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Assessment of energy and material recovery from winery wastes

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Abstract

Wine-making process generates a great amount of organic wastes (grape stalks, grape marcs, wine lees and waste activated sludge). They are characterized by seasonality, high biodegradability e polyphenols content, hence their environmental disposal without any treatment could be environmentally hazardous. Anaerobic digestion is a suitable biological process able to treat winery wastes because it produces renewable energy and reduces green house gases emission. Digestate had a higher available nutrient content than fed substrates, then it is a potential fertilizer. The aim of this study is to evaluate winery wastes anaerobic digestion feasibility using batch and semi-continuous tests at mesophilic and thermophilic temperature. Treatment of a sole substrate and utilization of co-substrates were considered (co-digestion). Three different approaches was analyzed: integration of wine lees treatment in digesters already built such as reactors located inside wastewater treatment plant and those treating livestock effluents (on-farm plants), and application of anaerobic digestion to support wine sector utilities and activities inside the cellar borders.

Batch tests showed that thermophilic specific methane production from winery wastes are comparable with mesophilic ones. Grape marc is the most abundant by-product and generates 0.36 $m^3CH_4/kgVS$, on the other hand it is available for few months after the grape harvest. Wine lees is produced throughout the year and specific methane production is 0.37 $Nm^3CH_4/kgVS$. Semi-continuous trials showed that treatment of a sole substrate is difficult to carry out because of low nutrients content and slow hydrolysis.

Anaerobic co-digestion of municipal waste activated sludge and wine lees was not steady operating with organic loading rate higher than 3.3 $kgCOD/(m^3d)$ because volatile fatty acids accumulated. Operating organic loading rate of 2.8 $kgCOD/(m^3d)$ where wine lees count for 70% of inlet COD, mesophilic and thermophilic process had specific biogas production of 0.38 $m^3/kgCOD$ e 0.40 $m^3/kgCOD$, respectively. Anaerobic digestion of wine lees with winery waste activated sludge confirmed results obtained in previous mesophilic trial, while thermophilic process failed. Observation of kinetic of anaerobic digestion operating with different hydraulic retention times (23 and 40 d) allowed for increase organic loading rate to 6.6 $kgCOD/(m^3d)$ and improve mesophilic process performances using an automatic feeding mode.

The problem observed operating at 55°C was due to missing micro-nutrients. Metals augmentation (Fe, Co and Ni) improved process stability and increased biogas production until 0.45 $m^3/kgCOD$. This approach produces renewable energy that could be used in winery wastewater treatment plant, the nutrient enriched supernatant supply nutrient in the aerobic biological reactor and digestate could be applied as fertilizer. Despite the positive purposes, raw effluent had residual phytotoxicity that doesn't meet legislation limits. In fact germination index indicated phytotoxicity using 30% v/v of digestate. A deeper study about effect of ammonium and copper, the main pollutant in digestate from winery wastes, indicated that they are not the main causes of toxicity and other compounds had a synergic effect increasing toxicity.

Finally, lab-scale tests demonstrate that maize silage, usually used as biomass to increase biogas production in on-farm plant, could be substitute with wine lees and catch crops. The feeding mixture had optimal

COD/N ratio and made the process more sustainable in terms of environmental and economical point of view. The process was steady and produced 0.32 m^3/kgCOD (73% methane).

1. Introduction

1.1. Winery wastes overview

Wine production is one of the most important agricultural activities in the world and it represents the largest agricultural sector of many European countries in terms of cultural heritage and economy. Vineyard area has been reduced in the world since 2000 mainly due to European grubbing-up. The reduction has been offset by increasing surface area intended to grape production in China, Argentina and Chile. International Organization of Vine and Wine (OIV, 2015) estimated that there were around 7,573 million of hectares of vineyard in the world in 2014. Half of this area is located in 5 Countries: Spain (14%), China (11%), France (10%), Italy (9%) and Turkey (7%).

The average grape production on surface basis was 9.7 tonnes/ha and, consequently, 736.7 million of grape were collected. The 41% of production derived from Europe in agreement with vineyard surface distribution. The favorable climate conditions, the redistribution of vineyards in Countries with appropriate regions and increasing yields allowed for production increase since 2000 (+1.1%).

