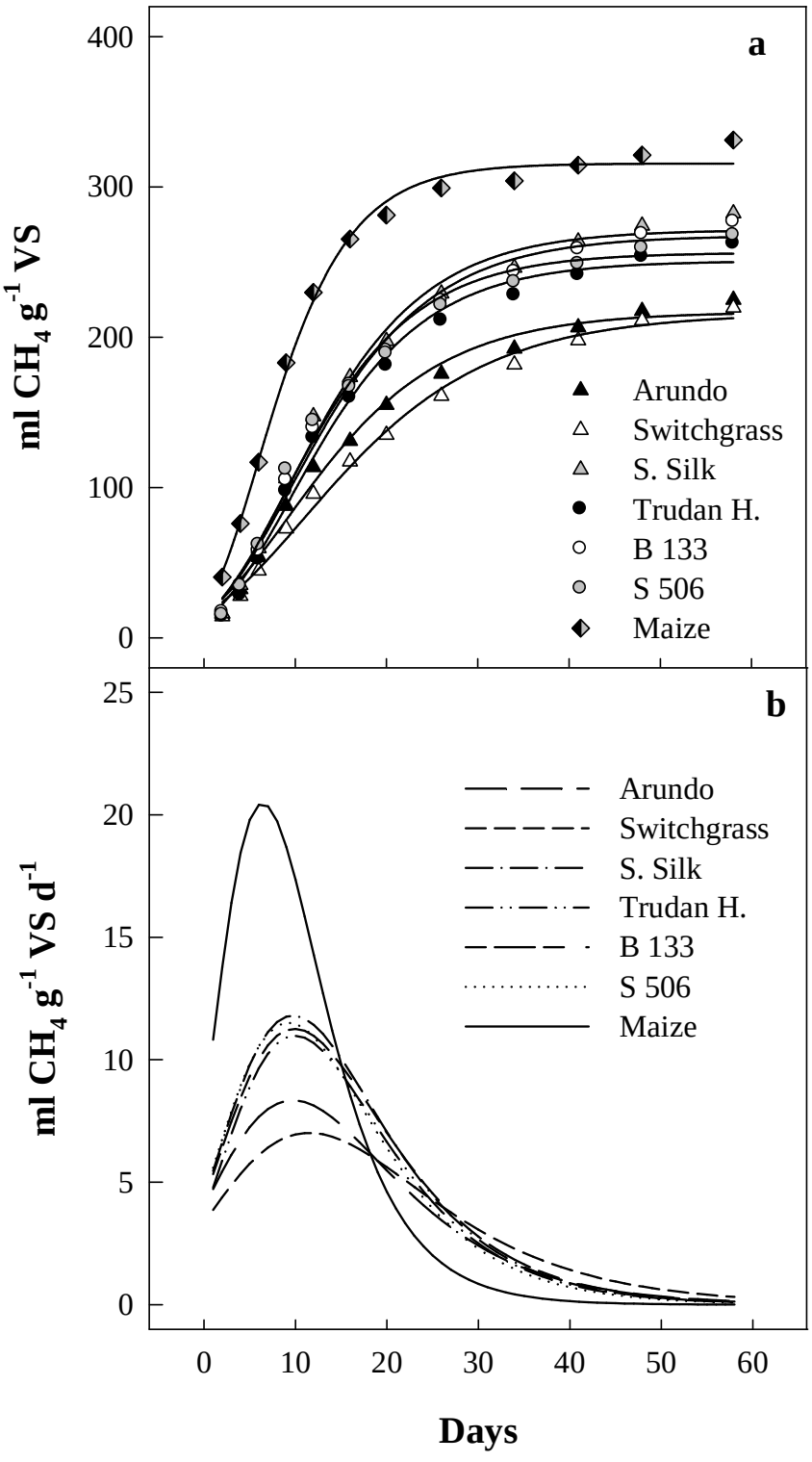

Figure 1.1 Cumulative methane yield during the anaerobic digestion of the seven crop samples (symbols) and fitted functions (lines) (a), and daily methane yield (b) estimated as the first derivative of fitted functions. Equation parameters and regression coefficients are reported in Table 1.3.

Table 1.3 Parameters of the Gompertz equation (potential CH_4 yield, substrate degradation rate and point of inflection) fitted in Figure 1.a and values of R^2_{adj}. In brackets, standard errors (n = 3).

Crop	$CH_{4;0}$	b	x_0	$R^2_{adj.}$
	ml g^{-1} VS	d	d	
Arundo	217 (5.3)	9.5 (0.9)	9.2 (0.5)	0.99**
Switchgrass	216 (5.5)	11.4 (0.9)	11.0 (0.6)	0.99**
S. Silk	271 (5.9)	8.5 (0.7)	9.2 (0.4)	0.99**
Trudan H.	251 (5.6)	8.4 (0.7)	9.4 (0.5)	0.99**
B 133	268 (5.5)	8.7 (0.7)	9.4 (0.4)	0.99**
S 506	256 (6.0)	8.2 (0.8)	8.7 (0.5)	0.99**
Maize	316 (3.7)	5.6 (0.3)	5.9 (0.2)	0.99**

$CH_{4;0}$, potential CH_4 yield; b, substrate degradation rate; x_0, point of inflection. **, significant at $P \leq 0.01$.

Differences of behaviour among the seven crops were also reflected in substrate degradation rate (b) and the point of inflection (x_0), the two traits providing a general picture of process kinetics (Table 1.3): the shortest time indicating easy substrate degradation was shown in Maize (b and x_0, 5.6 and 5.9 days, respectively). The three sorghum hybrids, S. Silk and Arundo exhibited a progressive slowdown in both traits (b between 8.2 and 9.5 days; x_0 between 8.7 and 9.4 days). At last Switchgrass showed the slowest kinetics (b and x_0, 11.4 and 11 days, respectively). The ca. 50% slower kinetics of Switchgrass vs. Maize is consistent with the ca. 30% lower potential CH_4 of the former vs. the latter crop, meaning that a recalcitrant substrate as Switchgrass takes more time to produce less methane than a more easily degradable substrate as Maize. This latter was substantially equivalent to glucose in terms of $CH_{4;0}$, b and x_0 (data not shown), indicating favourable conditions during the incubation.

Daily methane yield (Fig. 1.1b) outlined a consistent picture with fitted methane yield (Fig. 1.1.a) and AD kinetics (Table 1.3): Maize exhibited the highest peak in daily CH_4 yield (20.4 ml CH_4 g^{-1} VS d^{-1}) after only six days of incubation. The three annual sorghum hybrids and S.

Silk attained an intermediate peak (average, 11.4 ml CH_4 g^{-1} VS d^{-1}) at a later time (after ca. ten days of incubation). Lastly, Arundo and especially Switchgrass featured the lowest peaks (8.3 and 7.0 ml CH_4 g^{-1} VS d^{-1}, respectively) at the same time as sorghum genotypes. In exchange for the steep initial phase, Maize plunged to negligible levels of daily methane yield (< 1 ml CH_4 g^{-1} VS d^{-1}) after only 30 days of incubation, whereas the other crops took about 40 days (Switchgrass, 45 days), to pass below this threshold (Fig. 1.1.b). In full scale biogas plants, this could reflect in a shorter hydraulic retention time for the reference feedstock (Maize), compared to the six alternative crops.

Wide differences in potential methane yield are reported in the literature among biomass crops alternative to maize. In the case of sorghum, higher values than in this experiment were often observed: +16% as average (Mahmood and Honermeier, 2012; Mahmood et al., 2013; Sambusiti et al., 2013) in forage × fibre genotypes (my data, 251 ml CH_4 g^{-1} VS in Trudan Headless); +15% as average (Jerger et al., 1987; Chynoweth et al., 1993; Mahmood and Honermeier, 2012; Mahmood et al., 2013), +35% (Bauer et al., 2010), -13% (Chapter #4), -1% (Sambusiti et al., 2013) and the same result (Chapter #3) in fibre genotypes (my data, 251 ml CH_4 g^{-1} VS in B 133). Lastly, sweet sorghum featured the largest difference between my data (256 ml CH_4 g^{-1} VS in Sucros 506) and those reported in other works: 400 ml g^{-1} VS (Jerger et al., 1987), 303 ml g^{-1} VS (Sambusiti et al., 2013) and 345 ml g^{-1} VS (Mahmood et al., 2013). However, the first work cited is quite old and modern hybrids as Sucros 506 are bred for a higher resistance to lodging, involving an increase in structural carbohydrates to the expenses of soluble sugars. In both this experiment and the cited sources, the three sorghum types (forage, fibre and sweet) outlined a similar potential in terms of specific methane yield, irrespective of their intended use.

In the cited works and in this experiment, the anaerobic digestion assay was always conducted under mesophilic conditions (between 35 and 38 °C) with an organic load around 4 g VS l^{-1}, whereas the incubation time varied from a minimum of 21 days (Mahmood and Honermeier, 2012; Mahmood et al., 2013) to a maximum of 60 days (Jerger et al., 1987). Further divergence is shown by the use of either fresh (Mahmood and Honermeier, 2012; Mahmood et al., 2013), dry (Sambusiti et al., 2013; this experiment) or ensiled biomass (Bauer et al., 2010): it is acknowledged that ensiled biomass yields an approximate 15% more specific methane than fresh biomass (Chynoweth et al., 1993), aggravating the difficulties in the interpretation of anaerobic digestion results.

Among perennial species, Switchgrass was recently investigated as it concerns methane production. Massé et al. (2011) found a range between 169 and 252 ml CH_4 g^{-1} VS depending on the time of harvest (summer and autumn). Frigon et al. (2012) evidenced a lower range: 95 to 152 ml CH_4 g^{-1} VS, also depending on the time of harvest (summer and winter) as well as on feedstock pre-treatment (various types). In both sources, higher outputs were obtained when Switchgrass was harvested still unripe in the summertime, as consequence of a lower lignification. The association between high specific methane yield and low fibre content in crop biomass supports this point (Bélanger et al., 2012). Compared to this, Switchgrass in this experiment featured a good potential CH_4 yield (216 ml CH_4 g^{-1} VS), despite the fact that it had been harvested at senescence, involving advanced lignification.

Lastly, in Arundo a potential yield of ca. 260 ml CH_4 g^{-1} VS was recorded with a single harvest at the same time of the year as in this experiment (Ragaglini et al., 2014); 190 ml CH_4 g^{-1} VS was achieved in Chapter #3, while the same result was obtained in Chapter #4. Moreover, in Chapter #2, 273 ml CH_4 g^{-1} VS were achieved with Arundo without pre-treatments, which is about 25% higher than in this work (217 ml CH_4 g^{-1} VS); this difference is mainly due to the termophilic conditions under which the cited experiment was conducted (53 °C vs. 35 °C in Chapter #4 and #3, respectively).

1.3.3 Biomass characteristics in view of methane production

The overall picture of biomass components (Table 1.2) is consistent with potential CH_4 yield, methanation rate constant and daily CH_4 yield registered in the seven crops (Fig. 1.1 and Table 1.3): Maize, the top performer, benefited from high proteins, lipids and starch, which are more easily transformed into methane than structural carbohydrates. Conversely, Maize was relatively poor in soluble sugars, another easily degradable fraction. The three sorghum hybrids ranking at intermediate levels of potential CH_4 were rich in soluble sugars, relatively well provided with proteins, lipids and structural carbohydrates, poor in starch. Arundo and Switchgrass lay at the bottom levels of potential CH_4 and were rich in structural carbohydrates, poor in all the other components.

Despite their recalcitrance, structural carbohydrates are the main sources of carbon for methane production in ligno-cellulosic biomass. Their degradation is hampered by AIL, as the three polymers (cellulose, hemicellulose and lignin) are strongly intermeshed and chemically bound (Pérez et al., 2002). In this experiment the role of AIL is highlighted by Sorghum Silk,

the species with a good level of potential CH_4 yield (Table 1.3) in spite of low amounts of easily degradable components (Table 1.2). However, S. Silk contained less AIL than the other two multi-annual species (Arundo and Switchgrass), which explains the higher methane potential attained by the former vs. the two latter species.

1.3.4 Energy assessments

Gross energy yield displayed a wide range between 286 GJ ha^{-1} in the top performer (Maize) and 158 GJ ha^{-1} of the two weakest producers (Switchgrass and S. Silk) (Fig. 1.2.a). This range corresponds to a methane output between 4600 and 8400 Nm^3 ha^{-1}. The two sorghum hybrids B 133 and S 506 yielded an approximate 10% and 20% less than Maize, respectively. Lastly, Trudan H. and the three multi-annual species were undifferentiated with an average 166 GJ ha^{-1}.

Cumulative energy demand, representing the difference between GE and NE in Fig. 1.2.a, ranged between 39 GJ ha^{-1} in Maize, the crop involving the most intensive management, and 15 GJ ha^{-1} in the three multi-annual species. The three sorghum hybrids showed an intermediate CED (21 GJ ha^{-1}), which is due to tillage being carried out every year as in Maize, while for the rest of crop husbandry annual sorghum is more similar to multi-annual species.

Net energy yield described the same pattern as gross energy yield (Fig. 1.2.a), although the gap between Maize, on one side, and B 133 and S 506, on the other side, was restrained thanks to sorghum's lower CED. As a result, the two aforementioned sorghum hybrids were not significantly different from Maize. In this experiment, NE values encompass a range of net methane output between 4200 and 7000 Nm^3 ha^{-1}.

In contrast to GE and NE, Maize displayed the lowest value of energy efficiency, statistically undifferentiated from Trudan H. (average of the two crops, 7.6 GJ GJ^{-1}) (Fig. 1.2.b). This was mainly due to a high CED in the former crop; to a low GE in the latter crop. Three heterogeneous crops, Switchgrass, S. Silk and S 506, featured an intermediate EE (average, 10.3 GJ GJ^{-1}). Lastly, Arundo and B 133 were shown the most efficient crops (average EE, 12.1 GJ GJ^{-1}), thanks to a very low CED in the case of Arundo; to a good NE in exchange for a modest CED in the case of B 133 (Fig. 1.2.b).

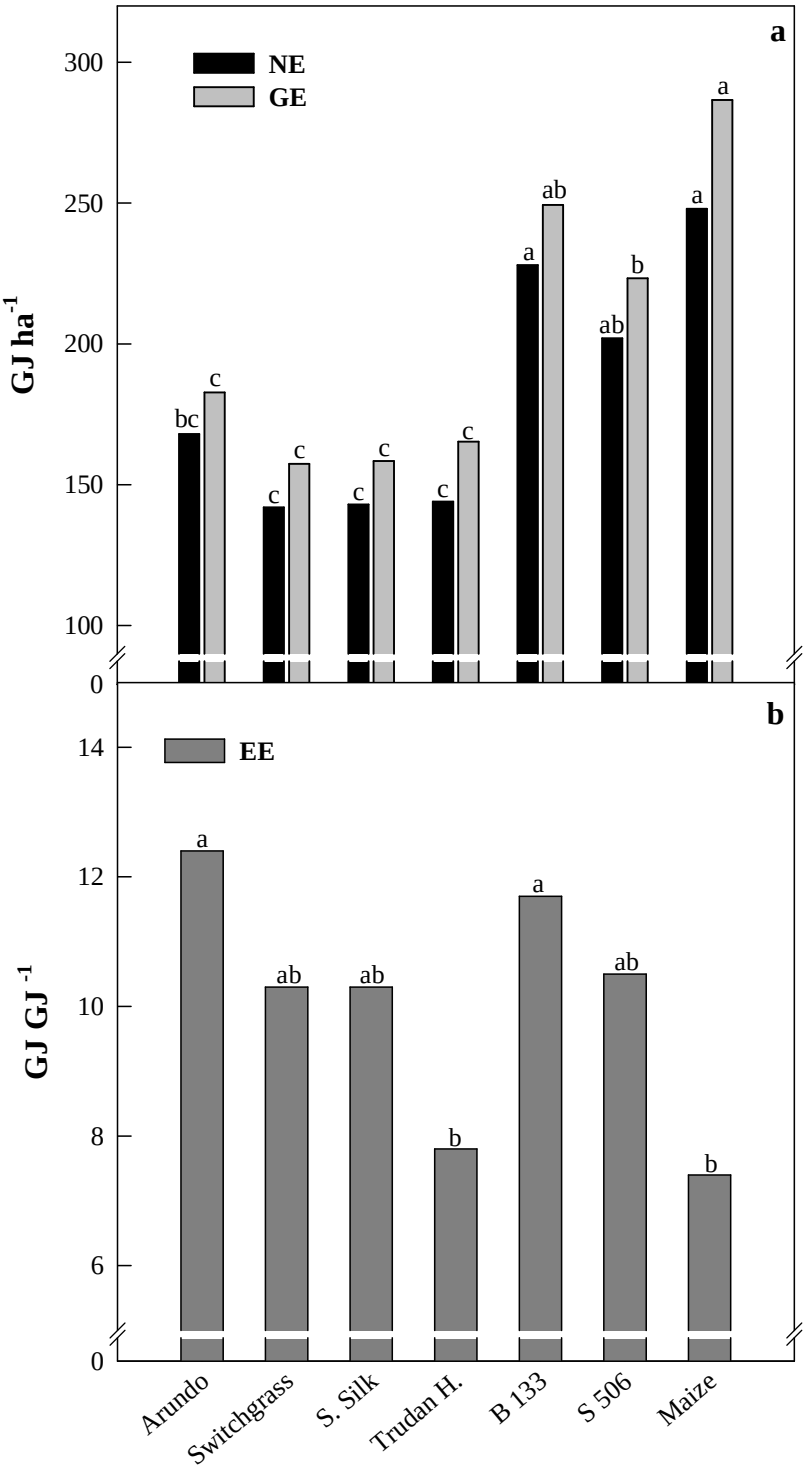

Figure 1.2 Gross energy yield (GE) and net energy yield (NE) (a), and energy efficiency (EE) (b) in the seven biomass crops. ANOVA always significant at $P \leq 0.01$. In each trait, different letters indicate statistically different means (SNK test; $P \leq 0.05$).

The only work focusing the energy balance of methane production using crop biomass (Switchgrass) combines the agricultural and industrial phase (Frigon et al., 2012), i.e. NE values result from GE - CED of biomass production and transformation into methane, and no direct comparison is made between Switchgrass and alternative species. The present work aims at filling this gap, providing useful data to support crop choice as the basis for a sustainable development of the biogas sector.

Wide differences are reported in the literature concerning gross energy yield or the equivalent trait, methane yield per hectare. A methane yield of 7288 Nm^3 ha^{-1} corresponding to a GE of 248 GJ ha^{-1} was recorded in Austria with ensiled sorghum, presumably a biomass (fibre or sweet) hybrid (Bauer et al., 2010); this data is very close to that of biomass sorghum in this experiment: 237 GJ ha^{-1} as the average of B 133 and Sucros 506. In another experiment a forage × fibre sorghum genotype achieved an average 129 GJ ha^{-1}, while a fibre genotype attained 175 GJ ha^{-1} (Mahmood and Honermeier, 2012); the two data are quite lower than those shown by the equivalent genotypes in this work (Trudan Headless and B 133, 165 and 250 GJ ha^{-1}, respectively). Gross energy yield in the cited experiments and in Mahmood et al. (2013) results from a higher specific methane yield than in this experiment, in exchange for a lower biomass yield.

In Switchgrass, GE ranging between 20 and 46 GJ ha^{-1} (Massé et al., 2011) and between 34 and 85 GJ ha^{-1} (Frigon et al., 2012) are reported, depending on harvest time and feedstock pre-treatment. The data obtained in this experiment with untreated Switchgrass (158 GJ ha^{-1}) largely exceeds those cited thanks to a much higher biomass yield per hectare.

In Arundo with a single harvest a methane yield up to ca. 9500 Nm^3 ha^{-1} is reported (Ragaglini et al., 2014). Conversely, no data of energy or methane yield per hectare is reported for Sorghum Silk. Based on my data, Arundo owns a ~15% margin over Switchgrass while S. Silk is at par. However, in this work Arundo suffers a wide gap from maize in both gross and net energy yield (-36% in GE and -32% in NE; Fig. 2.2.a). The prospects for Arundo to substitute maize appear therefore associated with the cultivation under low use of external inputs (water, fuels, etc.). In this light, biomass pre-treatments are seen as a valuable tool to improve Arundo's modest bio-degradability (Chapter #2), bridging the gap with maize.

In contrast to net energy yield, energy efficiency outlines good prospects for alternative crops to replace maize also in high fertility soils as in this experiment. The three multi-annual species and especially Arundo appear best suited for this task (average EE, +48% over maize; Fig. 2.2.b). In maize, the energy demand due to irrigation (4.5 GJ ha^{-1}) and additional N

supply (8.6 GJ ha^{-1}) represents a constraint the crop cannot overcome despite its good biomass and methane potential.

1.4 Conclusions

The seven crops investigated in this experiment featured remarkable differences in field biomass production, methane yield in the anaerobic digestion and the subsequent CH_4 output per hectare. The six crops alternative to maize outlined a less favourable composition in view of anaerobic digestion, as the result of a lower weight of the grain component which is rich in easily degradable fractions (proteins, lipids and starch). Not surprisingly, therefore, three alternative crops (Arundo and sorghum hybrids B 133 and S 506) yielded within ±10% dry biomass compared to maize, whereas none of them fell within -10% from maize in terms of potential methane yield. However, hybrid sorghum B 133 turned out to be quite competitive with Maize in terms of methane yield per hectare; conversely multi-annual species expressed a modest competitiveness due to deficiencies in both biomass and ultimate methane yield. Nevertheless, multi-annual species retain a special interest in view of the limited need of external inputs (energy, fertilizers, water, etc.) for their cultivation, reflecting in a lower environmental impact. To reduce the gap separating multi-annual species from maize, various strategies may be envisaged. Among them, it appears that the harvest stage should be better tailored to combine good biomass yield with lower recalcitrance to degradation. The implementation of biomass pre-treatments is also regarded as a promising approach, although the prospects for pre-treatment adoption depend on a careful evaluation of the energy and economic trade-off, in order to assure efficient and profitable processing.

CHAPTER 2

Effects of hydrothermal pre-treatments on Arundo biodegradability and methane production

Based on: Di Girolamo, G., Grigatti, M., Barbanti, L., Angelidaki, I., 2013.
Effects of hydrothermal pre-treatments on Giant reed (*Arundo donax*) methane yield.
Bioresource Technology 147, 152-159.

Abstract

Twelve hydrothermal pre-treatment combinations of temperature (150 and 180 °C), time (10 and 20 min) and acid catalyst (no catalyst; H_2SO_4 at 2% w/w immediately before steam cooking or in 24-hour pre-soaking) were tested to assess their effects on methane yield of Arundo biomass vs. untreated control. A batch anaerobic digestion was conducted with 4 g VS l^{-1} at 53 °C for 39 days. Untreated biomass exhibited a potential CH_4 yield of 273 ml g^{-1} VS; the four pre-treatments without acid catalyst achieved a 10%, 7%, 23% and 4% yield gain in the respective temperature/time combinations 150 °C/10 min, 150 °C/20 min, 180 °C/10 min and 180 °C/20 min. Conversely, the eight pre-treatments with H_2SO_4 catalyst incurred a methanogenic inhibition in association with high SO_4^{2-} concentration in the hydrolysate, known to enhance sulphate reducing bacteria. Furfurals were also detected in the hydrolysate of five strong pre-treatments with H_2SO_4 catalyst.

2.1 Introduction

The need to restrain CO_2 emissions and the increase in oil price drive the growing use of alternative energy sources (EU directive 2009/28/EC). Dedicated crops for energy uses may play a role in the abatement of GHG emission by reducing the dependence on fossil fuels. However, the introduction of energy crops in a scenario of decreasing food stocks is feared to compete for land with food crops, in turn leading to food price increases (FAO, 2008). Cultivating plant species suited for marginal lands is a proposed measure to alleviate this constraint; among them Arundo has showed a good adaptability and biomass production in limiting environmental conditions such as low water and fertilizer supply (Lewandowski et al., 2003). Arundo is a ligno-cellulosic perennial rhizomatous grass diffused in the Mediterranean area, which is considered a promising crop in terms of energy production in southern Europe (Lewandowski et al., 2003; Angelini et al., 2005). In ligno-cellulosic substrates, lignin is the most recalcitrant component to anaerobic biodegradadition (Taherzadeh and Kirimi, 2008), also hampering cellulose and hemicellulose biodegradability.

Various energy conversion technologies are diffused at present; among them AD with production of energy carrier biogas is acknowledged to be highly efficient (Petersson et al., 2007). In the anaerobic digestion of ligno-cellulosic materials, hydrolysis may be constrained by high lignin content and cellulose crystallization, resulting in low biogas output. Hence pre-

treatments are envisaged to overcome this constrain, such as thermo-chemical, including hydrothermal treatment.

In this method, the biomass is treated for a variable time with high temperature (160-260 °C) determining a pressure increase (0.7-4.8 MPa) (Kumar et al., 2009a); then the material is exposed to atmospheric pressure which determines hemicellulose and lignin degradation, increasing cellulose hydrolysis. The outcome mainly depends on residence time, temperature, particle size and moisture content (Sun and Cheng, 2002). Basically, pre-treatment effects may be enhanced by the addition of an acid or alkaline catalyst (Zimbardi et al., 2007) which promotes increased hemicellulose degradation in a shorter time at lower temperature (Kumar et al., 2009a).

This field of investigation is still widely unexplored as it concerns biomass of dedicated crops such Arundo. Owing to this species' characteristics (Scordia et al., 2012), it is sensed that methane output from its biomass could be enhanced by pre-treatments. To fill this gap of knowledge, a laboratory experiment was set up addressing hydrothermal pre-treatments and the subsequent AD of treated and untreated Arundo biomass. Aim of the experiment was to evaluate the effects of pre-treatments at varying time, temperature and catalyst on biomass degradability, potential yield and related traits in the anaerobic digestion.

2.2 Material and methods

2.2.1 Crop management

Arundo was grown at the experimental farm, University of Bologna (Italy), in Cadriano (BO, 44° 33' N, 11° 21' E, 32 m above sea level). At the end of the growth season (October), the crop was cut from the base and weighed; fresh biomass yield, total solids (TS; 48 h at 105 °C) and dry biomass yield per hectare were determined. Representative biomass samples were oven-dried (60 °C) and ground at 2 mm for chemical analysis.

2.2.2 Pre-treatments

Steam cooking as hydrothermal pre-treatment was studied as a function of three factors: temperature (150 and 180 °C), time (10 and 20 min) and acid catalyst (no catalyst; H_2SO_4 at 2% w/w TS either immediately prior to steam cooking or in a 24-hour pre-soaking before steam cooking), totalling 12 combinations plus the untreated control (Table 2.1).

Figure 2.1 describes the procedure followed in biomass pre-treatments, the fate of the resulting fractions and the analysis carried out during the whole process; the analytical procedures are described in section 2.2.4. More in detail, a consistent amount of substrate (100 g TS) at a given particle size (ca. 10 mm) was used in all treatments. 100 g TS was supplied with 2% w/w H_2SO_4 to final volume of 500 ml just prior to steam cooking (Steam 2, 4, 6 and 8) or impregnated with the substrate for 24 h before excess liquid (pre-hydrolysate) removal by filtration and steam cooking (Pre-soaking 1, 2, 3 and 4). The steam equipment was composed of a 12 l steam generator, a 2.7 l pressure vessel and three valves (steam introduction, steam release and collection of the liquid fraction after the pre-treatment). The pressure vessel was provided with a removable upper lid to introduce the sample and collect the solid fraction after the pre-treatment. A data logger monitored temperature of steam generator and pressure vessel at 30 s intervals. When the steam temperature reached the scheduled level (150 or 180 °C), steam introduction valve was opened. Within 30-40 s, the scheduled temperature was reached in the pressure vessel and maintained for all treatment duration. Thereafter, steam release valve was slowly opened until atmospheric pressure was restored, and the liquid fraction (hydrolysate) originating from steam condensation was collected through the bottom valve. Then the steam equipment was left to cool to ambient temperature, after which the solid fraction was collected.

Table 2.1 Conditions applied in the twelve hydrothermal pre-treatments vs. the untreated control. Acid catalyst (H_2SO_4) was supplied at the same level (2% w/w) either in pre-soaking, suspending 100 g TS of Arundo with H_2SO_4 in 500 ml for 24 h, or to solid samples of Arundo just before steam cooking. This was conducted at low and high temperature (150 and 180 °C), for a short and long time (10 and 20 min).

Pre-treatments	**H_2SO_4 (2% w/w)**		**Steam cooking**	
	in pre-soaking	**direct supply**	**temp. (°C)**	**time (min)**
Untreated	-	-	-	-
Steam 1	-	-	150	10
Steam 2	-	+	150	10
Steam 3	-	-	150	20
Steam 4	-	+	150	20
Steam 5	-	-	180	10
Steam 6	-	+	180	10
Steam 7	-	-	180	20
Steam 8	-	+	180	20
Pre-soaking 1	+	-	150	10
Pre-soaking 2	+	-	150	20
Pre-soaking 3	+	-	180	10
Pre-soaking 4	+	-	180	20

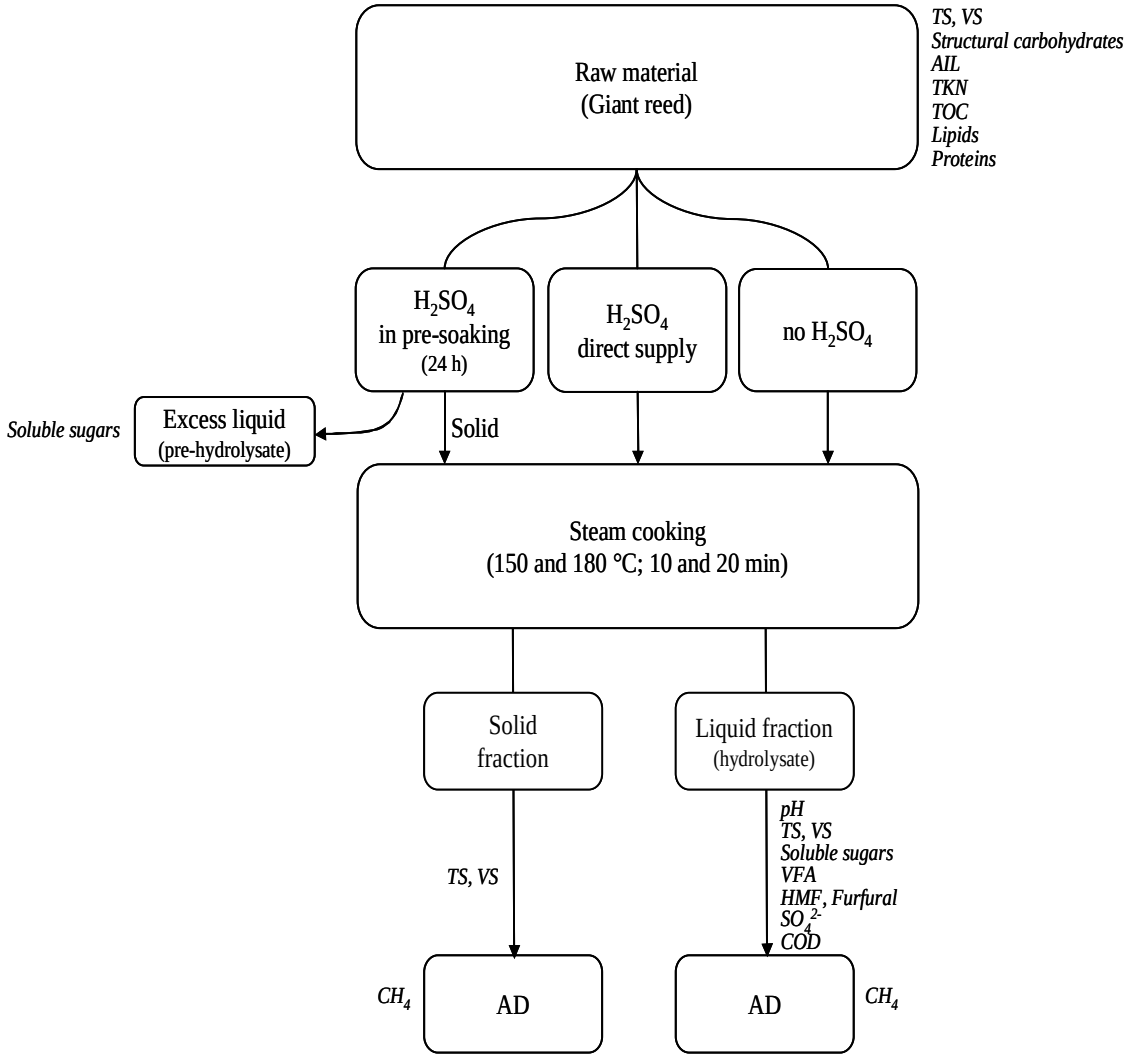

Figure 2.1 Scheme of pre-treatment procedure and analysis carried out on the raw material (Arundo), on the solid and liquid fraction resulting from hydrothermal pre-treatments and during the anaerobic digestion (AD) assay. TS, total solids; VS, volatile solids; Structural carbohydrates, hemicellulose and cellulose; AIL, acid insoluble lignin; TKN, total kjeldahl nitrogen; TOC, total organic carbon; Soluble sugars, glucose, xylose and arabinose; VFA, volatile fatty acids; HMF, 5-hydroxymethyl furfural; COD, chemical oxygen demand; CH_4, methane yield.

2.2.3 Methane potential assay

Methane yield was assessed in a batch anaerobic digestion. The assay was conducted at 53 °C in 323 ml glass bottles at a 4 g l^{-1} volatile solids (VS) loading for 39 days, suspending the samples in 80 ml of inoculum diluting by water (80% v/v) to a final volume of 100 ml. The high inoculum rate was adopted to avoid potential inhibition determined by high organic load or insufficient nutrients or alkalinity (Angelidaki and Sanders, 2004; Angelidaki et al., 2009). An effluent of anaerobic digestion (TS, 3.6%; VS, 41.2% TS; alkalinity, 31 g $CaCO_3$ l^{-1}; pH, 8.0) derived from a full scale biogas plant operating under thermophilic conditions was used as inoculum; the plant was fed with an approximate 75% animal manure and 25% industrial food waste. The following controls were added to the 13 treatments with Arundo: blank (inoculum alone) and positive control (Avicel PH 101; 4 g VS l^{-1}). After filling, the bottles were flushed with N_2 for 3 min to ensure anaerobic conditions and kept sealed for the whole incubation. The assay was done in triplicate. The solid and liquid fraction obtained from hydrothermal pre-treatments were incubated separately, but the final methane production was cumulated. Conversely, the excess liquid removed after pre-soaking (pre-hydrolysate) was analyzed to determine the soluble sugars released by structural carbohydrates, then was discarded without testing it via AD (Fig. 1). The incubation of both solid and liquid fraction lasted until the plateau (CH_4 production increase < 5%), totalling 39 days. Sixteen times (2-4 day interval) during the incubation CH_4 output was monitored as described by Hansen et al. (2004) and Angelidaki et al. (2009), by means of a gas chromatographer (GC) (Shimadzu GC 14A) equipped with flame ionization detector (FID) and expressed as ml CH_4 g^{-1} VS. Methane production from the sole inoculum (blank) was subtracted from methane produced in the samples with Arundo. CH_4 data are expressed at STP (Standard Temperature and Pressure; 273 K, 100 kPa).

2.2.4 Chemical analysis and analytical tools

The following analyses were carried out on raw biomass (untreated) and the 12 pre-treated substrates (Steam 1-8; Pre-soaking 1-4) at various steps of the experiment (Fig. 2.1).

2.2.4.1 Raw biomass

Arundoraw biomass was analysed for TS (48 h 105 °C), VS (4 h 550 °C), total nitrogen (TKN, by Kjeldahl-N method), total organic carbon (TOC, by the dichromate oxidation

method), lipid fraction (by the Soxhlet method with diethyl ether) and proteins by eq. 4. Soluble sugars (glucose, xylose and arabinose) contained in structural carbohydrates (hemicellulose and cellulose) were also determined by HPLC (Aminex XPX-87H column (300 × 7.8 mm) at 63 °C; mobile phase, 4 mM H_2SO_4; flow rate, 0.6 ml min^{-1}) provided with a refractive index detector (RID 1362A), according to the National Renewable Energy Laboratory (NREL) guidelines (Sluiter et al., 2011). Briefly, a acid hydrolysis (72% w/w H_2SO_4 at 30 °C for 60') was followed by sample autoclaving at 121 °C for 60' at a weaker acidity (4% w/w H_2SO_4). Then the hydrolysate was collected by vacuum filtration with glass micro-fibre filter (Whatman GF/C, Ø 47 mm). On the separated solid residue the acid-insoluble lignin (AIL) was determined (24 h at 550 °C). Thereafter hemicellulose in the raw biomass was calculated as (xylose + arabinose)*0.88; cellulose as glucose*0.9.

2.2.4.2 Hydrolysate

The liquid fraction following hydrothermal pre-treatments (hydrolysate) was analyzed for TS (48 h at 105 °C), VS (4 h at 550 °C) and pH. Soluble sugars (glucose, xylose and arabinose) were determined on the hydrolysate by means of HPLC (at the same conditions reported above) while the solid residue was used to determine acid-insoluble lignin (AIL) after 550 °C for 24 h. Moreover, 5-hydroxymethyl furfural (HMF) and furfural were measured by UV-detector after HPLC determination at the same conditions.

VFA were determined by a GC (Shimadzu) equipped with a FID; the separation was performed with a Zebron-FFAP capillary column (0.53 mm I.D × 1 μm). Prior to gas chromatography, 1.5 ml of hydrolysate was placed in an Eppendorf vial and acidified with H_3PO_4 34% v/v before centrifugation (12,000 rpm for 10 min), in order to convert VFA (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and hexanoic acid) to their acidic form saturating the basic sites on the analytical column: a 100 μl injection standard (4-methyl valeric acid, 1.1 mM) was added to 1 ml of sample in a GC vial.

SO_4^{2-} was determined in the hydrolisate of selected samples by Dionex ion chromatography ICS-150. After injection, the anion was separated by a Phenomenex STAR-ION_A33 column at 35 °C; a 3.5 mM Na_2CO_3 plus 1 mM $NaHCO_3$ solution was used as eluent at a flow rate of 1.5 ml min^{-1}. After separation, SO_4^{2-} was detected and measured by a conductivity detector.

Chemical oxygen demand (COD; g O_2 l^{-1}) was determined by the dichromate method for water analysis.

All the chemical traits except VFA and pH were analyzed in triplicates.

2.2.4.3 Calculations

Soluble sugars (glucose, xylose and arabinose) released by pre-treatments in the hydrolysate were expressed as % of the respective amounts contained in structural carbohydrates (cellulose in the case of glucose; hemicellulose in the case of xylose and arabinose) of the raw material by means of the following equation:

$$\text{Soluble sugars (\%)} = \frac{\text{Sugars}_{\text{released}}}{\text{Carbohydrate}_{\text{structural}}} * 100 \qquad \text{[eq. 1]}$$

where $\text{Sugar}_{\text{released}}$ is the amount of one such soluble sugar detected in the hydrolysate and $\text{Carbohydrate}_{\text{structural}}$ is the amount of the same sugar in structural carbohydrates of the raw material.