Most of the grapes (40 million of tonnes) were pressed to produce wine, must and juice. Worldwide wine generation was 270 million of hectoliters in 2014, on average with the values recorded since 2000 (257-296 mhl). At the moment France is the biggest wine producing country (46.7 Mhl), followed by Italy (44.7 Mhl), Spain (38.2 Mhl) and USA (22.3 Mhl).

Less than 4 million of tonnes were not fermented and generated most and juice. The remaining part of grape (45% of total grape) were destined to consumption of fresh grape (25 million of tonnes) and resins (5 million of tonnes). Production of fresh and dried grapes were concentrated in China, Turkey, Iran and India.

Generally the food and beverage production makes important contributions to a range of environmental impacts, and wine sector is not an exception. Due to strong relationship between wine production and the surrounding area, traditionally and among the population, wine-making process is perceived to be environmentally friendly and the producers generally pay attention to sustainability. The several life cycle assessment analyses (Point et al., 2012) about wine production are a proof of environmental heed. To date, the main environmental impacts kept in consideration include water consumption and pollution, soil degradation, damage to vegetation and to ecosystems, odors and air emissions, noise from vehicles and equipment, introduction into environment of pesticides and heavy metals. As indicated in Figure 1 the waste management was not considered, however the reduction of waste and the recovery of by-products are important tools to minimize the environmental impacts, as foreseen in ISO 14000 (Oliveira and Duarte, 2016).