The theoretical methane potential ($B_{0,th}$; ml^{-1} g VS) of the untreated substrate was calculated based on the stoichiometric conversion of organic matter:

$$B_{0,th} = 415 * \text{carbohydrates} + 496 * \text{proteins} + 1014 * \text{lipids} \qquad \text{[eq. 2]}$$

where carbohydrates represent the total carbohydrates content calculated as proposed by Hansen et al. (1998):

$$\text{Carbohydrates} = \text{VS} - (\text{proteins} + \text{lipids}) \qquad \text{[eq. 3]}$$

Proteins represent the total protein content calculated as:

$$\text{Proteins} = \text{TKN} * 6.25 \qquad \text{[eq. 4]}$$

Substrate biodegradabilty (BD) was determined by comparing the cumulated methane yield at the end of the incubation with the theoretical potential:

$$BD\,(\%) = \frac{CH_{4;\,end}}{B_{0;\,th}} * 100 \qquad \text{[eq. 5]}$$

where $CH_{4;\,end}$ (ml g^{-1} VS) is cumulated methane yield at the end of the incubation or, in those treatments where eq. 8 was successfully fitted, potential methane yield, i.e. the function asymptote; $B_{0,th}$ (ml g^{-1} VS) is the theoretical methane potential according to eq. 2.
Methanogenic inhibition was calculated as:

$$Inhibition\,(\%) = 100 - \frac{CH_{4;\,end\ Pre\text{-}treatment}}{CH_{4;\,end\ Untreated}} * 100 \qquad \text{[eq. 6]}$$

where $CH_{4;\,end\ Pre\text{-}treatment}$ and $CH_{4;\,end\ Untreated}$ are the respective $CH_{4;\,end}$ of pre-treated and untreated samples. Negative values indicate methanogenic enhancement.
The combined severity factor (log R'_0) expressing the overall severity of pre-treatment conditions was calculated as proposed by Kabel et al. (2007):

$$\log R'_0 = (10^{-pH}) * t * \exp\left(\frac{T - 100}{14.75}\right) \qquad \text{[eq. 7]}$$

where, pH is the hydrolysate pH, t is pre-treatment time (min) and T pre-treatment temperature (°C).

2.2.5 Statistical analysis

In all traits normal distribution and equal variance of data were controlled through the Kolmogorov-Smirnov and Bartlett tests, respectively. Data were then submitted to one way analysis of variance (ANOVA) through the CoStat 6.3 software (CoHort Software, Monterey, CA, U.S.A.). The SNK test at $P \leq 0.05$ was adopted to separate means of statistically significant traits. Pearson's correlation (r) was assessed among selected traits.
Methane yield cumulated over time in selected treatments was fitted by means of the Sigma Plot 10 software (Systat Software Inc., Chicago, Illinois, U.S.A.), according to an exponential rise to max equation:

$$CH_{4\,m} = CH_{4;0}\,(1-e^{-kt}) \qquad \text{[eq. 8]}$$

where $CH_{4\,m}$ is the fitted methane yield (ml g^{-1} VS) at a given time (d), $CH_{4;\,0}$ is the potential methane yield (ml g^{-1} VS), i.e. the function asymptote; *k* is the methanation rate constant (d^{-1}) and t is time (d).

2.3 Results and discussion

2.3.1 Biomass yield and characteristics

Dry biomass yield of Arundo at the 9th growth season (2010) was 26.8 Mg ha^{-1}, fitting in the normal range observed for this species in Italian environments (13-47 Mg ha^{-1}, Angelini, et al., 2005). The air-dried biomass had 93.3% TS and 87.2% VS (on a TS basis). TS were composed of 329, 233 and 259 g kg^{-1} cellulose, hemicellulose and AIL, respectively. Those amounts are in agreement with values reported for this species by Scordia et al. (2012). TOC and TKN values were 446 and 6 g kg^{-1} TS, respectively, determining a 56.9 C/N ratio. Proteins and lipids were 51 and 9 g kg^{-1} TS, respectively. Therefore Arundo was particularly deprived of these two important components compared to maize (reference proteins and lipids, 75 and 20 g kg^{-1} TS, respectively), which is the biomass crop most commonly used as feedstock for AD in Italy.

2.3.2 Hydrolysate composition

Addition of the acid catalyst determined a TS increase in the hydrolysate, indicating a stronger biomass degradation (Table 2.2): pre-treatments without catalyst (Steam 1, 3, 5 and 7) averaged 1.62% TS; those with H_2SO_4 supplied prior to steam cooking (Steam 2, 4, 6 and 8), 1.98% TS; those with H_2SO_4 in pre-soaking (Pre-soaking 1-4), 2.36% TS. Pre-soaking also showed a higher VS content (average, 93.1% TS) than the rest of pre-treatments (average, 70.2% TS).

The amount of soluble sugars released in the hydrolysate also varied depending on treatments (Table 2.2): glucose consistently showed lower values in Pre-soaking 1-4 (average, 7.3 g kg^{-1} TS), compared to the remaining pre-treatments (average, 10.9 g kg^{-1} TS). An opposite pattern was observed in xylose and arabinose, whose mean values in Pre-soaking 1-4 (43.8 and 17.4 g kg^{-1} TS for the two respective sugars) were much higher than in the other pre-treatments (12.6

and 3.5 g kg^{-1} TS, respectively). Hence it appears that pre-soaking exerted a strong action on hemicellulose, whereas cellulose was modestly affected. The significant decrease of glucose determined by pre-soakings vs. the other treatments (average, -3.7 g kg^{-1} TS) may be partly explained by pre-hydrolysate removal from the system (Fig. 2.1): analysis of the filtered liquid removed after Pre-soaking 1-4 showed an average 1.6 g glucose kg^{-1} TS. However, the amount of removed glucose corresponds to a potential CH_4 yield of only 2.4 ml g^{-1} VS. As general, it is perceived that the low glucose concentration observed in this experiment was probably due to the crystalline and thermo-resistant structure of cellulose (Kaparaju et al., 2009a), involving only a modest release at temperatures (150 – 180 °C) at which hemicellulose is already being dissolved. This is consistent with the fact that pentosans (C5 sugars), namely xylose and arabinose, are more susceptible to thermal degradation than hexosans (C6 sugars), i.e. glucose (De Bari et al., 2013).

As a result, the amount of glucose released in the hydrolysate hardly averaged 3% of this sugar's initial amount in Arundo cellulose (329 g kg^{-1}) (Fig. 2.2). Xylose and arabinose outlined modest values in Steam 1-8: 8% and 5% of initial hemicellulose content (233 g kg^{-1} TS), respectively. Much higher values were evidenced for the two sugars in Pre-soaking 1-4 (respective averages, 34% and 25%). Suryawati et al. (2009) observed up to 1.9% and 25.2% of glucose and xylose release, respectively, in different conditions of hydrothermal pre-treatments for Switchgrass (*Panicum virgatum*).

HMF and furfural were not found in 7 pre-treatments out of 12 (Table 2.2). In practice, the two compounds were only detectable in pre-treatments including acid catalyst and either high temperature (Steam 8, Pre-soaking 3-4), or low temperature associated with long treatment time (Steam 6 and Pre-soaking 2). It appears therefore that only a strong combination of pre-treatment factors leads to HMF and furfural accumulation. The two noxious compounds have already been seen to originate from the degradation of glucose and xylose following hydrothermal pre-treatments (Larsson et al., 1999). The relatively low concentration of HMF and furfural found in this experiment (average, 0.27 and 0.17 g kg^{-1} TS, respectively) was probably due to their high volatility hampering full recovery in the hydrolysate (Kaparaju et al., 2009b). These concentrations are quite lower than those (about 2 g kg^{-1} for both HMF and furfural) shown to curb methane yield (Barakat et al., 2012). However, it is perceived that even 0.2 g kg^{-1} TS of either compound may adversely affect methane production rate, since methane producing *Archaea* require a period of adaptation (Benjamin et al., 1984).

Acetic acid was the most abundant VFA produced during hydrothermal pre-treatment, while other VFA were observed in negligible amounts (data not shown). Acetic acid was positively correlated with xylose and arabinose (Fig. 2.3), due to the fact that acetic acid is released from acetyl groups contained in the side chains of hemicellulose (Kaparaju et al., 2009b) which is mainly composed of xylose and arabinose. Therefore pre-treatments releasing high amounts of the two sugars also displayed high levels of acetic acid: this is the case of Pre-soaking 1-4 compared to the rest of pre-treatments (average acetic acid, 18.8 and 2.4 g kg^{-1} TS in the two respective groups).

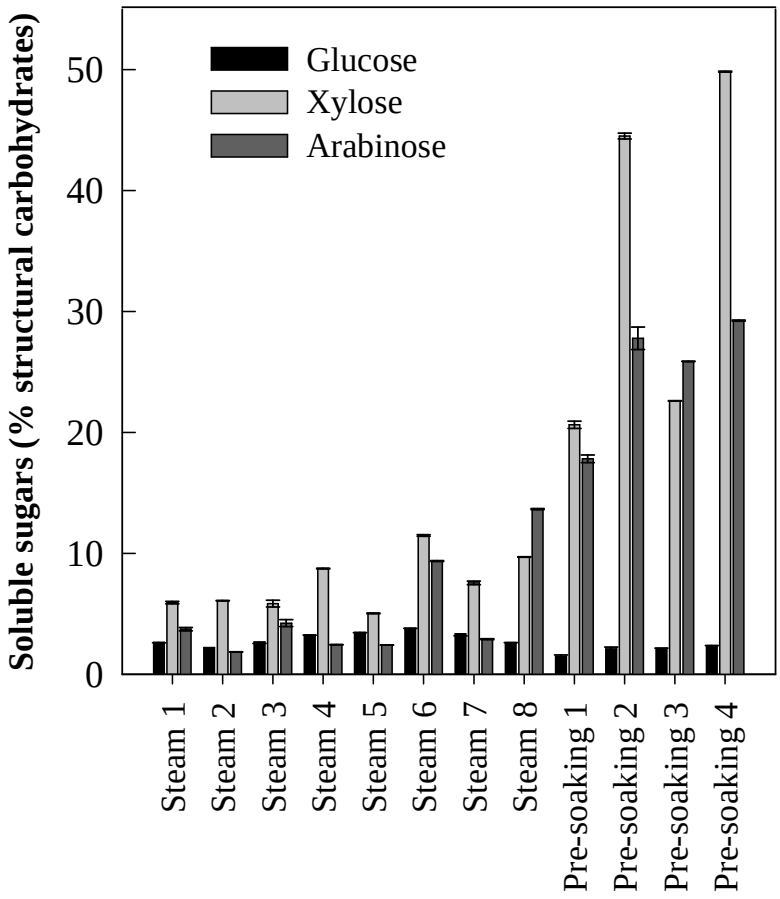

Figure 2.2 Sugars released in the hydrolysate as % of the respective amounts contained in structural carbohydrates of untreated Arundo. Vertical bars, ± standard errors (n = 3). Pre-treatment conditions are described in Table 2.1.

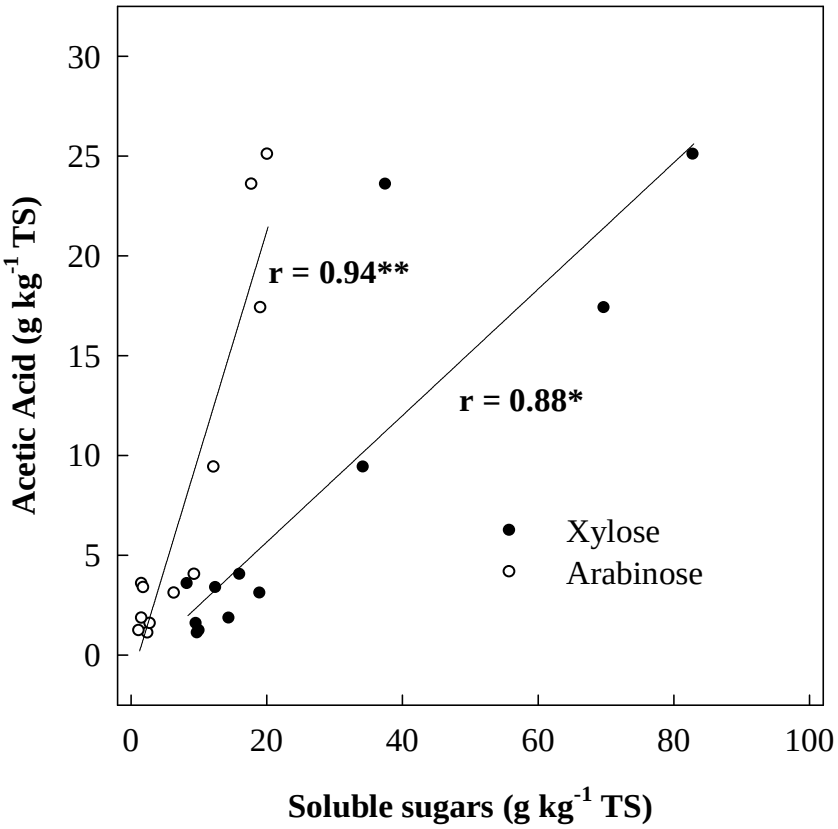

Figure 2.3 Correlations between the amount of xylose, arabinose and acetic acid released by hydrothermal pre-treatments in the hydrolysate (n = 12).

Table 2.2 Characteristics of the hydrolysate after hydrothermal pre-treatments. Pre-treatments conditions are described in Table 2.1.

Pre-treatments	TS	VS	Glucose	Xylose	Arabinose	HMF	Furfural
	%	% TS	g kg^{-1} TS				
Steam 1	1.5 f	72.2 b	9.6 c	9.9 fg	2.6 g	0 d	0 f
Steam 2	2.1 d	56.1 d	8.0 d	10.1 fg	1.3 h	0 d	0 f
Steam 3	1.4 g	72.3 b	9.7 d	9.7 fg	2.9 g	0 d	0 f
Steam 4	2.0 de	64.0 c	11.8 b	14.6 ef	1.7 h	0 d	0 f
Steam 5	1.6 f	73.4 b	12.6 b	8.4 g	1.7 h	0 d	0 f
Steam 6	1.9 e	74.6 b	13.9 a	19.1 d	6.5 f	0.22 c	0.12 d
Steam 7	2.0 de	72.7 b	12.1 b	12.6 eg	2.0 h	0 d	0 f
Steam 8	2.0 de	76.3 b	9.5 c	16.1 de	9.4 e	0.36 a	0.07 e
Pre-soaking 1	2.2 c	94.4 a	5.4 e	34.3 c	12.3 d	0 d	0 f
Pre-soaking 2	2.4 ab	90.5 a	7.6 d	69.8 b	19.2 b	0.22 c	0.21 b
Pre-soaking 3	2.3 bc	93.0 a	7.8 d	37.6 c	17.9 c	0.30 b	0.14 c
Pre-soaking 4	2.5 a	94.3 a	8.6 cd	82.9 a	20.2 a	0.24 c	0.32 a

TS, total solids; VS, volatile solids; HMF, 5-hydroxymethyl furfural. ANOVA always significant at $P \leq 0.01$. In each trait, different letters indicate statistically different means (SNK test; $P \leq 0.05$).

2.2.3 Methane yield during the incubation

Hydrothermal pre-treatments determined a variable effect on methane yield during the incubation: two basically different behaviours were shown by pre-treatments either enhancing or inhibiting bio-methanation. Pre-treatments without acid catalyst (Steam 1, 3, 5 and 7) enhanced the trait with respect to untreated Arundo (Fig. 2.4 and Table 2.4): CH_4 production was initiated after 2 days of incubation and the cumulated yield followed a similar pattern in these four pre-treatments, although different final levels were attained (Fig. 2.4). In non-inhibited treatments the exponential rise to max function explained a very high share of the total variation (R^2 always at 0.99**; Table 2.3): the untreated achieved a potential methane yield of 273 ml g^{-1} VS (Table 2.3); Steam 7 was only slightly higher (+4%), but showed a much faster kinetics (*k*, 0.15 vs. 0.07 d^{-1}; Table 2.3): a CH_4 yield of 250 ml g^{-1} VS was reached in only 15 days of incubation (Fig. 2.4). Steam 1, 3 and 5 achieved a significant gain in potential methane yield (+10%, +7% and +23% vs. the untreated, respectively), depicting similar kinetics (Table 2.3 and Fig. 2.4). Facing these increases of methane yield, the modest biodegradability of Arundo (64% in untreated) was augmented up to 79% in Steam 5 (Table 2.4).

Contrasting this general picture of smooth CH_4 production, pre-treatments with acid catalyst (Steam 2, 4, 6, 8 and Pre-soaking 1-4) were detrimental to AD as they soon incurred a methanogenic inhibition depressing cumulated methane yield (data not shown); this in turn hampered the possibility to fit curves describing CH_4 trends in time. As a result, biodegradability fell from 64% of untreated biomass to 30% in pre-treatments with H_2SO_4 supplied just prior to steam cooking (average of Steam 2, 4, 6 and 8); to only 5% in pre-treatments with H_2SO_4 pre-soaking for 24 h before steam cooking (average of Pre-soaking 1-4) (Table 2.4). The loss of biodegradability corresponds to a final methanogenic inhibition averaging 53% and 93% in the two aforementioned groups of treatments with respect to untreated Arundo (Table 2.4).

Table 2.3 Equation parameters and regression coefficients of the exponential rise to max functions fitted in Figure 4 for the untreated control and the four pre-treatments without acid catalyst. In brackets, standard errors (n = 3). Pre-treatment conditions are described in Table 2.1.

Pre-treatments	**$CH_{4;0}$** (ml g^{-1} VS)	***k*** (d)	**$R^2_{adj.}$**
Untreated	273 (6.8)	0.07 (0.004)	0.99**
Steam 1	301 (6.4)	0.08 (0.005)	0.99**
Steam 3	293 (4.4)	0.10 (0.005)	0.99**
Steam 5	337 (8.6)	0.08 (0.005)	0.99**
Steam 7	283 (4.0)	0.15 (0.008)	0.99**

$CH_{4;\,0}$, potential methane yield; *k*, methanation rate constant; VS, volatile solids. ** means significant at $P \leq 0.01$.

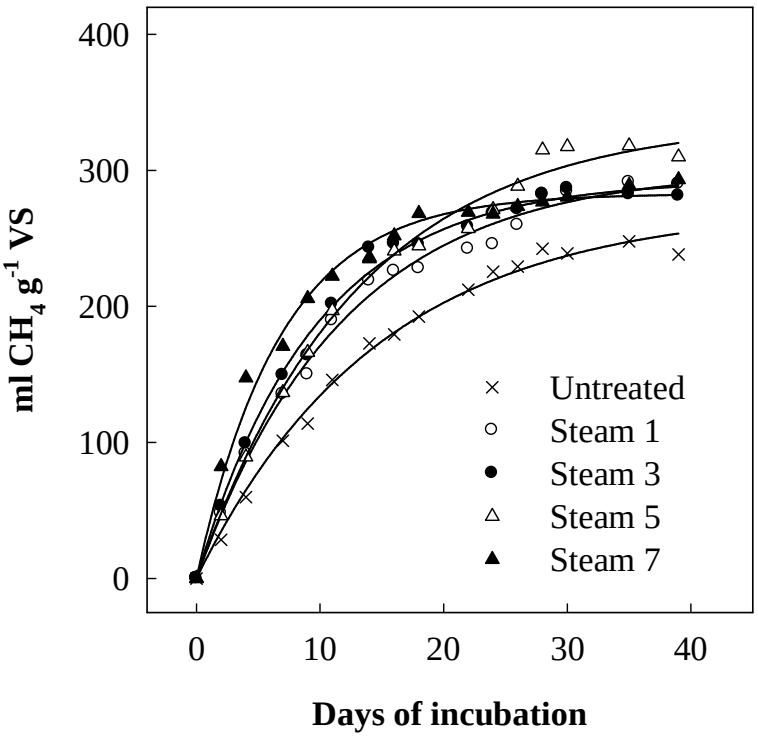

Figure 2.4 Cumulative methane yield during the anaerobic digestion of the untreated control and the four pre-treatments without H_2SO_4 catalyst (symbols), and fitted functions (lines). Equation parameters and regression coefficients are reported in Table 2.3. Pre-treatment conditions are described in Table 2.1.

Table 2.4 Biodegradability (BD) and methanogenic inhibition in untreated and pre-treated samples. Negative values of inhibition indicate methanogenic enhancement. Pre-treatment conditions are described in Table 2.1.

Pre-treatments	**BD** (%)	**Inhibition** (%)
Untreated	64	-
Steam 1	71	-10
Steam 2	33	49
Steam 3	69	-7
Steam 4	33	49
Steam 5	79	-23
Steam 6	32	50
Steam 7	67	-4
Steam 8	24	63
Pre-soaking 1	6	91
Pre-soaking 2	4	94
Pre-soaking 3	0	100
Pre-soaking 4	8	87

This strong inhibition was associated with high SO_4^{2-} concentration in the hydrolysate, most likely boosting the activity of sulphate reducing bacteria (SRB) which are known to negative affect anaerobic digestion (O'Reilly and Colleran, 2006). Chen et al. (2008) reported two stages of methanogenic inhibition associated with sulphur: first, the competition for organic and inorganic substrates from SRB curbs methane production; then the build up of sulphides as a product of SO_4^{2-} reduction becomes toxic to various groups of micro-organisms. Several factors affect the competition between SRB and methane producing *Archaea*. The main ones are: physical structure of microbial cultures, substrate type and concentration, pH, temperature, biomass type, sulphate concentration, long chain fatty acids, chemical oxygen demand (COD)/SO_4^{2-} ratio, sulphide toxicity, trace elements and other nutrients (Sousa et al.,

2009; Patidar and Tare, 2005). Among them, SO_4^{2-} and the COD/SO_4^{2-} ratio are seen the two most prominent traits associated with high methanogenic inhibition. Moset et al. (2012) observed a decrease of methane yield up to 96% when digesting pig slurry rich in SO_4^{2-} (2.4 g l^{-1}). Likewise, Jeong et al. (2008) evidenced a 40% reduction of methane yield using activated sludge with low COD/SO_4^{2-} ratio (below 11.6). Compared to this, in this experiment SO_4^{2-} in the hydrolysate achieved 1.8 and 3.0 g l^{-1} in the average of Steam 2, 4, 6 and 8, and Pre-soaking 1-4, respectively; the COD/SO_4^{2-} ratio settled at 7.5 and 6.2 in the two respective groups (data not shown). Therefore both traits showed levels which are consistent with the strong inhibition incurred in the eight treatments involving acid catalysis. As a result of high SO_4^{2-} concentration stimulating acidification, pH in the hydrolysate fell from 6.1 (average of Steam 1, 3, 5 and 7) to 1.7 (average of Steam 1, 3, 5 and 7), to only 1.3 (average of Pre-soaking 1-4) (data not shown).

The combined severity factor (log R'_0), originally correlated with fractions (e.g., sugars, lignin, furfurals) deriving from biomass pre-treatment (Kabel et al., 2007), makes it possible to summarize in a single trait the effect of time, temperature and pH in the hydrolysate. This experiment showed a good correlation between this trait and methanogenic inhibition/enhancement (r = 0.90**) (Fig. 2.5): a log R'_0 value between -4 and -2 was associated with methanogenic enhancement (average inhibition, -12%); between 0.5 and 2.5, with inhibition (average, 73%). Therefore the combined severity factor represents a simple trait which could be used to anticipate the effect of methanogenic inhibition, although further studies are needed to support this point.

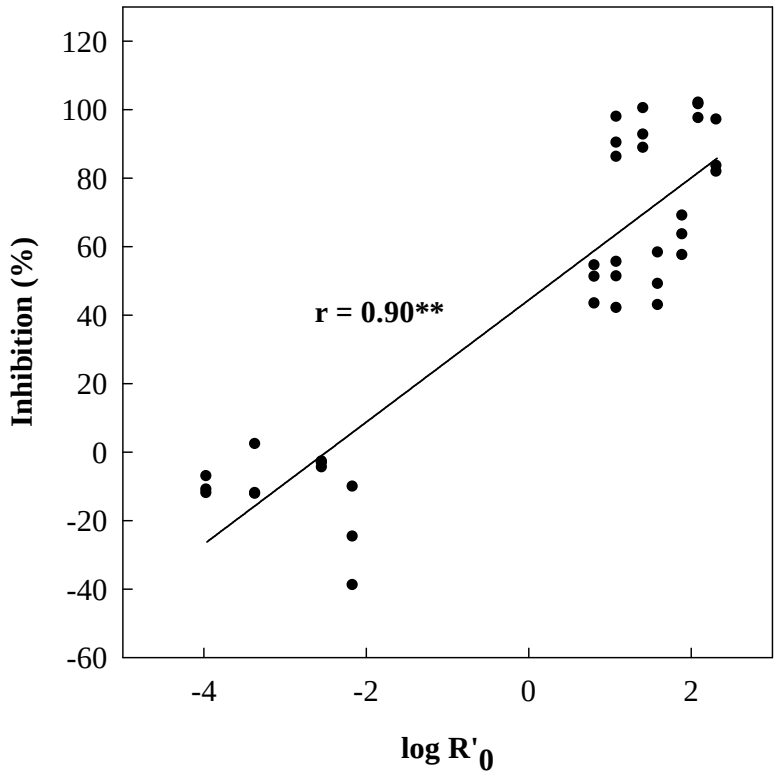

Figure 2.5 Correlation between combined severity factor (log R'_0) and methanogenic inhibition in hydrothermal pre-treatments (n = 36). Negative values of inhibition indicate methanogenic enhancement.

There is no general consensus about how time, temperature and catalyst should be arranged in pre-treatment composition, which is consistent with the large variation in their results. As an example, in cereal straw a much higher increase in methane yield (40% over an untreated level of 240 ml CH_4 g^{-1} VS) was obtained under mild treatment conditions (90 °C for 30 min) by Menardo et al. (2012), whereas a modest increase (20% over 180 ml CH_4 g^{-1} VS) was observed in the same substrate under stronger conditions (200 °C for 10 min) by Chandra et al. (2012a). A large variation is also shown in biomass species, although no data is yet available in literature, concerning Arundo after pre-treatments. However, an increase of 30% over 190 ml CH_4 g^{-1} VS (pre-treated with NaOH 0.15 N at 25 °C for 24 h) and 62% over 218 ml CH_4 g^{-1} VS (pre-treated with maleic acid 0.6 M at 25 °C for 24 h), were obtained in Arundo (Chapter #3 and #4, respectively). Several other species have been tested in experiments dealing with hydrothermal pre-treatments in AD, resulting in contrasting results: this is the case of perennial ryegrass (*Lolium perenne*) staging a good yield increase (39%

over 325 ml CH_4 g^{-1} VS) at 100 °C for an unspecified time with a 5% NaOH addition (Xie et al., 2011), compared to Switchgrass (*Panicum virgatum*) showing a modest increase (24% over 112 ml CH_4 g^{-1} VS) at 121 °C for 15 min with a 7% NaOH addition (Frigon et al., 2012). However, it is perceived that the large variation in CH_4 outputs among biomass crops, beside specific differences depends on plant stage and associated traits: in the work of Xie et al. (2011), perennial ryegrass was harvested early in the growth season, presumably before heading thus with a modest lignification; conversely in the work of Frigon et al. (2012) Switchgrass was set for winter harvest involving crop weathering, loss of leaves and advanced lignification (AIL, 207 g kg^{-1} TS), three factors leading to poor biodegradability. Compared to these two cases, Arundo in this experiment displayed a lignin content (AIL, 259 g kg^{-1} TS) even higher than Switchgrass, whereas its potential methane yield (337 ml g^{-1} VS in Steam 5) fit closer to that of pre-treated perennial ryegrass (452 ml g^{-1} VS) (Xie et al., 2011) than Switchgrass (139 ml g^{-1} VS) (Frigon et al., 2012). Hence it appears that the relationship between biomass traits and CH_4 production needs to be more deeply investigated to better understand biomass behaviour during anaerobic digestion.

2.4 Conclusions

In this experiment hydrothermal pre-treatments without acid catalyst contributed to methane yield of Arundo (average, +12%), whereas pre-treatments with H_2SO_4 underwent a strong methanogenic inhibition. The boundary between beneficial and detrimental pre-treatments appears tenuous and difficult to seize, although parameters are proposed to more accurately foretell pre-treatment effects, such as the combined severity factor. The paucity of systematic studies on pre-treatment time, temperature and acid catalysis in the scientific literature on ligno-cellulosic biomasses is a further element explaining the current difficulties in dealing with pre-treatment design and implementation. More to this, when improvements of final methane production are obtained, it is necessary to evaluate their viability in full scale biogas plants. These issues combined represent the current frontier in the research on anaerobic digestion. Future progress may be envisaged, amid other strategies, in bland pre-treatments (e.g., lower temperatures and weaker catalysts) as a potential means to improve net energy gain and methane production efficiency.

CHAPTER 3

Effects of alkaline pre-treatments on composition, structure and methane output of Arundo, biomass sorghum and barley straw

Based on: Di Girolamo, G., Grigatti, M., Bertin, L., Capecchi, L., Ciavatta C., Barbanti, L., 2014. **Mild alkaline pre-treatments loosen fibre structure enhancing methane production from biomass crops and residues.** Biomass and Bioenergy (*Submission, Under revision*).

Abstract

Three ligno-cellulosic substrates representing varying levels of biodegradability (Arundo, B 133 sorghum and Barley straw) were combined with mild alkaline pre-treatments (untreated, NaOH 0.05, 0.10 and 0.15 N at 25 °C for 24 hours) to study pre-treatment effects on physical-chemical structure, anaerobic digestibility and methane output of the three substrates. The most recalcitrant substrate (Arundo) staged the highest increase in cumulative methane yield in batch AD (58 days; 35 °C; 4 g VS l^{-1}): +30% with NaOH 0.15 N over 190 ml CH_4 g^{-1} VS in untreated Arundo. Conversely, the least recalcitrant substrate (B 133 sorghum) exhibited the lowest gain (+10% over 248 ml CH_4 g^{-1} VS), while an intermediate behaviour was shown by Barley straw (+23% over 232 ml CH_4 g^{-1} VS). Pre-treatments also speeded AD kinetics and reduced technical digestion time (i.e., the time needed to achieve 80% methane potential), which are the premises for increased production capacity of full scale AD plants. Fibre components (cellulose, hemicellulose and acid insoluble lignin determined after acid hydrolysis) and substrate structure (Fourier transform infrared spectroscopy and scanning electron microscopy) outlined remarkable reductions of the three fibre components after pre-treatments, supporting claims of loosened lignin binding over cellulose and hemicellulose. Hence mild alkaline pre-treatments demonstrated to improve the biodegradability of ligno-cellulosic substrates to an extent proportional to their recalcitrance, contributing to mitigate the food vs. non-food controversy raised by the use of cereals as feedstocks for biogas production in AD plants.

3.1 Introduction

The continuous increase of GHG emission to the atmosphere and the concern for energy security have strengthened the interest RES (UNEP, 2011). To promote their development, the European Commission has set a double target of 20% energy consumption from RES and 20% reduction of GHG emission by 2020 (EREC, 2010). In this frame, biogas production through AD of biomass is seen as an economically viable and environmentally friendly technology that is growing worldwide, but at the same time may spur the competition for available land with food crops, since the most suitable substrates for AD are starch crops as cereals (Murphy et al., 2011). Ligno-cellulosic substrates, such as biomass crops suited for marginal lands and agricultural residues, are seen to potentially alleviate this contrast.

Ligno-cellulosic substrates are mainly composed of cellulose, hemicellulose and lignin in a proportion depending on plant species and organ. Cellulose and hemicellulose are easily degraded by AD, whereas lignin is a recalcitrant component, also hampering the activity of hydrolytic enzymes (Tarherzadeh and Karimi, 2008). As a result, hydrolysis is the rate-limiting step during AD of ligno-cellulosic substrates (Lu et al., 2007). To overcome this drawback, pre-treatments are envisaged, which are able to loosen the bindings between lignin and the two structural carbohydrates, easing microbial access and subsequent degradation (He et al., 2008; Hendriks and Zeeman, 2009). This in turn leads to increases in hydrolysis rate, digestion efficiency and biogas production, and to concurrent decrease in the hydraulic retention time.

Pre-treatments may be grouped into mechanical (e.g., milling), thermal (e.g., liquid hot water), chemical (acid or alkaline) and biological (e.g., enzymatic) treatments, or a combination of them. Acid pre-treatments require high temperature and pressure during treatment; they determine the hydrolysis of hemicellulose in monomeric units, while rendering cellulose accessible for enzymatic attack. However, acid agents, especially sulphuric acid, are corrosive and toxic, requiring expensive processing for acid neutralization or post treatment recovery (Agbor et al., 2011). Thereby, alkaline pre-treatments are often favoured to treat ligno-cellulosic substrates, as they may be performed at lower temperature and pressure than acid pre-treatments. Conversely, alkaline pre-treatments necessitate hours or days, instead of minutes as acid pre-treatments. Various alkali agents can be used (sodium, calcium and potassium hydroxides), but sodium hydroxide has been most studied. Alkaline pre-treatments involve the saponification of the ester bonds between hemicellulose and lignin, loosening linkages between lignin and structural carbohydrates (Sun and Cheng, 2002). In most studies, alkaline pre-treatments are performed under severe conditions of temperature and alkali concentration, as a way to achieve top CH_4 production. Few studies hint at the investment needed to implement this complex technology at a small plant scale, in the frame of the current EU guidelines. More to this, few studies compare pre-treatment efficiency among different types of ligno-cellulosic biomass, paying little attention to the opportunity of applying treatments to substrates, regardless of their biodegradability.

Given the concerns for the sustainable development of the biogas sector, at the light of the current EU policy supporting small plants and the use of agricultural residues in lieu of cereals as AD feedstocks, we have studied an affordable technology with low energy requirement applied to substrates at varying biodegradability. In this frame, a laboratory

experiment was set up to investigate mild alkaline pre-treatments, i.e. pre-treatments carried out at low temperature and modest NaOH concentration, on two dedicated biomass crops and one agricultural residue. Aim of the experiment was to evaluate pre-treatment effects on chemical composition, physical structure and methane production of the three substrates, in view of pre-treatment implementation in AD plants. In a broader perspective, this experiment aimed at overcoming the uncertainties in pre-treatment effects on ligno-cellulosic substrates, relieving the constraints hampering their adoption in small- to medium-scale AD plants that rely on standard technology.

3.2 Material and methods

3.2.1 Substrates

Two biomass crops and one agricultural residue were used as substrates in this experiment. The two crops were Arundo (multi-annual specie) and B 133 hybrid sorghum (a fibre genotype); Barley straw was the agricultural residue. The two crops were grown at the experimental farm, University of Bologna, in Cadriano (BO, 44° 33’ N, 11° 21’ E, 32 m above sea level). Representative biomass samples were oven-dried (60 °C) and ground at 2 mm for chemical analysis, pre-treatments and subsequent AD assay.

3.2.2 NaOH pre-treatment

Alkaline pre-treatments for chemical analysis and AD were conducted in glass flasks (Pyrex 100 ml) at 25 °C in the dark, over 24 h under continuous stirring (120 rpm). Each dry substrate was accurately mixed in a flask with an appropriate amount of NaOH solution at three increasing levels (0.05, 0.10 and 0.15 N) to maintain 10% total solids (TS); the corresponding NaOH to substrate loading was 2, 4 and 6% (w/w). An aliquot of pre-treated substrates was used for the AD assay (section 2.3), while the remaining solid and liquid fraction were separated by vacuum pump with glass micro-fibre filter (Whatman GF/C, Ø 47 mm). Then the solid fraction was washed with deionized water and oven dried for 48 h at 60 °C, before being subjected to compositional analysis.

3.2.3 Anaerobic digestion

Untreated and pre-treated substrates were digested in a batch mode, using the inoculum collected from a full scale biogas plant operating at 55 °C, fed with maize silage and fresh vegetable residues. The inoculum was incubated at 35 °C in the dark with repeated manual stirring to adapt to mesophilic conditions until the end of biogas emission (ten days). The starved inoculum had the following characteristics: TS, 35 mg g^{-1}; volatile solids (VS), 29 mg g^{-1} fresh weight; total alkalinity, 31 g $CaCO_3$ l^{-1}; pH, 8.0. The AD assay was conducted at 35 °C with an organic load of 4 g VS l^{-1} for each substrate in 100 ml serum bottles filled with 48 ml of inoculum and 12 ml of deionised water to reach a final volume of 60 ml. A high inoculum rate (80% v/v), corresponding to a 5.8:1 inoculum to substrate ratio (VS/VS), was adopted to avoid potential inhibition determined by high organic load, insufficient nutrients or alkalinity (Angelidaki and Sanders, 2004; Angelidaki et al., 2009). Additional serum bottles were set up as controls: blank (inoculum alone); blank plus NaOH addition at the three levels of normality; glucose at the same organic load as substrates with and without NaOH additions. After filling, the bottles were flushed with N_2 for 60s to ensure anaerobic conditions, capped with butyl rubber stoppers and sealed with aluminium crimps. The assay was conducted in triplicate, totalling 60 serum bottles.

3.2.4 Analytical methods

3.2.4.1 Chemical analyses

Prior to AD, the three untreated substrates were subjected to the assessment of the following traits: TS (48 h at 105 °C); VS (4 h at 550 °C); total Kjeldahl nitrogen (TKN) through titration with 0.1 M H_2SO_4 after steam distillation of samples following hot digestion with 96% H_2SO_4; proteins by multiplying TKN * 6.25; lipids by Soxhlet method with diethyl ether; starch by the amyloglucosidase-α-amylase method (McCleary et al., 1997).

Structural carbohydrates (cellulose and hemicellulose) and lignin were determined in untreated and pre-treated solid substrates by the National Renewable Energy Laboratory (NREL) method (Sluiter et al., 2011). Thus, samples (150 mg) were first hydrolyzed with 1.5 ml of 72% w/w of H_2SO_4 at 30 °C for 60 min in a water bath, then diluted to reach a final H_2SO_4 concentration of 4% by adding 42 ml of deionized water and kept to autoclave at 121 °C for 60 min. The insoluble residue was separated from the supernatant by vacuum filtration with glass micro-fibre filter (Whatman GF/C, Ø 47 mm). This insoluble residue was washed

with 25 ml of deionized water and placed in a crucible. The crucible and glass micro-fibre filter were dried at 105 °C for 12 h to determine the amount of acid insoluble residue (AIR), thereafter they were placed in a muffle furnace at 550 °C for 24 h to determine acid insoluble lignin (AIL).