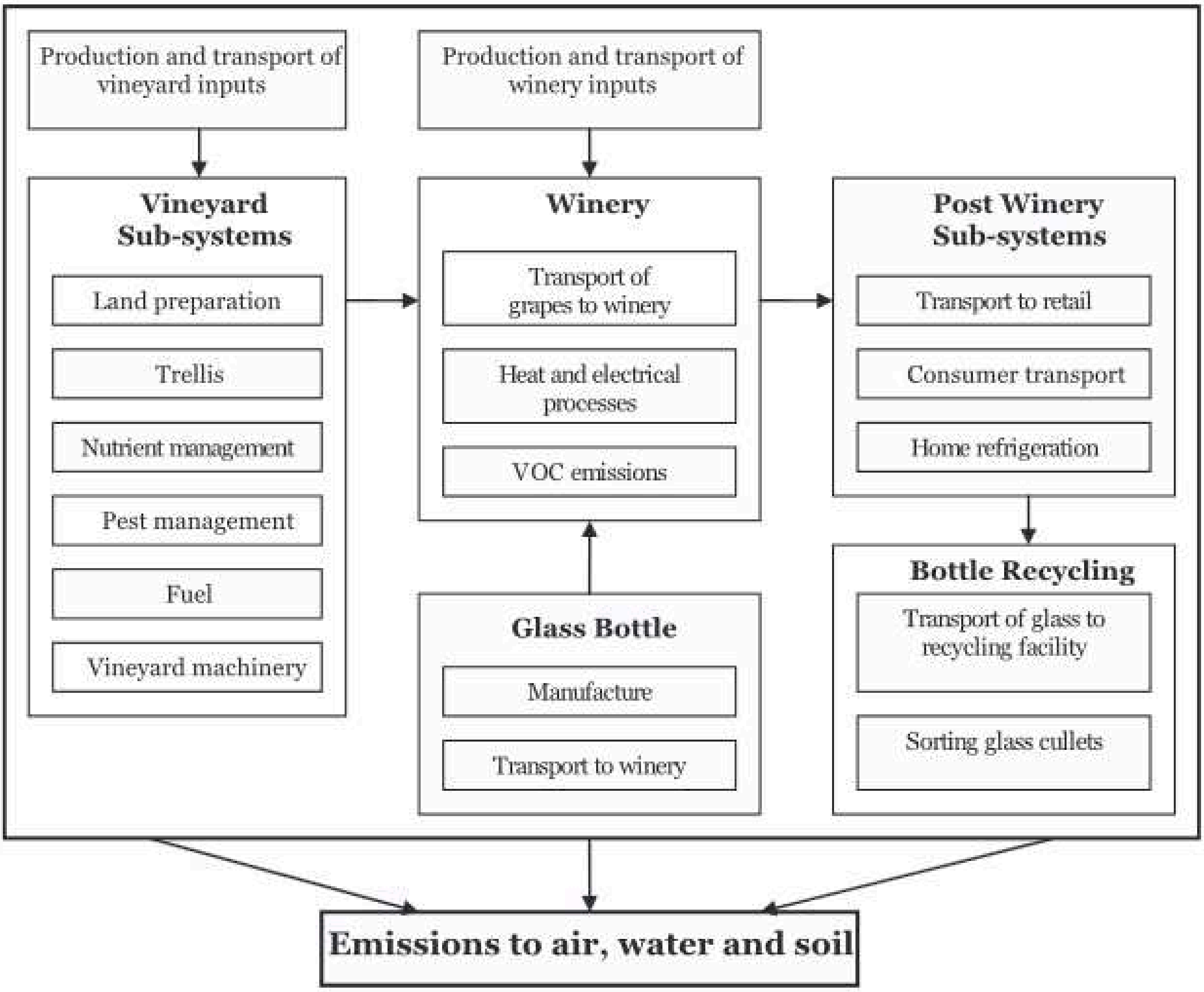

Figure 1 Example of system flow diagram of wine's life cycle. (Point et al., 2012)

In the wine-making process, two different periods are usually considered: harvest and post-harvest. The first one lasts about three months and is performed in autumn, it generates the 60-70% of the total waste streams. The main wastes include stems or stalks from de-stemmed grape, grape marcs, lees from most fermentation (Figure 2).

Wastewater derives from cleaning practices and is generated in both the considered periods. It is the most abundant waste flow (Table 1) and has to be treated before emission into the environment, usually by aerobic treatment processes. This kind of processes consumes a great deal of energy and generates another solid waste known as winery sludge.