Monomeric sugars (glucose, xylose and arabinose) in the supernatant after acid hydrolysis were determined by means of HPLC (Shimadzu with LC-10 AT pump) equipped with a Biorad Aminex HPX-87H column (300 x 7.8 mm) and a refractive index detector (Shimadzu RID-10A). H_2SO_4 4 mM at a flow rate of 0.6 ml min^{-1} was used as the mobile phase; the temperature of the column and detector were maintained at 63 and 50 °C, respectively. Thereafter, cellulose and hemicellulose content of untreated and pre-treated solid sample were calculated by the following equations, subtracting the amount of starch from the sulphuric acid glucan (starch + cellulose) value to obtain the actual amount of cellulose (Sluiter et al., 2010):

$$\text{Cellulose (g g}^{-1}\text{ VS)} = \frac{\text{gluc (g l}^{-1}) * \text{Volume}_{\text{final}}\text{ (ml)} * 0.90 * \text{SRS}_{\text{gluc}}}{\text{Weight}_{\text{sample}}\text{ (g)} * \text{VS}} - \text{starch (g g}^{-1}\text{ VS)} \quad \text{[eq. 1]}$$

$$\text{Hemicellulose (g g}^{-1}\text{ VS)} = \frac{\text{xyl (g l}^{-1}) * \text{Volume}_{\text{final}}\text{ (ml)} * 0.88 * \text{SRS}_{\text{xyl}}}{\text{Weight}_{\text{sample}}\text{ (g)} * \text{VS}} + \frac{\text{ara (g l}^{-1}) * \text{Volume}_{\text{final}}\text{ (ml)} * 0.88 * \text{SRS}_{\text{ara}}}{\text{Weight}_{\text{sample}}\text{ (g)} * \text{VS}} \quad \text{[eq. 2]}$$

where: gluc, xyl, ara and starch are the concentrations of each respective sugar; $Volume_{final}$ is the volume of supernatant after acid hydrolysis; 0.90 and 0.88 are the coefficients used to anhydrous correction for C-6 (glucose) and C-5 (xylose and arabinose), respectively; SRS is the sugar recovery standard of each sugar; $Weight_{sample}$ is the amount of substrate.

All analytical determinations were performed in duplicate.

3.2.4.2 Biogas measurement and analyses

The incubation lasted until the plateau in CH_4 production (increase < 5%), totalling 58 days. Twelve times (day 2, 4, 6, 9, 12, 16, 20, 26, 34, 41, 48 and 58), biogas production was quantified with a water-displacement system constituted of a 1 l Schott bottle and a graduated cylinder (Mariotte bottle). This was connected to the batch with a syringe needle only for the time needed to measure water displacement (ca. 10 s). After equilibrium and reading, the

water displacement apparatus was disconnected. Then the gas in the bottle headspace was analyzed for the biogas components (H_2, O_2, CH_4 and CO_2) through an Agilent microGC 3000A (Agilent Technologies, Milan, Italy) coupled with thermal conductivity detector (TCD) under the following conditions: injector temperature, 90 °C; column temperature, 60 °C; sampling time, 20 s; injection time, 50 ms; column pressure, 25 psi; run time, 44 s; carrier gas, N_2. CH_4 production was calculated based on volume displacement and percent methane content at each current reading and its previous reading (Lou et al., 2012):

$$CH_4 = \left[\frac{(A+B)*\%CH_{4t}}{100}\right] - \left[\frac{B*\%CH_{4(t-1)}}{100}\right] \qquad \text{[eq. 3]}$$

where: A = displaced gas volume; B = headspace gas volume; t = current sampling time; t-1 = previous sampling time.

CH_4 production from the sole inoculum was subtracted from CH_4 produced in each sample. CH_4 data are expressed at STP (Standard Temperature and Pressure; 273 K, 100 kPa) and are reported on a VS basis (ml g^{-1} VS).

3.2.4.3 FTIR analysis

The structure of untreated and pre-treated solid fraction was analyzed with Fourier transform infrared spectrometry (FTIR; Tensor 27, Bruker Co., Billerica, MA, USA).

Each spectrum was obtained with an average of 32 scans at room temperature and a resolution of 4 cm^{-1}, in the wavenumber range from 600 to 4000 cm^{-1}.

Based on absorbance readings at specific bands of the spectrum, the following traits were calculated, which refer to the relationships amid fibre components: Total Crystallinity Index (TCI), calculated as the 1375 to 2900 cm^{-1} peak ratio (Nelson and O'Connor, 1964), which reflects the overall degree of order of cellulose; Lateral Order Index (LOI), calculated as the 1430 to 898 cm^{-1} peak ratio (Hurtubise and Krässig, 1960), indicating the amount of crystalline vs. amorphous cellulose (i.e., their ratio). Depending on LOI and cellulose, the amount of crystalline cellulose may be calculated as: cellulose * LOI/(1+LOI) (Monlau et al., 2012). Lastly, the relationship between lignin and the two structural carbohydrates was interpreted through the H lignin/H carbohydrates ratio (Monlau et al., 2012), calculated as the ratio of the 1510 cm^{-1} peak to the sum of 1630, 1430, 1375, 1158 and 898 cm^{-1} peaks.

3.2.4.4 Scanning electron microscopy

Differences in ligno-cellulosic structure between untreated and pre-treated (NaOH at 0.15 N) substrates were observed under a scanning electron microscopy (SEM; Philips 515, Eindhoven, NL) at 10.4 kV with a magnification ×1000. Dry samples were mounted on aluminium stubs with double stick tape, coated with a gold-palladium using an ion sputtering unit EMITECH K500 (Emitech, Ashfors, UK) film to improve their conductivity. Pictures were taken with a digital camera Nikon 5400 Coolpix (Nikon, Chiyoda-ku, Tokyo, Japan).

3.2.5 Data analysis

To better assess pre-treatment effects on AD, data of cumulative CH_4 yield were fitted by means of a first order kinetics, i.e. an exponential rise to max equation with the Sigma Plot 10 statistical software package (Systat Software Inc., Chicago, IL, USA):

$$CH_{4\,m} = CH_{4;0}\,(1-e^{-kt}) \qquad \text{[eq. 4]}$$

where $CH_{4\,m}$ is the cumulative methane yield (ml g^{-1} VS) at a given time (d), $CH_{4;\,0}$ is the potential methane yield (ml g^{-1} VS), i.e. the function asymptote; *k* is the methanation rate constant (d^{-1}) and t is time (d).

Technical digestion time (T_{80}), indicating the time needed to produce 80% of potential methane yield (Palmowski and Müller, 2000), was calculated with an inverse function of equation 4.

Chemical traits were submitted to a one-way ANOVA for the three substrates; cumulative CH_4 yield was submitted to a two-way completely randomized ANOVA for substrates, pre-treatments and their interaction, through the CoStat 6.3 software (CoHort Software, Monterey, CA, USA). The Student - Newman-Keuls (SNK) test at $P \leq 0.05$ was adopted to separate means of statistically significant traits.

A principal component analysis (PCA) was run on cellulose, hemicellulose, AIL and cumulative CH_4 yield, in order to evaluate the relationship between structural components and methane output. In this analysis, the twelve combinations of substrates (Arundo, B 133 and Barley straw) per NaOH levels (0, 0.05, 0.10 and 0.15 N) were considered as single cases. Before analysis, the factors were subjected to a rotation, in order to maximize the amount of

explained variance (varimax normalized). The analysis was run with the Statistica 5.0 software (StatSoft, Tulsa, OK, USA)

3.3 Results and discussion

3.3.1 Chemical composition of raw substrates

The characteristics of the three substrates are shown in Table 3.1. Volatile solids (VS) varied within a tight range (between 923 and 937 mg g^{-1} TS for Barley straw and B 133, respectively. The C/N ratio was always higher than the optimum range for AD (from 15 to 30; Li et al., 2013), reflecting a low nitrogen content (data not shown). Proteins were low in Barley straw (26 mg g^{-1} VS); twice as high in Arundo and B 133 (51.7 mg g^{-1} VS as average). A varying amount of lipids was found: 9.5, 12.2 and 16.3 mg g^{-1} VS in Arundo, Barley straw and B 133, respectively. Starch, the polysaccharide accumulated in storage organs as grains, was very low in Barley straw (16.2 mg g^{-1} VS), intermediate in Arundo (38.5 mg g^{-1} VS), relatively high in B 133 (53.3 mg g^{-1} VS). However, even in the richest substrate (B 133) lipids and starch were low compared to whole-plant maize (average, 34 and 320 mg g^{-1} VS in the two respective components; NRC, 2001), which is the reference crop used as AD feedstock. Cellulose and hemicellulose, the main carbon sources for AD in ligno-cellulosic substrates, accounted for ca. 50% of total VS. B 133 showed a lower cellulose and hemicellulose content (278 and 205 mg g^{-1} VS, respectively) than Arundo and Barley straw (average, 323 and 221 mg g^{-1} VS for the two respective carbohydrates). AIL outlined a similar ranking as the two structural carbohydrates.

Table 3.1 Chemical composition of the three substrates.

Substrate	VS	C/N	Proteins	Lipids	Starch	Cellulose	Hemicell.	AIL
	mg g^{-1} TS		mg g^{-1} VS					
Arundo	925 b	56.9 b	51.2 a	9.5c	38.5 b	329 a	222 a	229 a
B 133	937 a	54.2 b	52.2 a	16.3 a	53.3 a	278 b	205 b	198 b
Barley straw	923 c	64.1 a	26.0 b	12.2 b	16.2 c	318 a	220 a	215 ab

TS, total solids; VS, volatile solids; Hemicell., hemicellulose; AIL, acid insoluble lignin. ANOVA always significant at $P \leq 0.01$. In significant traits, letters indicate statistically different data according to the SNK test ($P \leq 0.05$).

3.3.2 Methane yield during the incubation

In this experiment, untreated and pre-treated substrates were mixed with the inoculum just prior to the beginning of the incubation, in order to better simulate the procedure of a full scale AD plant with standard technology.

Similar trends of cumulative CH_4 yield were observed in untreated and pre-treated substrates during the 58 days of AD (Fig. 3.1). In particular, a steep CH_4 production was observed at the beginning of the incubation, while a temporary decline was shown after 10 days in Arundo (Fig. 3.1a), B 133 (Fig. 3.1b) and, to a lesser extent, Barley straw (Fig. 3.1c). This slowdown might be caused by the depletion of the easily degradable fraction of VS (Ahn et al., 2009), requiring a period of adaptation for micro-organisms to degrade the remaining, more recalcitrant fraction.

Thereafter, CH_4 yield diverged to a varying extent during the incubation (Fig. 3.1), indicating a different effect of NaOH addition in each specific substrate. The significant substrate × treatment interaction at the end of the incubation supports this point (Fig. 3.2). In untreated substrates, Arundo showed the lowest cumulative CH_4 yield (190 ml g^{-1} VS), followed by Barley straw (232 ml g^{-1} VS) and B 133 (248 ml g^{-1} VS). The ca. 20% lower yield of Arundo vs. B133 and Barley straw is consistent with the ca. 10% higher lignin content (Table 3.1), suggesting a higher recalcitrance to AD.

In treated substrates, cumulative CH_4 yield of Arundo augmented in parallel with the increase in NaOH concentration: 216, 229 and 246 ml g^{-1} VS at the three levels of NaOH, corresponding to a 14, 21 and 30% respective gain over the untreated substrate (Fig. 3.2). Cumulative CH_4 yield of Barley straw increased by 15% at NaOH 0.05 N and by ca. 23% at NaOH 0.10 and 0.15 N, leading to the highest CH_4 yield in the experiment (286 ml g^{-1} VS) (Fig. 3.2). In B 133 a moderate increase was obtained only at the two highest NaOH levels, achieving an average CH_4 yield of 273 ml g^{-1} VS (+10% over the untreated substrate) (Fig. 3.2). It is worth noticing that in statistical terms Arundo, despite a steep increase in cumulative CH_4 yield, necessitated 0.10 and 0.15 N NaOH to attain the yield of untreated Barley straw and B 133, respectively (Fig. 3.2).

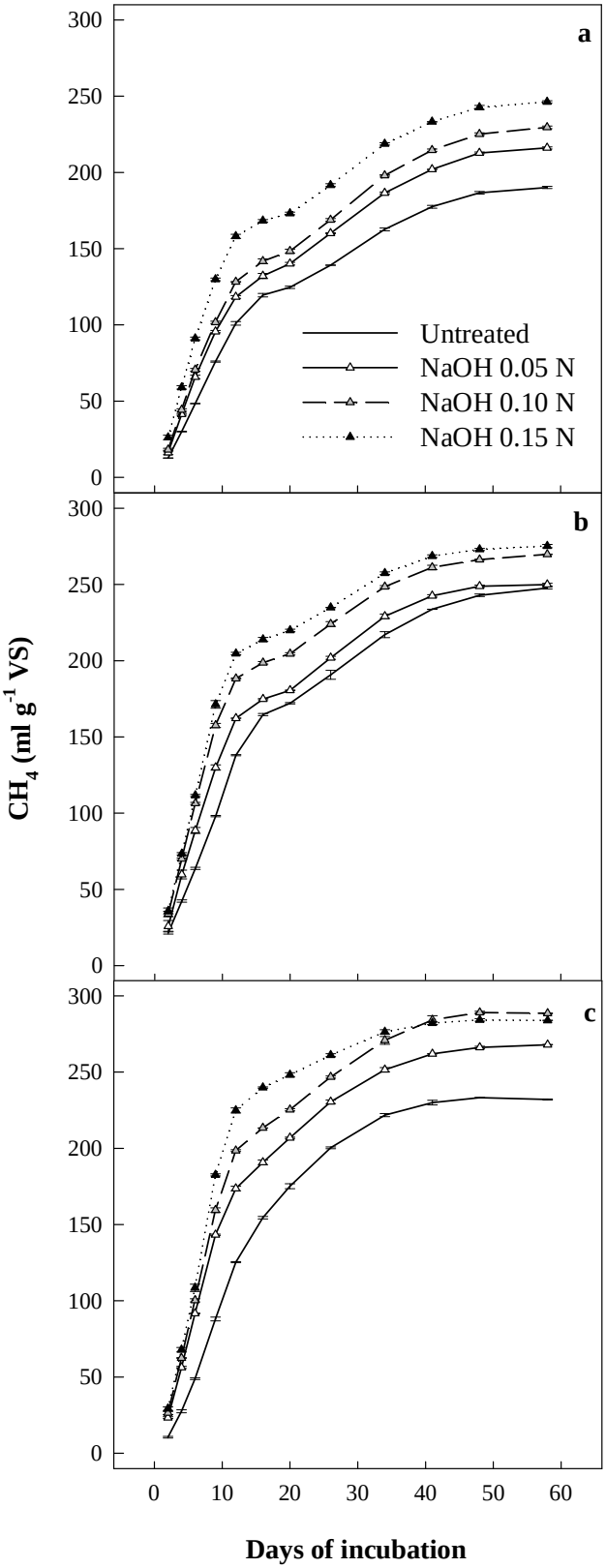

Figure 3.1 Cumulative methane yield of untreated and NaOH pre-treated Arundo (a), B 133 sorghum (b) and Barley straw (c) during the anaerobic digestion assay. Vertical bars, ± standard errors (n = 3).

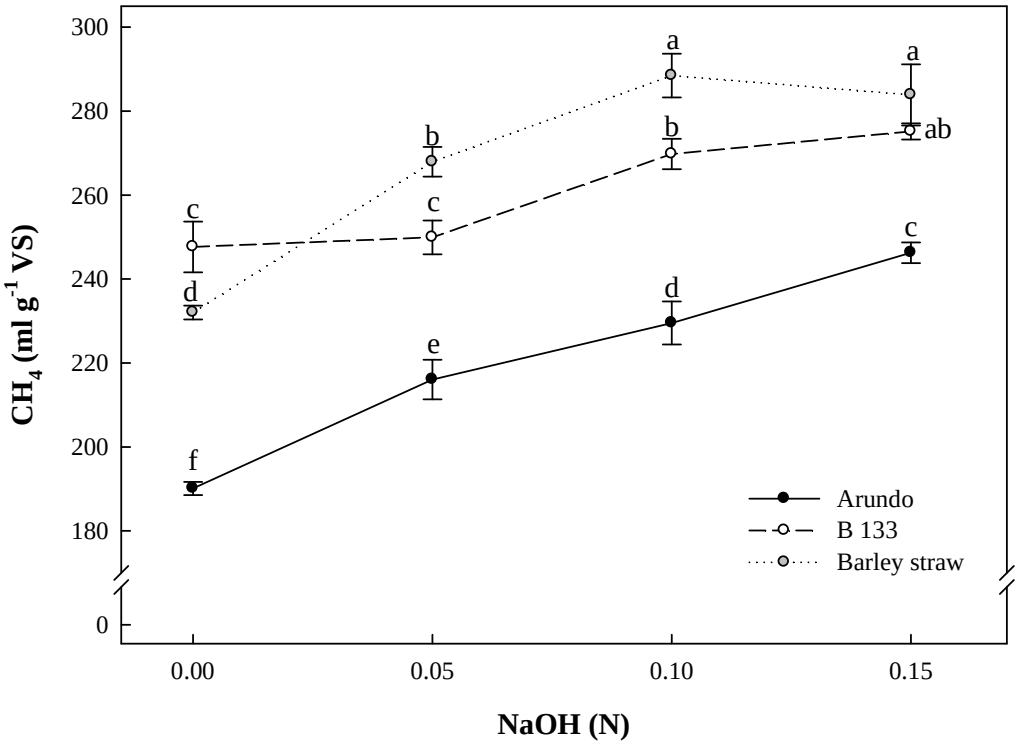

Figure 3.2 Significant substrate × treatment interaction on cumulative CH_4 yield at the end of the incubation. Different letters indicate statistically different data according to the SNK test ($P \leq 0.05$). Vertical bars, ± standard errors (n = 3).

In all treatments, the first order kinetics explained a high share of the total variation of cumulative CH_4 yield (Fig. 3.1) (R^2 always $\geq$ 0.97**; Table 3.2). The benefits of NaOH treatments were also proved in terms of process kinetics: untreated Arundo, B 133 and Barley straw showed similar values of the methanation rate constant (average *k*, 0.053 d^{-1}; Table 3.2). Pre-treatments determined a faster kinetics, although Arundo staged a lower *k* increase (+45% with NAOH 0.15 N vs. untreated substrate) than B 133 and Barley straw (average, +83%) (Table 3.2). A significant correlation was shown between NaOH concentration and methanation rate constant (r = 0.80**; data not shown), indicating a good responsiveness of this trait to NaOH addition.

Technical digestion time (T_{80}) was also reduced after pre-treatments (Table 3.2). After NaOH treatments, B 133 and Barley straw showed shorter T_{80} than Arundo, which is consistent with the higher *k* values of two former substrates. Especially in Barley straw, NaOH at 0.15 N reduced T_{80} by an approximate 50% vs. the untreated substrate. In this experiment, the

association of shorter T_{80} and higher CH_4 yield at T_{80}, calculated through (eq. 4), determined a much higher amount of CH_4 produced per day of incubation: up to + 129% in Barley straw with NaOH at 0.15 N vs. the untreated substrate (data not shown). This represents the best premise for a reduction of the hydraulic retention time, concurrently increasing the digestion capacity of commercial AD plants (Zheng et al., 2009).

A vast literature deals with alkaline pre-treatments aimed at enhancing CH_4 output from AD of ligno-cellulosic materials. However, results are influenced by many factors (especially type of substrate, time, temperature and amount of alkaline agent), and their interpretation is not easy. Further divergence is shown by the variable amount of liquid in which the substrate is soaked at a given ratio with the alkali agent, involving a variable availability of the agent for hydrolysis. The inconsistency in treatment conditions and their results reflects in the performances of agricultural residues, which are the substrates most frequently associated with alkaline pre-treatments in the literature. A 73% increase in methane yield was obtained in maize stover pre-treated with 2% NaOH at 20 °C for 3 days (Zheng et al., 2009), while a 37% increase was obtained in the same substrate pre-treated with 5% NaOH at 20 °C for 1 day (Zhu et al., 2010). Compared to these, in this experiment a similar substrate as Barley straw, pre-treated with 2% NaOH (0.10 N) at 25 °C for 1 day, staged only a 24% increase of methane yield. It is perceived that this large variation in pre-treatment efficiency is associated with lignin content. In fact, the higher increase obtained by Zheng et al. (2009) is associated with a lower lignin than in Zhu et al. (2010) and in Barley straw of this experiment (94 vs. 229 and 215 mg g^{-1} VS, in the three respective works). This point is further supported by Monlau et al. (2012), who evidenced only a 6% increase of methane yield in sunflower stalks with a high lignin content (337 mg g^{-1} VS), after pre-treatment with 4% NaOH at 55 °C for 24 h. Beside crop residues, several biomass crops have been tested in experiments with NaOH pre-treatments, resulting in contrasting results: this is the case of switchgrass showing a good yield increase (24% over a base yield of 112 ml CH_4 g^{-1} VS) after 15 min at 121 °C with 7% NaOH (Frigon et al., 2012), compared to wheat (whole plant) staging a much higher increase (54.5% over 261 ml CH_4 g^{-1} VS) after 1 h at 75 °C with 8% NaOH (Taherdanak and Zilouei, 2014). Sambusiti et al. (2013) observed a modest CH_4 increase (7% over 270 ml CH_4 g^{-1} VS) with sorghum B 133 pre-treated with 10% NaOH at 55 °C for 12 h, compared to the same hybrid in this experiment, showing an increase of 11% over 248 ml CH_4 g^{-1} VS with 6% NaOH at 25 °C for 24 h (Fig. 3.2).