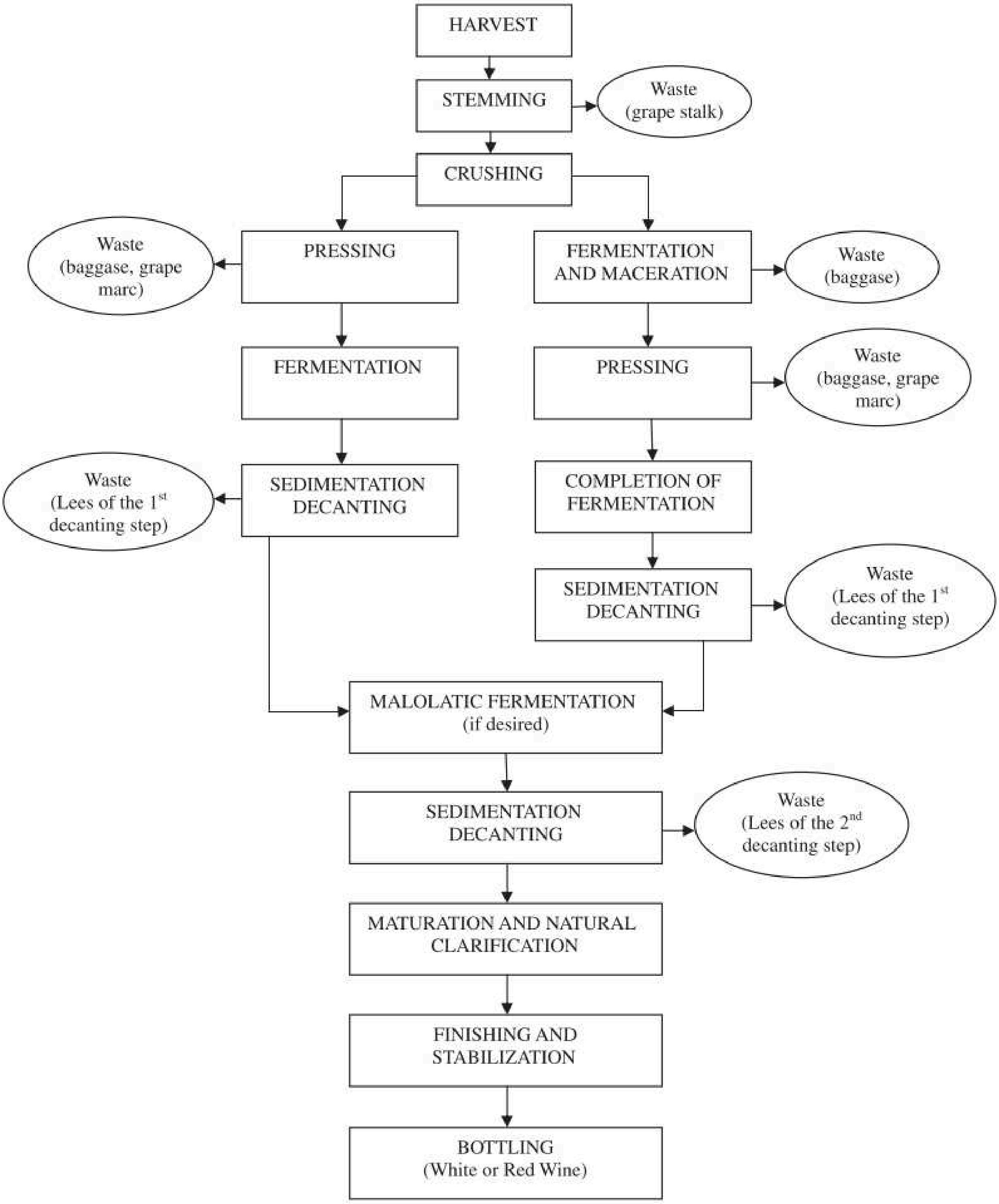

Figure 2 Diagram of the wine-making process (Devesa-Ray et al., 2011)

Grape stalks, that is the grape skeleton, are obtained by grape stripping operation. Wadhwa and Bakshi (2013) estimated production of 5 tonnes/ha/year. This by-product has a high degree of fibers (lignin and cellulose) and a high percentage of nutritive mineral elements, especially nitrogen and potassium. One of the widely diffuse practice is spreading them directly on fields or after composting, in order to recover this substrate as fertilizer (Diaz et al., 2002).

Table 1 Winery wastewater production for Country

Country	Production	Reference
Portugal	1.65 m^3/1000kg grape	Oliveira and Duarte, 2016
	2.2 L/L wine	
	2-15 L/L wine	Oliveira et al., 2009
Italy	0.98-1.13 L/L wine	Lucas et al., 2010
	1.4 L/L wine	Bolzonella et al., 2010
	0.2-6 L/L wine	Berta et al., 2003
	0.7-14 L/L wine	Andreottola et al., 2009
Chile	1 -6 L/L wine	Aybar et al., 2007
South Africa	1.5-6 m^3/1000kg grape	Walsdorff et al., 2005
	1.3-7 m^3/1000kg grape	Duarte et al., 2004
	1.6-8.0 L/L wine	Fernandez et al., 2007
	3-5 m^3/1000kg grape	Kumar et al., 2006
Greece	1-4 L/L wine	Vlyssides et al., 2005

Grape marc or pomace consist of grape skin, pulp and seeds collected after grape juice extraction (Barcia et al., 2014). Grape marc is produced after pressing the previously crushed grapes, in white wine production technology, or after the maceration phase concurrent with fermentation step, in red wine production technology. It is characterized by a high organic content, is rich in carbohydrates (Corbin et al., 2015) and phenolic compounds (Deng et al., 2011).

Wine lees are the residue collected after grape juice fermentation or wine aging at the bottom of recipients. They are characterized by high organic matter content, acid pH values and low electrical conductivity (Bustamante et al., 2008). The organic matter consists of many soluble compounds (ethanol, malic and tartaric acid and sugars), substances partially adsorbed on particles such as polyphenols and microbial biomass (mainly yeasts) (Pérez-Serradilla and de Castro, 2008).

The amount of wastes produced depends on grape cultivar and wine-making process(Table 2). Other wastes are produced from correlated activities: vine shoots originated from plants trimming, waste activated sludge from bioreactor treating winery wastewater, and exhausted grape marcs deriving from fermented grape marc. Traditionally grape stalks are used as fertilizer but Bustamante et al. (2008) reported that properties of winery wastes (low pH, high organic matter cont, presence of polyphenols and limiting concentration of micro-nutrients) make them incompatible with agricultural requirements and therefore they must be conditioned before to use. Composting became an important biotechnology to stabilized the grape stalks but also it can be applied also to other by-products such as lees, grape marcs. Several authors described the best condition to obtained a stabilized substrate and reduce the hazard (Bustamante et al., 2007; Devesa-Rey et al., 2011; Diaz et al., 2002). The application of compost in vineyards with poor soil (Bertran et al., 2004)represents a method to nutrients recycle, moreover it increases the percentages of organic matter, nutrient levels (providing a slow fertilization action over a long period), microbial biomass and improves the soils' physical properties (aeration, water holding capacity, etc.) (Ribereau-Gayon et al., 2006). On the other hand the composting is an energy consuming method and represent a management cost for the wineries.

Table 2 Production of organic wastes from wine-making process

Waste	Amount	Reference
Grape skin	1.95 kg/100kg grape	Ribereau-Gayon et al., 2006
Grape stalks	3 kg/100kg grape	
Grape seeds	4.5 kg/100kg grape	
Grape stalks	3 kg/100kg grape	Oliveira and Duarte, 2016
Lees	5 kg/100kg grape	
Grape marc	12 kg/10kg grape	
Grape stalks	3 kg/100kg grape	Oliveira and Duarte, 2014
Grape marc	13 kg/100kg grape	
Lees	6 kg/100kg grape	
Grape stalks	2.5-7.5 kg/100kg grape	Wadhwa and Bakshi, 2013
Grape marc	24-45 kg/100kg grape	
Grape seed	3-6 kg/100kg grape	
Grape marc	14 kg/100kg grape	Celma et al., 2007
Lees	14 kg/100kg grape	

Other traditional system to recycle is the production of spirit and bio-ethanol through fermentation and distillation (Rodriguez et al., 2010), in turn, this process generates other residues that have to treated: exhausted grape marc and vinasses. Distilleries are able to recover tartaric acid, oil (Fiori, 2010; Molero Gómez et al., 1996), and polyphenols (Conde et al., 2011; Vatai et al., 2009), but at the moment the applied technologies are expensive and not-sustainable.

An overview of technologies utilized to recover materials from winery wastes is summarized in Table 3. Many studies evaluated composting, but also innovative methods were proposed such as production of enzymes, extraction of polyphenols, oil and lactic acids. Lees are one of by-products less valorized although they are available also in the post-harvest period. They are used as a supplement in animal feed but have a poor nutrients value (Maugenet, 1973) or as source of tartrates.

As suggested by Dimou et al. (2015) the best approach to valorize winery wastes should be the bio-refinery one where more processes are integrated. In this contest the energy production by anaerobic digestion should be taken into consideration because it could sustain the energy consuming processes, remove pollutants and generates an interesting fertilizer

Table 3 Technologies applied to winery wastes to recover energy and material (Devesa-Ray et al., 2011)

Residue	Treatment	Product	References
Lees, vine shoots, grape stalks, grape marc	Composting	Plant substrate	Paradelo et al., 2010; Nogales et al., 2005; Diaz et al., 2002
Grape marc	Composting	Plant substrate	Bustamante et al., 2010, 2007; García-Martínez et al., 2009; Manios et al., 2007; Moldes et al., 2007a; Pardo et al., 2007; Nogales et al., 2005; Diaz et al., 2002
Grape marc	Composting	Substrate for mushroom cultivation	Pardo et al. 2007
Grape marc	Vermicomposting	Plant substrate	Gómez-Brandón et al., 2011;, Paradelo et al., 2009b
Hydrolyzed grape marc, lees	Composting	Plant substrate	Paradelo et al., 2010
Grape marc	Composting	Adsorbent for colored compounds	Paradelo et al., 2009
Grape marc	Solid state fermentation	Bio-ethanol	Rodriguez et al., 2010
Vinification lees	Extraction of tartaric acid	Nutritional supplement for *Debaryomyces hansenii*	Salgado et al., 2010
Grape marc	Extraction	Tannins as wood adhesives	Jiang et al., 2011
Grape marc	Extraction	Polyphenols	Conde et al., 2011; Ping et al., 2011; Vatai et al., 2009
Grape marc seed	Extraction	Oil	Fiori, 2010;Molero Gómez et al., 1996
Vinification lees	No treatment	Nutritional supplement for Lactobacillus	Bustos et al., 2004a,b
Vinasse	Alkali treatment; microwave;	Lactic acid	Liu et al., 2010