Table 3.2 Potential methane yield ($CH_{4;\,0}$), methanation rate constant (*k*) and R^2 of first-order kinetics fitted to data of cumulative methane yield (Fig. 3.1), and technical digestion time (T_{80}). In brackets, standard errors (n = 3).

Substrate	**Pre-treatments**	**$CH_{4;\,0}$**	***k***	**R^2**	**T_{80}**
		ml g^{-1} VS	d^{-1}		days
Arundo	untreated	201 (6.0)	0.051 (0.003)	0.99**	32 (0.6)
	NaOH 0.05 N	224 (6.2)	0.055 (0.003)	0.99**	29 (0.1)
	NaOH 0.10 N	236 (6.9)	0.059 (0.004)	0.99**	20 (0.2)
	NaOH 0.15 N	243 (6.7)	0.074 (0.006)	0.98**	22 (0.5)
B 133	untreated	259 (6.9)	0.055 (0.004)	0.99**	29 (0.4)
	NaOH 0.05 N	252 (5.8)	0.073 (0.005)	0.99**	22 (0.8)
	NaOH 0.10 N	266 (6.0)	0.087 (0.006)	0.98**	19 (0.4)
	NaOH 0.15 N	272 (6.3)	0.095 (0.007)	0.98**	17 (0.4)
Barley Straw	untreated	257 (12.5)	0.052 (0.006)	0.98**	31 (0.5)
	NaOH 0.05 N	274 (5.7)	0.074 (0.004)	0.99**	22 (0.6)
	NaOH 0.10 N	293 (7.2)	0.078 (0.005)	0.98**	21 (0.7)
	NaOH 0.15 N	289 (8.6)	0.098 (0.009)	0.97**	16 (0.3)

3.3.3 Changes in fibre composition of pre-treated substrates

In ligno-cellulosic substrates as dedicated crops and agricultural residues, layers of lignin shield cellulose and hemicellulose from enzymatic attack during anaerobic digestion. Alkaline pre-treatment can release these layers, increasing the solubility of structural components (Gierer, 1985). In this experiment, pre-treated substrates underwent compositional changes improving biodegradability and, ultimately, methane production. However, the reduction in the three structural components depicted different patterns at varying NaOH concentration in the three substrates (Fig. 3.3). B 133 showed the highest solubilisation of cellulose (from 16 to 34% at the three NaOH levels), compared to Arundo (from 11 to 19%) and Barley straw (from 1 to 10%).

B 133 also showed a major hemicellulose reduction (from 18 to 42%), compared to Arundo and Barley straw (from 10 to 23% and from 11 to 16% in the two respective substrates). The cleavage of cellulose ester-linked structure and the subsequent increase in its solubility has already been proved in the literature (Xiao et al., 2001). However, it is sensed that hemicellulose is already more accessible for AD than cellulose, as the modest increase in methane yield achieved with B 133 (+10%) shows.

Lastly, in contrast to the two structural carbohydrates, the strongest lignin (AIL) reduction after NaOH pre-treatments was observed in Barley straw (from 6 to 25%), compared to B 133 (from 6 to 13%) and Arundo (from 0 to 10%).

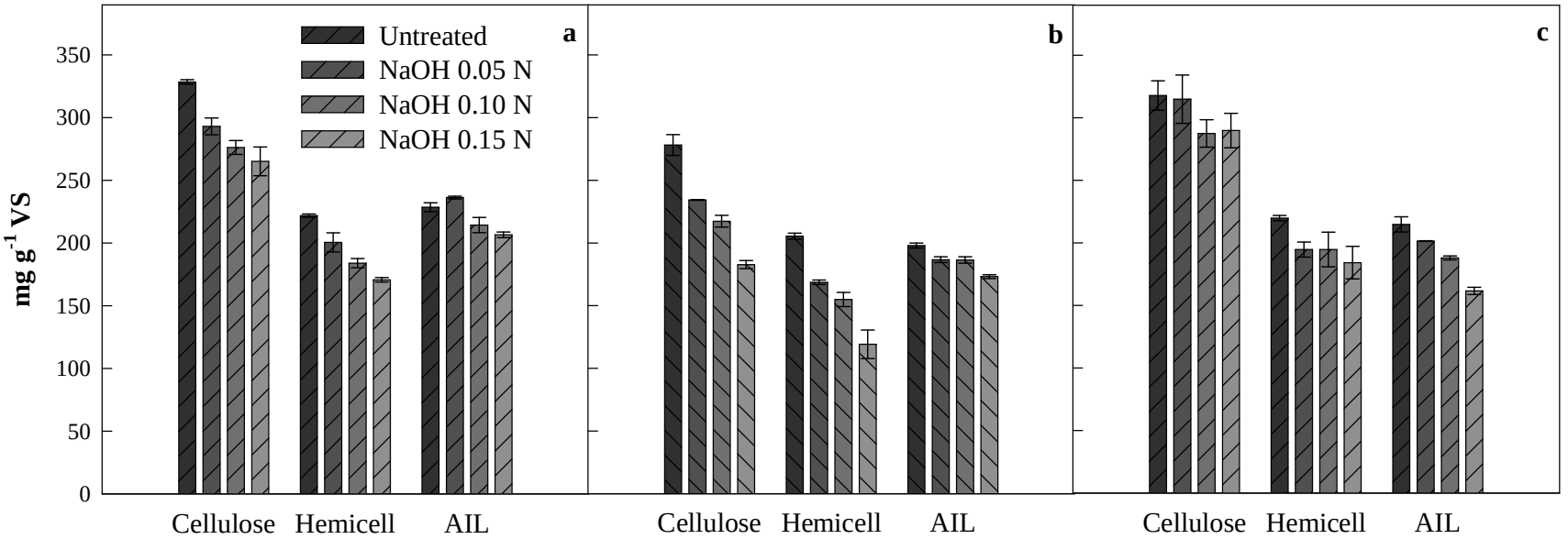

Figure 3.3 Fibre composition of untreated and NaOH pre-treated Arundo (a), B 133 sorghum (b) and Barley straw (c). Vertical bars, ± standard errors (n = 2).

3.3.4 Changes of fibre structure of untreated and pre-treated substrates

FTIR analysis has already been used to study the structural characteristics of ligno-cellulosic material (Alemdar and Sain, 2008). Twelve bands of particular relevance in the wavenumber range from 600 to 4000 cm^{-1}, were analyzed. Their spectra referring to untreated and NaOH 0.15 N pre-treated substrates are displayed in Fig. 3.4, and assigned to the functional groups and linkages reported in Table 3.3, according to the cited sources. In this experiment, the solubilisation of structural carbohydrates and lignin suggested by fibre composition (Fig. 3.3) is supported by FTIR analysis, showing a decreasing intensity of their absorption bands at increasing NaOH concentration (Table 3.4).

TCI and LOI, the two traits representing cellulose crystallinity, exhibited a substantial steadiness or a modest increase after NaOH addition at the highest level (Table 3.5). This is reflected in the amount of crystalline cellulose, calculated on the basis of LOI: crystalline cellulose consistently decreased after pre-treatments, staging the strongest decrement in B 133, followed by Arundo (Table 3.5). However, this decline was concurrent with that of total cellulose (Fig. 3.3), indicating that the crystalline form consistently represented ca. 60% of cellulose in untreated and pre-treated substrates.

It appears, therefore, that the treatments applied in this experiment promoted cellulose hydrolysis but did not influence the proportion between crystalline and amorphous form, in contrast to the decreases of LOI observed in ligno-cellulosic substrates by Isroi et al. (2012), Teghammar et al. (2012) and, to a lesser extent, Monlau et al. (2012) and Taherdanak and Zilouei (2014). Conversely, the H lignin/H carbohydrates ratio decreased after pre-treatment, proving that NaOH addition had exerted a stronger effect on lignin than structural carbohydrates (Table 3.5). Barley straw was the substrate showing the largest relative loss in this trait (-18%), followed by B 133 (-15%) and lastly Arundo (-5%). Hence there is no simple relationship between initial H lignin/H carbohydrates level and its abatement after pre-treatment, on one side, and CH_4 yield increase (Fig. 3.2). Nevertheless, it may be evinced that, although FTIR data refer to the surface of investigated substrates, this is sufficient to support increased enzymatic attack starting from the surface of digesting materials. Compared to my data, Monlau et al. (2012) evidenced either steadiness (alkaline pre-treatment) or increase (thermal pre-treatment under acidic conditions) in H lignin/H carbohydrates ratio of sunflower stover.

The structural change observed with FTIR analysis was also supported by SEM (Fig. 3.5): the surface of untreated substrates appears very compact with lignin sheltering hemicellulose and cellulose. NaOH treatment determined a weakening of fibre structure accompanied with a partial loosening of the linkages between lignin and the two structural carbohydrates and a general swelling, as also observed by Krishania et al., 2013; Salehian et al., 2013; Taherdanak and Zilouei, 2014.

Hence, in agreement with the literature the reductions of absorbance and the SEM images prove that alkaline pre-treatments could actually disrupt lignin structure, loosening its binding with cellulose and hemicellulose. This in turn could augment the accessible surface of ligno-cellulosic material for enzymatic attack (Pavlostathis and Gossett, 1985), positively reflecting on substrate biodegradability, speed of the hydrolytic phase and kinetics of methane production.

Table 3.3 Characteristic bands of absorbance in FTIR spectra, respective functional groups and assignment to fibre fractions.

Wavenumber (cm^{-1})	**Functional group**	**Assignment**	**Reference**
3175	–OH stretching intramolecular H bonds	Cellulose	Taherdanak and Zilouei, 2014
2900	C–H stretching	Cellulose	Gastaldi et al., 1998
1720	C=O stretching acetyl or carboxylic acid	Hemicellulose and lignin	Sun et al., 2005 He et al., 2008;
1610	C=C stretching of aromatic ring	Lignin	Sene et al., 1994
1598	C=C stretching of aromatic ring	Lignin	Sun et al., 2005
1510	C=C stretching of aromatic ring	Lignin	Corredor et al., 2009
1430	$–CH_2$ bending	Cellulose	Liang and Marchessault, 1959
1375	C–H deformation	Cellulose	Yang et al., 2009
1315	$–CH_2$ wagging vibrations	Cellulose and hemicellulose	He et al., 2008
1230	C–O–H deformation, C–O of phenolics and C–C–O stretching of esters	Hemicellulose and lignin	Sene, et al., 1994
1158	C–O–C stretching	Cellulose and hemicellulose	He et al., 2008
898	Glucose ring stretch, C–H deformation	Cellulose	Steward et al., 1995

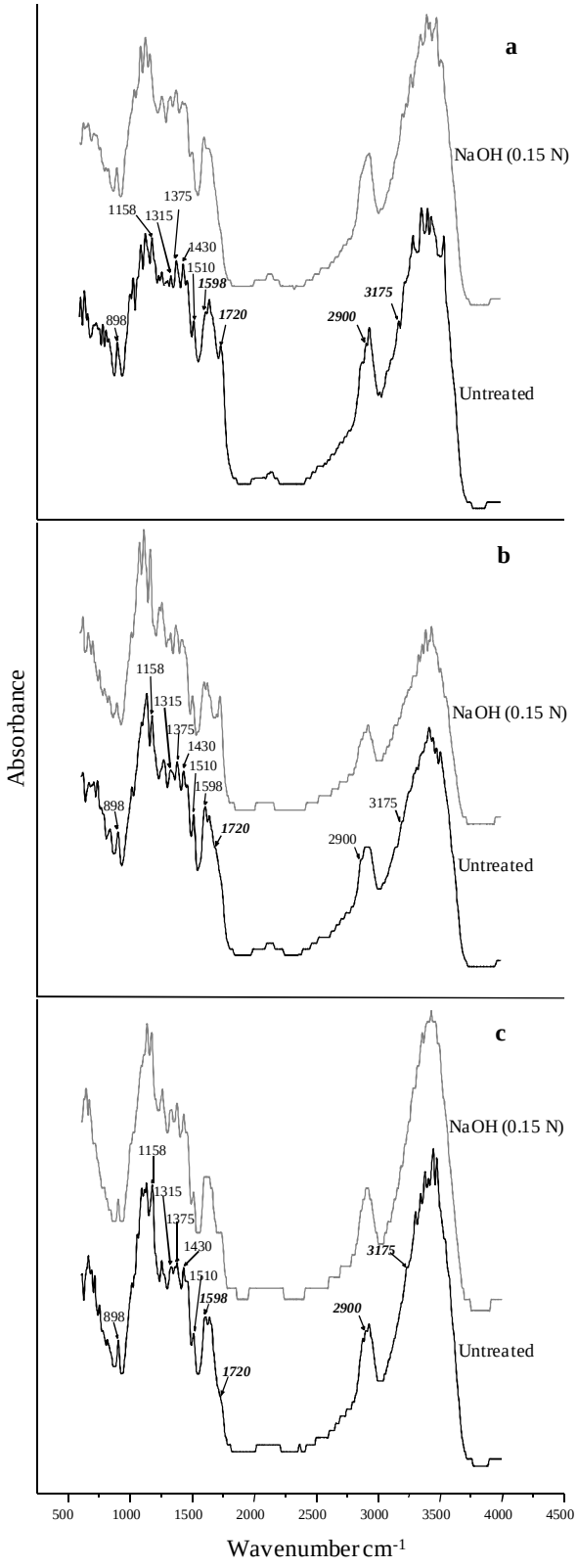

Figure 3.4 Fingerprint range from 600 to 4000 cm^{-1} of the FITR spectra of untreated (black line) and NaOH 0.15 N pre-treated substrates (gray line) of Arundo (a), B 133 sorghum (b) and Barley straw (c). In bold italic, bands faded after alkaline pre-treatment.

Table 3.4 Absorbance related to bands of the fingerprint range from 600 to 4000 cm^{-1} in untreated and pre-treated substrates.

Substrate	Pre-treatments	Wavenumbers (cm^{-1})											
		3175	**2900**	**1720**	**1610**	**1598**	**1510**	**1430**	**1375**	**1315**	**1230**	**1158**	**898**
Arundo	untreated	0.37	0.33	0.30	0.37	0.37	0.38	0.45	0.47	0.42	0.46	0.45	0.34
	NaOH 0.05 N	0.34	0.30	0.28	0.32	0.33	0.34	0.42	0.43	0.40	0.41	0.46	0.28
	NaOH 0.10 N	0.32	0.29	0.17	0.32	0.33	0.31	0.39	0.41	0.39	0.37	0.45	0.28
	NaOH 0.15 N	0.24	0.22	0.13	0.24	0.26	0.24	0.30	0.32	0.31	0.29	0.35	0.21
B 133 sorghum	untreated	0.27	0.25	0.20	0.31	0.31	0.31	0.38	0.40	0.37	0.36	0.42	0.28
	NaOH 0.05 N	0.18	0.17	0.19	0.23	0.23	0.22	0.29	0.32	0.31	0.31	0.37	0.20
	NaOH 0.10 N	0.18	0.16	0.19	0.23	0.22	0.21	0.29	0.31	0.29	0.31	0.35	0.20
	NaOH 0.15 N	0.16	0.15	0.11	0.21	0.21	0.20	0.27	0.30	0.28	0.28	0.37	0.19
Barley straw	untreated	0.26	0.23	0.12	0.25	0.25	0.23	0.32	0.33	0.31	0.30	0.40	0.21
	NaOH 0.05 N	0.16	0.15	0.07	0.15	0.15	0.14	0.20	0.21	0.20	0.19	0.27	0.13
	NaOH 0.10 N	0.14	0.13	0.09	0.14	0.14	0.13	0.20	0.21	0.20	0.20	0.25	0.12
	NaOH 0.15 N	0.07	0.07	0.06	0.09	0.09	0.07	0.11	0.12	0.11	0.12	0.18	0.08

Table 3.5 Total Crystallinity Index (TCI), Lateral Order Index (LOI), crystalline cellulose (mg g^{-1} VS) and H lignin/H carbohydrates, based on absorbance data at specific bands of FTIR spectra in untreated and NaOH 0.15 N pre-treated substrates. In brackets, standard errors (n = 2).

Substrate	Pre-treatment	TCI	LOI	Crystalline cellulose	H lignin/H carbohydrates
Arundo	untreated	1.41 (0.01)	1.32 (0.01)	187 (1)	0.178 (0.001)
	NaOH 0.15 N	1.45 (0.08)	1.42 (0.05)	156 (4)	0.170 (0.005)
B 133 sorghum	untreated	1.57 (0.04)	1.38 (0.01)	161 (4)	0.175 (0.004)
	NaOH 0.15 N	1.96 (0.16)	1.43 (0.13)	107 (2)	0.148 (0.002)
Barley straw	untreated	1.40 (0.05)	1.49 (0.03)	190 (9)	0.150 (0.007)
	NaOH 0.15 N	1.85 (0.39)	1.52 (0.04)	175 (10)	0.124 (0.009)

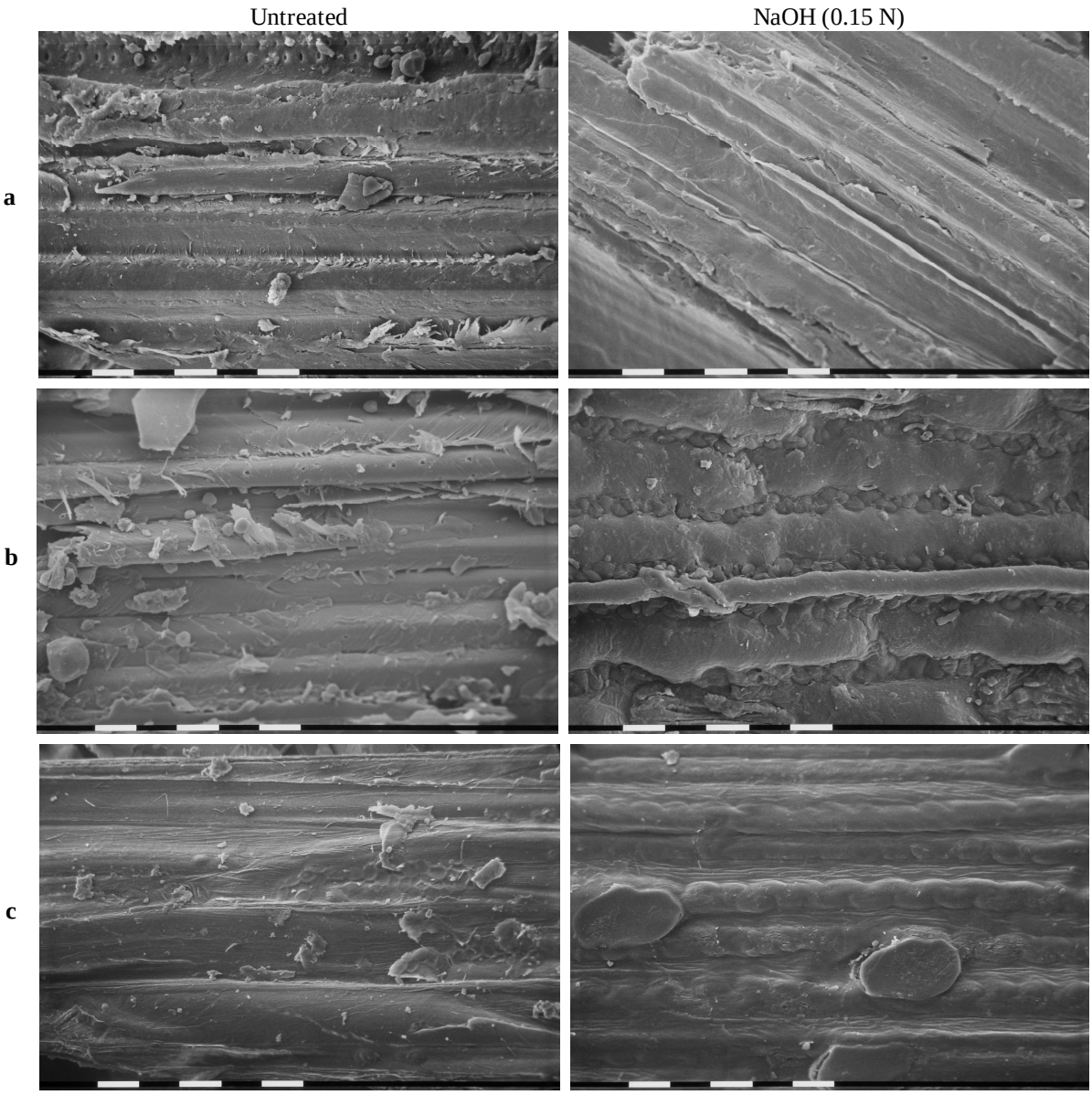

Figure 3.5 SEM images of untreated and NaOH 0.15 N pre-treated Arundo (a), B 133 sorghum (b) and Barley straw (c). Bars = 10 µm.

3.3.5 Relationship between structural components and CH_4 yield

The PCA between structural components and cumulative CH_4 yield of untreated and pre-treated substrates recovered 95.7 % of the total variance, almost equally distributed between the two principal components (Fig. 3.6). In the plot of factor loadings (Fig. 3.6, above), PC 1 was mainly described by cellulose and hemicellulose (loading, 0.96 and 0.92, respectively), whereas AIL and cumulative CH_4 yield were not significantly represented in this component (respective loading, 0.35 and -0.23). Conversely, these two traits were significantly ascribed to PC 2 (loading, 0.90 and 0.95, respectively). It appears therefore that methane output was unrelated to cellulose and hemicellulose, whereas it was adversely related to lignin content. In other words, cellulose and hemicellulose reductions in pre-treated samples appear insufficient to support the increase of methane yield, while the decrease of AIL offers a better clue to explain it. It may be evinced that cellulose and hemicellulose could be degraded even without AIL removal from the substrate, although AIL degradation paved the access to structural carbohydrates (Fig. 3.5), speeding incubation kinetics and enhancing CH_4 yield.

The plot of factor scores consistently depicts substrate behaviour (Fig. 3.6, below): untreated and mildly pre-treated (i.e., NaOH 0.05 N) Arundo were clustered high in the positive side of PC 2, approximately in the same position as AIL in the factor loading plot (Fig. 3.6, above). In the positive side of PC 2 but closer to neutrality, lay strongly pre-treated (i.e., NaOH 0.10 and 0.15 N) Arundo and untreated Barley straw, indicating a persisting, although weaker constraint to CH_4 output. Barley straw outlined a stronger advantage from NaOH addition than Arundo, performing a steady shift towards the most negative region of PC 2 (Fig. 3.6 bottom), in the same area as CH_4 in the factor loading plot (Fig. 3.6, above). Lastly, untreated and pre-treated B 133 clustered around the origin of the axes (Fig. 3.6, below), showing little benefit from NaOH addition.

It may be evinced that, although structural components showed intensive cellulose and hemicellulose hydrolysis and AIL removal after pre-treatments (Fig. 3.3), PCA proved that only AIL was inversely related to CH_4 output, at least in the two more recalcitrant substrates (Arundo and B133).

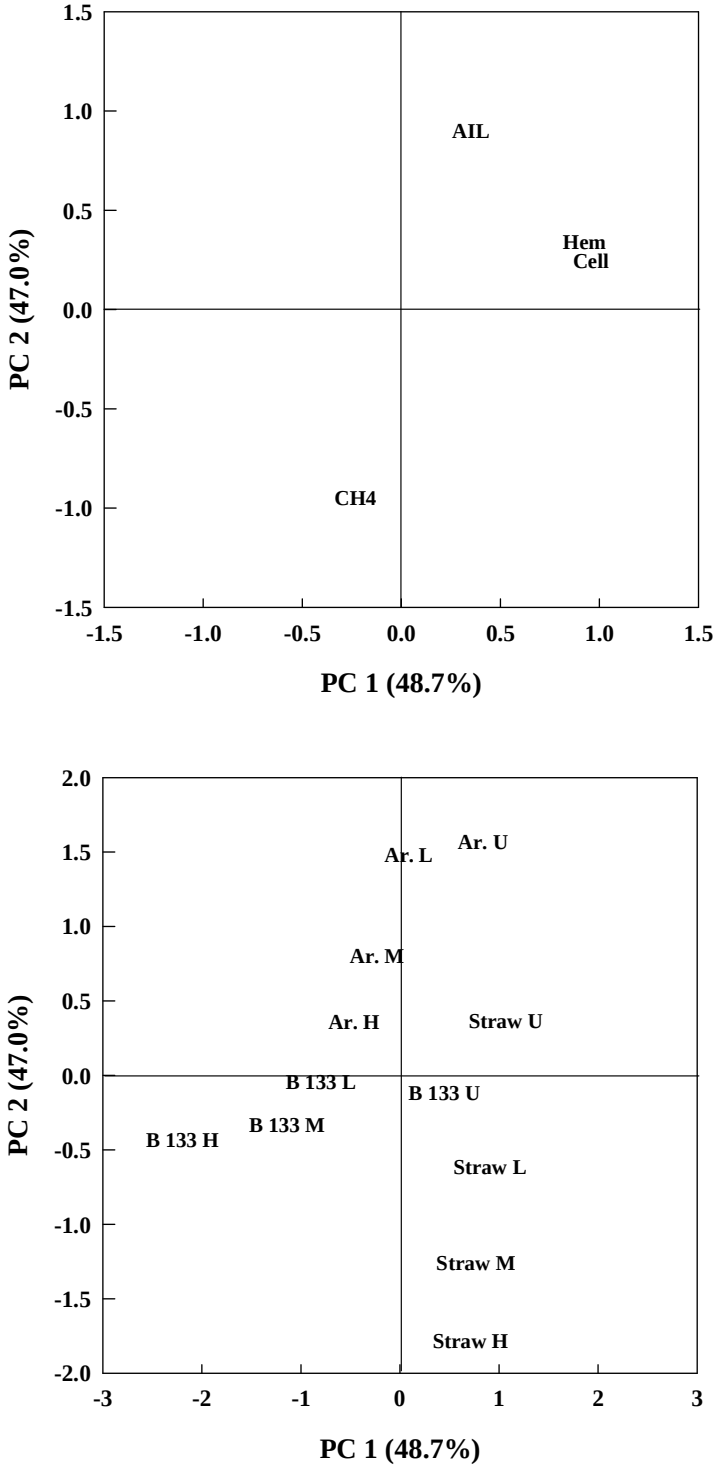

Figure 3.6 Factor loadings (above) and scores (below) of the principal component analysis performed on untreated and NaOH pre-treated substrates. Cell, Hem, AIL and CH_4 mean cellulose, hemicellulose, acid insoluble lignin and cumulative CH_4 yield at the end of the incubation, respectively. Ar., B 133 and Straw indicate Arundo, B 133 sorghum and Barley straw, respectively. U, L, M and H mean untreated and pre-treated with low (0.05 N), medium (0.10 N) and high (0.15 N) levels of NaOH, respectively. PC 1 and 2, 1st and 2nd principal component; in brackets, percent of the total variation explained by each PC.

3.4 Conclusions

In this experiment, mild alkaline pre-treatments at low temperature proved efficient in enhancing methane yield of three ligno-cellulosic substrates to an extent that was directly related to their recalcitrance to bio-degradation. Pre-treatment benefits were also shown in terms of process kinetics and technical digestion time, achieving comparable results with stronger physical-chemical treatments reported in the literature. Compositional analysis associated with FTIR and SEM procedures revealed remarkable changes in physical and chemical structure of the three substrates after pre-treatment, supporting claims of enhanced biodegradability. This was particularly true in the case of the more recalcitrant Arundo and Barley straw, whereas B 133 sorghum took a modest advantage from NaOH addition. This sorghum could therefore represent a valid alternative to cereals as AD feedstock without pre-treatments. These results appear of particular interest from a practical point of view, as mild pre-treatments that could routinely be implemented in small size biogas plants represent an affordable technology to increase methane output. This contributes to the biogas sector sustainability and further development, mitigating the food vs. non-food controversy in current bio-energy discussion.

CHAPTER 4

Effects of a biomimetic catalyst on the composition, structure and methane output of Arundo, biomass sorghum and barley straw

Abstract

A biomimetic catalyst using a dicarboxylic acid was tested in this study. Pre-treatments at 25 °C for 24 h with two levels of maleic acid (0.3 and 0.6 M) and a combination of sulphuric acid (0.04 M) and maleic acid (0.3 and 0.6 M) were tested to study pre-treatment effects on substrate composition, structure and methane yield of three different ligno-cellulosic substrates (Arundo, Barley straw and B 133 sorghum). Methane production was evaluated in batch AD at 35 °C for 51 days with 4 g VS l^{-1} as organic load. The most recalcitrant substrate (Arundo) staged the highest increase in cumulative methane yield with maleic acid at 0.6 M (+62% over 218 ml CH_4 g^{-1} VS of the untreated substrate). Conversely, the least recalcitrant substrate (B 133 sorghum) exhibited the lowest gain (+36% over 284 ml CH_4 g^{-1} VS), while an intermediate behaviour was shown by Barley straw (+41% over 269 ml CH_4 g^{-1} VS). These large increases in CH_4 output determined by pre-treatments may be explained by the concurrent reduction of structural carbohydrates, especially hemicellulose (-43% in B 133 sorghum pre-treated with maleic acid 0.6 M). Hence, the bio-mimetic approach demonstrated to improve biodegradability of ligno-cellulosic substrates, especially in recalcitrant substrates as Arundo.

4.1 Introduction

Current energy policies are focusing on the use RES to mitigate the global warming caused by the CO_2 emission and reduce the dependency on fossil fuel. Among RES, agricultural biomasses have the largest potential and can be considered as one of the options for meeting the energy/fuels demand in a sustainable manner (Koçar and Civaş, 2103). However, the use of agricultural crops (starch crops, rapeseed, etc.) for bio-energy chains, may increase competition for arable land currently used for food production. To alleviate the debate concerning the land use change, ligno-cellulosic biomass (i.e. energy crops and agricultural residues) can be used as energy sources (Valentine et al., 2012) through AD, substituting crop components as starch and oil that have a primary use in food production. This in turn alleviates the controversy regarding the use of land for energy to the detriment of food supply. Ligno-cellulosic biomass is composed of holocellulose (cellulose and hemicellulose) and lignin, in a different proportion and relationship according to plant species. Among these fractions, lignin is fairly resistant to AD, retarding or preventing the hydrolysis of carbohydrates (Gallert and Winter, 2005). Therefore, during anaerobic digestion of ligno-

cellulosic substrate, hydrolysis is considered the rate limiting step (Pavlostathis and Giraldo-Gomez, 1991), influencing the kinetics and, consequently, production of biogas. To overcome the recalcitrance of ligno-cellulosic substrates, a pre-treatment may help to loosen fibre structure, remove or rearrange lignin fraction and hydrolyze cellulose and hemicellulose (Moiser et al., 2005), resulting in faster hydrolysis and improved methane yield.

Pre-treatments can be grouped into physical, chemical, biological, and their combination (Moiser et al., 2005). Among them, chemical pre-treatments with the use of dilute sulphuric acid have been widely investigated (Moiser et al., 2005; Taherzadeh and Karimi, 2008; Bruni et al., 2010; Brodeur et al., 2011; Lee and Jeffries, 2011; Monlau et al., 2013), performing satisfactory results. The main reactions that occur during dilute sulphuric acid pre-treatment are the hydrolysis of hemicellulose (Hendriks and Zeeman, 2009), a partial hydrolysis of cellulose and a solubilisation of lignin, leading to changes in the structure of biomass (Fernandes et al., 2009). The main drawbacks from the use of sulphuric acid are corrosion of the equipment and hydrogen sulphide formation during AD, as reported in Chapter #2, lowering the quality of biogas. To avoid these constraints, the use of organic instead of mineral acids could be envisaged in the frame of a biomimetic approach, i.e. one that mimics natural enzymes. In fact, it was observed that cellulolytic and hemicellulolytic enzymes catalyse hydrolysis through a general acid-base mechanism by means of two carboxylic acids present on the amino acids of the enzyme active site (Lu and Moiser, 2007). Accordingly, dicarboxylic acids having a similar catalytic structure as the enzymes, were proposed as biomimetic catalyst (Guo et al., 2012), and they are more selectively for β-(1,4)-glycolic bonds when compared to sulphuric acid (Lu and Moiser, 2007). Among the dicarboxylic acids, maleic acid showed the most favourable catalysis selectively (Moiser et al., 200). However, even if maleic acid is easier to handle than sulphuric acid due to its lower strength, this approach appears more expensive compared to sulphuric acid. For that reason Guo et al. (2012) suggested a combination of mineral and organic acid to develop improved biomimetic acid catalyst, integrating the lower cost of sulphuric acid with the advantage of biomimetic acids.

With this premise, a vast literature studied the biomimetic approach to enhance hemicellulose hydrolysis, making cellulose more accessible for enzymatic attack in view of improving bioethanol output (Guo et al., 2012; Lee and Jeffries, 2011; Scordia et al., 2010; Scordia et al., 2011; Scordia et al., 2013), while only a study evaluated this approach on methane potential (Fernandes et al., 2009). Therefore, the objective of this work was to investigate bland pre-

treatments with organic acid (maleic acid) and the combination of mineral and organic acid (sulphuric + maleic acid) on the same three ligno-cellulosic substrates subjected to mild alkaline pre-treatments in Chapter #3. The effects of pre-treatments were evaluated on chemical composition, physical structure and methane yield of the three substrates.

4.2 Material and methods

Three substrates (Arundo, Barley straw and biomass sorghum B 133) were tested in this Chapter; more details on substrate characteristics are given in Chapter # 3 (section 3.3.1).

Pre-treatments were carried out at 25 °C for 24 hours with four different acid solutions: organic acid as maleic acid at two increasing concentrations (0.3 and 0.6 M), and two combinations of mineral acid as sulphuric acid at fixed molarity (0.04 M) with the two concentrations of maleic acid (Table 4.1). The sulphuric acid concentration was chosen based on a previous preliminary experiment.

AD of untreated and pre-treated substrates was conducted for 51 days at 35 °C and 4 g l^{-1} VS as organic load, using a starved inoculum with the following characteristics: TS, 32 mg g^{-1}; volatile solids (VS), 26 mg g^{-1} fresh weight; total alkalinity, 29 g $CaCO_3$ l^{-1}; pH, 7.8.

Chemicals analysis, biogas measurement and FTIR were followed as reported in Chapter #3 (section 3.2.4.1, 3.2.4.2 and 3.2.4.3, respectively). At the end of incubation, the amount of cumulative CH_4 yield was submitted to a two-way completely randomized ANOVA for substrates, pre-treatments and their interaction, through the CoStat 6.3 software (CoHort Software, Monterey, CA, USA). The Student - Newman-Keuls (SNK) test at $P \leq 0.05$ was adopted to separate means of statistically significant traits.

Table 4.1 Conditions applied in the pre-treatments: O and M mean organic and mineral acid, corresponding to maleic and sulphuric acid, respectively; L and H means low and high concentration of maleic acid, respectively. Pre-treatments were conducted at 25 °C for 24 h.

Treatment	Maleic acid	Sulphuric Acid
	Molarity	
Untreated	-	-
OAc.L	0.3	-
OAc.H	0.6	-
M+OAc.L	0.3	0.04
M+OAc.H	0.6	0.04

4.3 Results and discussion

4.3.1 Methane yield during the incubation

At the end of AD, the cumulative CH_4 yield of untreated and pre-treated substrates is illustrated in Figure 4.1. In the first 4 days of incubation, the CH_4 yield was similar in all untreated and pre-treated substrates. Thereafter, a steep increase especially in pre-treated substrates was observed, followed by a temporary slowdown after 10 days. Untreated substrates outlined a steady methanation rate (Fig. 4.1). At 10 days, ca. 50% of cumulative CH_4 yield was produced as average in untreated substrates, vs. 68% in substrates pre-treated with OAc.L and M+OAc.L, and 76% with OAc.H and M+OAc.H (Fig. 4.1). The rapid CH_4 production in pre-treated substrates at the beginning of the incubation might be due to the conversion of easily degradable compounds, indicating an increase of the overall biodegradability after pre-treatment. In fact, Lee and Jeffries (2011) reported that a pre-treatment with the same dicarboxylic acid as in this experiment (maleic acid) released a remarkable amount of monomer sugars derived from structural carbohydrates (i.e. hemicellulose and cellulose). Conversely, a temporary slowdown of CH_4 production after 10 days was likely due to a period of adaptation for micro-organisms to degrade the recalcitrant fraction.

In each substrate, cumulative CH_4 yield in the five treatments (untreated and the four pre-treated) diverged to a varying extent during the incubation. As a result of this, cumulative CH_4 yield at the end of the incubation was significantly affected by the substrate × treatment interaction, meaning that pre-treatments exerted a different effect on final CH_4 output depending on substrate (Fig. 4.2). In untreated substrates, Arundo showed a lower CH_4 yield (218 ml g^{-1} VS) than Barley straw and B 133 (average, 276 ml g^{-1} VS).

In treated substrates, Arundo achieved a similar CH_4 yield in pre-treatments at OAc.L and M+OAc.L (average, 272 ml g^{-1} VS), followed by M+OAc.H and OAc.H (330 and 354 ml g^{-1} VS, respectively; Fig. 4.2). This corresponds to a 24, 51 and 62% respective gain in CH_4 yield vs. untreated Arundo. Likewise, pre-treatments in Barley straw outlined a similar CH_4 yield in OAc.L and M+OAc.L (average, 323 ml g^{-1} VS), as well as in OAc.H and M+OAc.H (average, 380 ml g^{-1} VS), increasing CH_4 output by 20 and 41%, respectively. Conversely, in B 133 a variable CH_4 yield was obtained after pre-treatments: 307, 335, 373 and 398 ml g^{-1} VS at M+OAc.L, OAc.L, M+OAc.H and OAc.H, respectively, achieving an increase between 8% (M+OAc.L) and 40% (OAc.H). It is worth noticing that B 133, a more biodegradable

substrate, had benefited less from pre-treatments than Barley straw and Arundo. Moreover, was not observed a remarkable difference between organic acid and combination mineral and organic acid pre-treatments (Fig. 4.2).

Fernandes et al. (2009) studied the effect of maleic acid (0.05 M) pre-treatment (at 150 °C for 30 min) on methane yield of three different substrates as hay, straw and bracken with different lignin content (25, 57 and 185 mg g^{-1} VS, respectively). They found that the methane yield of hay and straw was not enhanced after pre-treatment, while they observed a 57% increase (over 110 ml CH_4 g^{-1} VS) in bracken, concluding that the effect of pre-treatment was more profound in ligno-cellulosic biomass with a higher lignin content. Compared to these, in this experiment a similar trend was observed: the methane yield after pre-treatment augmented in parallel with the increase lignin content (41, 31 and 25% CH_4 yield increase with a lignin content of 229, 215 and 198 mg g^{-1} VS in Arundo, Barley straw and B 133, respectively).

In general, in this thesis the biomimetic catalyst was the best pre-treatment, leading to a CH_4 increase by 2 fold compared to NaOH pre-treatment (+32% vs. +16% methane increase, as average of all substrates and pre-treatments) and by 3 fold compared to hydrothermal pre-treatment (+32% vs. +11%).

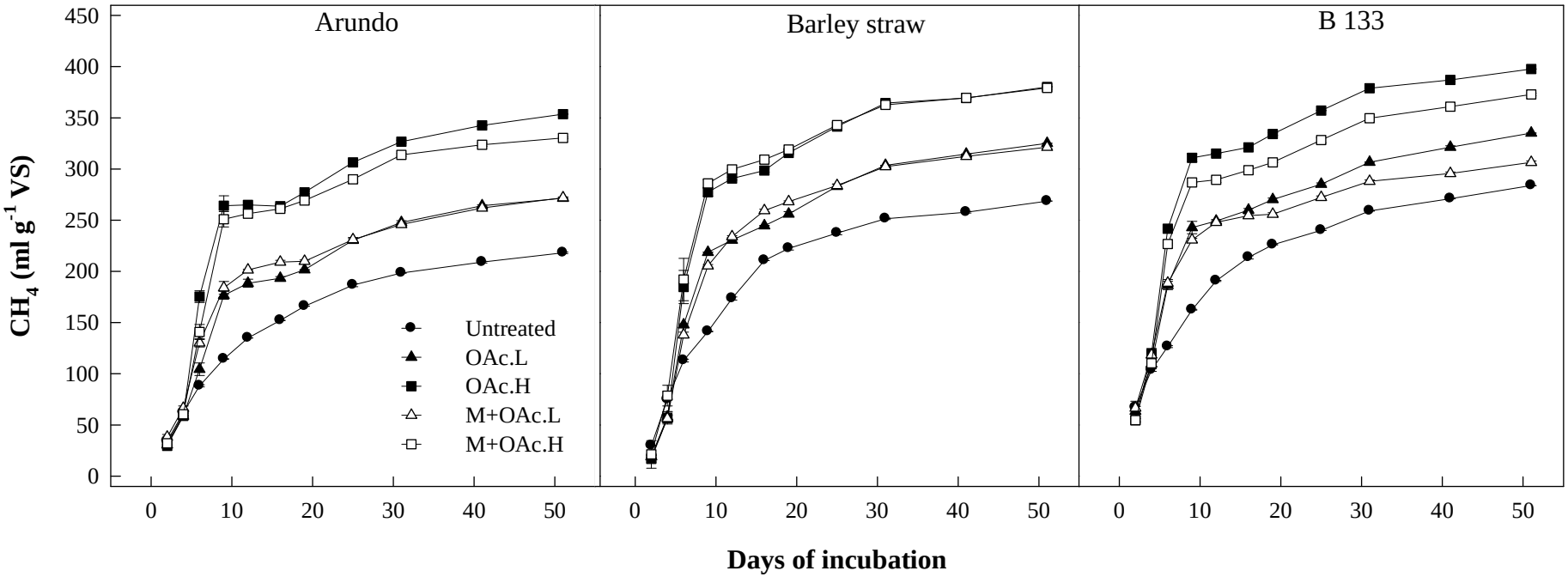

Figure 4.1 Cumulative methane yield of untreated and pre-treated Arundo, Barley straw and B 133 sorghum during the anaerobic digestion assay. OAc. organic (maleic) acid; M+OAc. mineral (0.04 M H_2SO_4) and organic acid; L and H mean low (0.3 M) and high (0.6 M) levels of maleic acid. Vertical bars, ± standard errors (n = 3).

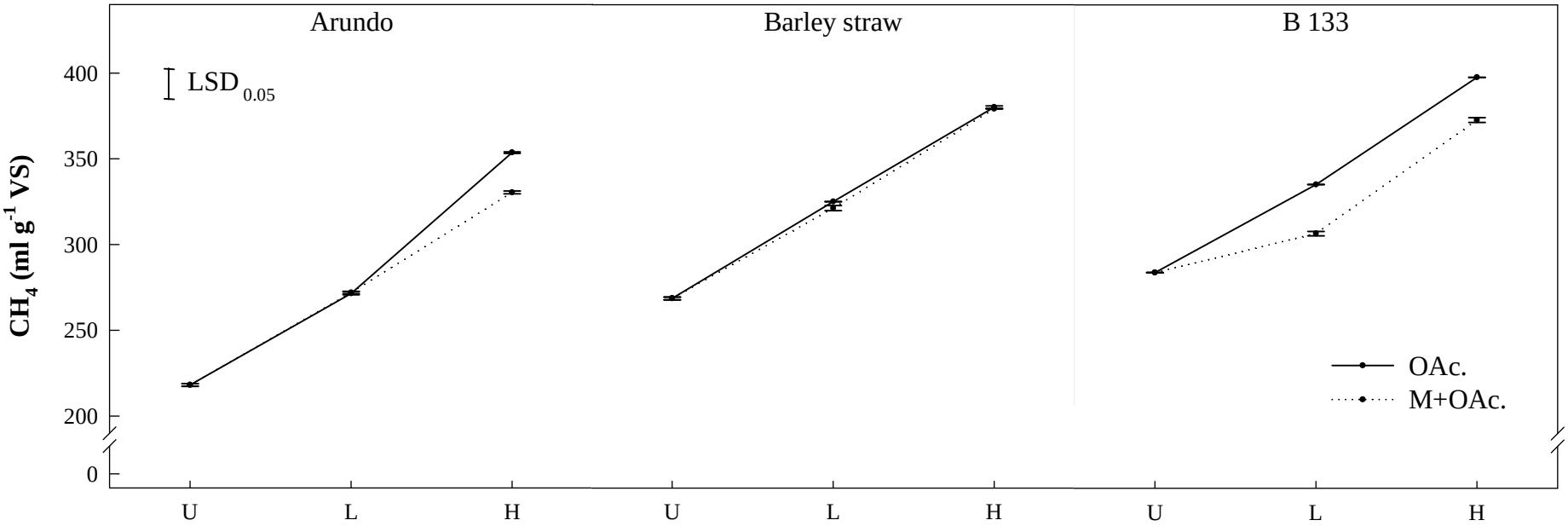

Figure 4.2 Significant substrate × treatment interaction on cumulative CH_4 yield at the end of the incubation of Arundo, Barley straw and B 133 sorghum. U, L and H mean untreated and pre-treated with low (0.3 M) and high (0.6 M) levels of maleic acid (OAc.) alone or in combination with sulphuric acid at 0.04 M (M+OAc.). $LSD_{0.05}$ (Least Significant Difference at $P \leq 0.05$) = 19.6 ml CH_4 g^{-1} VS. Vertical bars, ± standard errors (n = 3).

4.3.3 Changes in fibre composition and structure of pre-treated substrates

Pre-treatments may alter the ligno-cellulosic structure, even disrupt structure, enhancing the bioconversion of ligno-cellulosic substrates. In this experiment the reduction of the three structural components (cellulose, hemicellulose and lignin) outlined different patterns depending on substrates; however, no relevant difference was observed at varying acid solution (Fig. 4.3): B 133 showed the highest solubilisation of cellulose and hemicellulose subjected to the four acid solutions (range, from 22 to 33% and from 40 to 47% in the two respective carbohydrates), compared to Arundo (from 20 to 23% and from 34 to 41%) and Barley straw (from 5 to 14% and from 22 to 30%). Lastly, the strongest lignin (AIL) reduction was observed in B 133 (23%, as average of the four pre-treatments), compared to Arundo (16%) and Barley straw (7%) (Fig. 4.3). In the literature, maleic acid has already been shown a good agent for hydrolyzing hemicellulose (Lu and Moiser, 2007 and 2008; Kootstra et al., 2009a). Hemicellulose reduction up to ca. 80% was obtained in corn stover pre-treated with maleic acid (0.05 M) combined with microwave heating (170 °C for 30 min), while only a 12% cellulose reduction was observed (Kootstra et al., 2009b). Likewise, Guo et al. (2012) reported a 80% hemicellulose reduction in *Miscanthus* after pre-treatment (170 °C for 6 min) with maleic and sulphuric acid at 0.53 and 0.075 M, respectively. In addition, they demonstrated that maleic acid is mainly active on the easily hydrolysable fraction of hemicellulose. In fact, hemicellulose of most ligno-cellulosic substrates may be present under two fractions, easy and hard to hydrolyze; the latter portion has been shown to account for 35% of the total (Jacobsen and Wyman, 2000). Compared to the cited works, this experiment exhibited up to 47% hemicellulose reduction, probably derived from the easily hydrolysable fraction. Beside its activity in the hydrolysis of hemicellulose, maleic acid proved also effective in reducing cellulose and lignin in this experiment (-19% and -15% in the two respective components, as average of all substrates and pre-treatments vs. untreated) (Fig. 4.3). Changes in fibre composition were also supported by FTIR analysis. In this experiment, bandsof absorbance are assigned to specific functional groups and linkages as in Table 3.3 (Chapter #3). Especially the bands assigned to hemicellulose and cellulose exhibited an absorbance decrease after pre-treatment (Table 4.2), confirming a solubilisation of the structural carbohydrates and a change in fibre structure (Sambusiti et al., 2013). TCI and LOI (see section 3.2.4.3 in Chapter #3 for their calculation) showed an increase after pre-treatment, indicating that only amorphous cellulose was solubilized (Table 4.2).

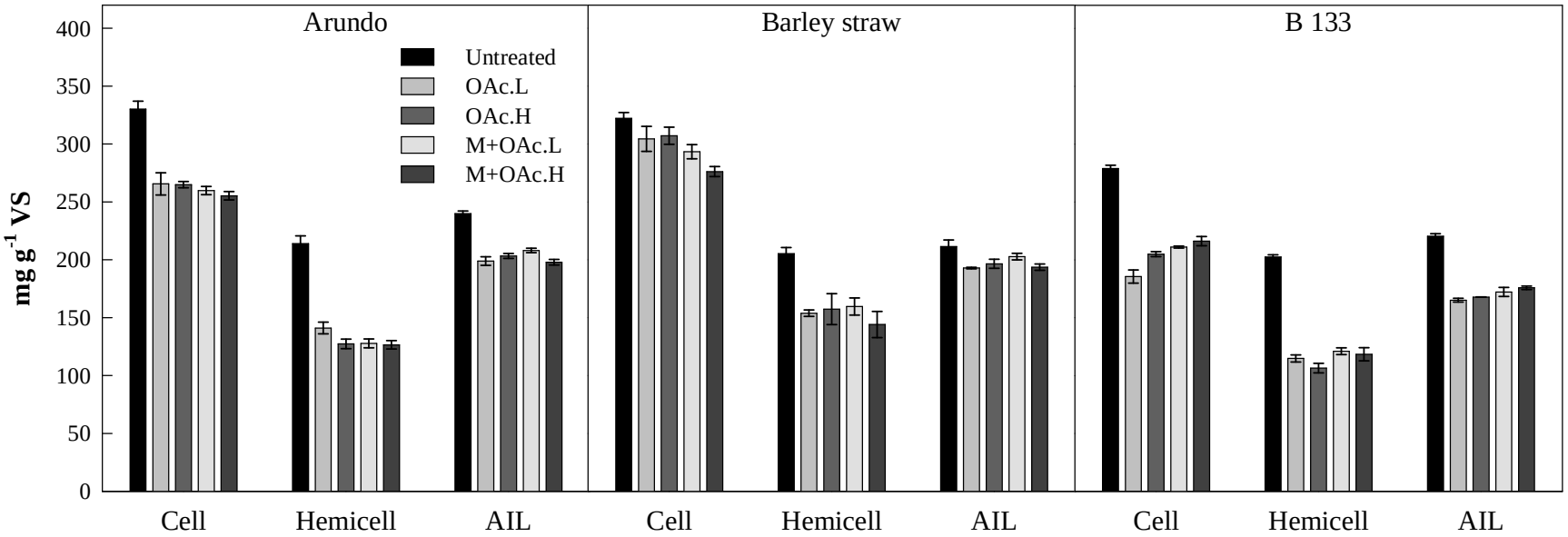

Figure 4.3 Fibre composition of untreated and pre-treated Arundo, Barley straw and B 133 sorghum. OAc. organic (maleic) acid; M+OAc. mineral (0.04 M H_2SO_4) and organic acid; L and H mean low (0.3 M) and high (0.6 M) levels of maleic acid. Cell, Hemicell and AIL mean cellulose, hemicellulose and lignin (acid insoluble lignin), respectively. Vertical bars, ± standard errors (n = 2).

Table 4.2 Absorbance related to bands assigned to hemicellulose and cellulose (functional groups and linkages reported in Table 3.3, Chapter #3). OAc. organic (maleic) acid; M+OAc. mineral (0.04 M H_2SO_4) and organic acid; L and H mean low (0.3 M) and high (0.6 M) levels of maleic acid. LOI (Lateral Order Index) and TCI (Total Crystallinity Index), based on absorbance data at specific bands of FTIR spectra. In brackets, standard errors (n = 2).

Substrate	Pre-treatments	Wavenumbers (cm^{-1})							LOI	TCI
		2900	1720	1430	1375	1315	1158	898		
Arundo	Untreated	0.33	0.32	0.45	0.47	0.42	0.45	0.34	1.32 (0.01)	1.41 (0.01)
	OAc.L	0.12	0.15	0.23	0.26	0.24	0.29	0.15	1.57 (0.15)	2.08 (0.05)
	OAc.H	0.16	0.20	0.28	0.32	0.32	0.21	0.16	1.76 (0.24)	2.00 (0.37)
	M+OAc.L	0.14	0.17	0.25	0.27	0.25	0.26	0.14	1.69 (0.08)	1.89 (0.01)
	M+OAc.H	0.08	0.12	0.16	0.17	0.15	0.19	0.09	1.76 (0.01)	2.03 (0.01)
Barley straw	Untreated	0.23	0.14	0.32	0.33	0.31	0.40	0.21	1.49 (0.03)	1.40 (0.05)
	OAc.L	0.19	0.18	0.30	0.33	0.30	0.34	0.19	1.57 (0.04)	1.77 (0.25)
	OAc.H	0.25	0.23	0.32	0.37	0.32	0.40	0.24	1.37 (0.05)	1.68 (0.34)
	M+OAc.L	0.14	0.11	0.22	0.24	0.22	0.27	0.13	1.70 (0.02)	1.79 (0.09)
	M+OAc.H	0.16	0.16	0.24	0.28	0.25	0.30	0.17	1.47 (0.10)	1.77 (0.14)
B 133 sorghum	Untreated	0.25	0.21	0.38	0.40	0.37	0.42	0.28	1.38 (0.01)	1.57 (0.04)
	OAc.L	0.17	0.20	0.28	0.33	0.31	0.38	0.20	1.41 (0.08)	2.05 (0.16)
	OAc.H	0.09	0.12	0.18	0.22	0.20	0.27	0.12	1.45 (0.05)	2.44 (0.23)
	M+OAc.L	0.14	0.19	0.28	0.33	0.31	0.42	0.19	1.50 (0.12)	2.42 (0.19)
	M+OAc.H	0.09	0.12	0.20	0.22	0.20	0.26	0.13	1.49 (0.05)	2.31 (0.18)

4.4 Conclusions

Maleic acid, representing a biomimetic catalyst, was proposed as biomass pre-treatment to enhance methane yield of ligno-cellulosic substrates, also in combination with a strong mineral acid (H_2SO_4). After pre-treatments, remarkable physical and chemical changes of fibre structure were observed, supporting enhanced substrate biodegradability. As a result, pre-treatments also improved cumulative methane yield of three ligno-cellulosic substrates to an extent that was directly related to their recalcitrance to bio-degradation. However, although a considerable methane increase was obtained, an economic evaluation is needed before the implementation of a biomimetic treatment in full scale biogas plants.

General conclusion

Methane production from ligno-cellulosic substrates (dedicated energy crops and agricultural residues) appears a favourable alternative to fossil fuels, while at the same time mitigating the "food vs. fuel" dilemma. The seven crops investigated in this thesis (Chapter #1) showed a remarkable difference in biomass yield, methane production and subsequent methane output per hectare. The six crops alternative to Maize outlined a less favourable composition (i.e. easily degradable fraction such as proteins, lipids and starch) in view of anaerobic digestion. However, three alternative crops (Arundo and sorghum hybrids B 133 and S 506) yielded within ±10% dry biomass compared to Maize, whereas none of them fell within -10% from maize in terms of potential methane yield. Therefore, the characteristics of these substrates may influence the methane yield. For example, biomass species as Arundo and Switchgrass having a low amount of easily biodegradability compounds (proteins, lipids, soluble sugars), associated with a high content of structural carbohydrates and lignin, have a slow methane production kinetics reflecting in low methane yield, compared to the group of the sorghum hybrids (intermediate amount of biodegradable compounds and structural carbohydrates) and, to a greater extent, Maize. However, sorghum B 133 turned out to be quite competitive with Maize in terms of methane yield per hectare, whereas multi-annual species expressed a modest competitiveness. Nevertheless, multi-annual species retain a special interest in view of the limited need of external inputs (energy, fertilizers, water, etc.) for their cultivation, reflecting in a lower environmental impact and a favourable energy balance. To reduce the gap separating multi-annual species from maize, various strategies may be envisaged, such as harvesting at an earlier stage or adopting pre-treatments to enhance biodegradability.

In order to improve methane yield of ligno-cellulosic substrates, pre-treatments are seen as a valuable tool in a portfolio of strategies supporting bio-energy growth. However, in the literature different types of pre-treatments were tested, but the high variability of pre-treatment conditions and substrate composition influence their results, i.e. their effectiveness in terms of methane yield increase. For example in this thesis, hydrothermal pre-treatments at high temperature and pressure contributed to methane yield of Arundo (average, +12%) provided that no acid catalyst was added to the substrate (Chapter #2). However, when improvements of final methane production are obtained, it is necessary to evaluate their viability in full scale biogas plants. These issues combined represent the current frontier in the research on anaerobic digestion. Future progress may be envisaged, amid other strategies, in

bland pre-treatments (e.g., lower temperatures and weaker catalysts) as a potential means to improve net energy gain and methane production efficiency. This may be the case of mild alkaline pre-treatments at low temperature (Chapter #3), which proved efficient in enhancing methane yield of three ligno-cellulosic substrates to an extent that was directly related to their recalcitrance to bio-degradation (+21, +20 and +7% as average of all pre-treatments, for Arundo, Barley straw and B 133 sorghum, respectively). Pre-treatment benefits were also shown in terms of process kinetics and technical digestion time, achieving comparable results with stronger physical-chemical treatments reported in the literature. Compositional analysis associated with FTIR and SEM procedures revealed remarkable changes in physical and chemical structure of the three substrates after pre-treatment, supporting claims of enhanced biodegradability. This was particularly true in the case of the more recalcitrant Arundo and Barley straw, whereas B 133 sorghum took a modest advantage from NaOH addition. Another bland pre-treatment tested in this thesis was maleic acid as a biomimetic catalyst (Chapter #4) that showed remarkable changes in physical and chemical fibre structure, supporting claims of enhanced substrate degradability. Hence also in this case pre-treatments proved able to enhance methane yield of three ligno-cellulosic substrates to an extent that was directly related to their recalcitrance to bio-degradation (+41, +31 and +24% as average of all pre-treatments, for Arundo, Barley straw and B 133 hybrid sorghum, respectively).

Contrasting the achievements of this thesis and other similar works, pre-treatment implementation in full scale anaerobic digestion plants is still limited due to operational parameters not yet standardised. Furthermore, a careful evaluation under the energetic, economic and environmental viewpoint is needed, before disseminating pre-treatment techniques to commercial biogas plants.

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