	fermentation		
Vinasse	Solubilization and precipitation	Tartaric acid	Rivas et al., 2006; Versari et al., 2001; Salgado et al., 2010
Lees, grape marc,	Yeast-induced fermentation	Protein	Silva et al., 2011
Vinasse, grape marc	Fermentation with *Trichoderma viride*	Biocontrol agent	Bai et al., 2008; Santos et al., 2008
Vinasse	Fermentation	Protein rich fungal biomass	Nitayavardhana and Khanal, 2010
Trimming vine shoots	Hydrolysis, fermentation of hemicellulosic sugars by L. pentosus	Lactic acid; biosurfactants	Bustos et al., 2004a, 2005b, 2007; Moldes et al., 2007b
Trimming vine shoots	Hydrolysis; delignification; simultaneous saccaharification and fermentation of cellulosic fraction	Lactic acid	Bustos et al., 2005a; Alonso et al., 2009
Trimming vine shoots	Hydrolysis and fermentation of hemicellulosic sugars with Lactobacillus and *Debaryomyces hansenii*	Lactic acid; xylitol; bio-surfactants	Rivas et al., 2007; Portilla et al., 2008a
Trimming vine shoots	Pre-hydrolysis and alkaline extraction	Ferulic acid, p-coumaric acid and other phenolic compounds	Max et al., 2009, 2010
Trimming vine shoots	Pulping process	Cellulose pulp	Jimenez et al., 2004, 2006
Trimming vine shoots	Carbon dioxide activation	Activated carbon	Nabais et al., 2010
Trimming vine shoots, together with other viticulture residues	Not determined	Source of nutrients for ruminants	Molina-Alcaide et al., 2008
Trimming vine shoots grape marc	Solid state fermentation with *Pleurotus*	Source of microbial and human food	Sánchez et al., 2002
Grape marc	Hydrolysis, fermentation with *L. pentosus*	Lactic acid bio-surfactants	Portilla et al. 2007, 2008b, 2009a

Grape marc	Hydrolysis, fermentation with *L. pentosus*	Bio-emulsifiers	Portilla et al., 2010
Grape seed oil	Fermentation with *Pseudomana aeruginosa*	Bio-surfactants	Wei et al., 2005
Grape marc	Fermentation with *Lactobacillus*	Anti- allergen	Tominaga et al., 2010
Grape marc	Solid state fermentation	Hydrolytic enzymes	Díaz et al., 2009, 2011; Botella et al., 2005
Grape marc, trimming vine shoots	Solid state fermentation with *Pleurotus*	Source of microbial and human food	Sánchez et al., 2002
Grape marc	No treatment	Adsorbent for metal effluent decontamination	Farinella et al., 2008
Grape marc, lees	Yeast-induced fermentation	Protein	Silva et al., 2011
Grape marc vinasse	Fermentation with *Trichoderma viride*	Bio-control agent	Bai et al., 2008; Santos et al., 2008

1.2. Treatment of winery wastes by anaerobic digestion

Generality about anaerobic digestion

Anaerobic digestion (AD) is a well known technology applicable to substrates with high organic content and represents a sustainable treatment system which is capable of minimizing energy consumption and generate energy (heat and electricity).

It is a biochemical process carried out by different consortia of microorganisms (Weiland, 2010) that involved a community of microorganisms that transform, in absence of oxygen, organic matter into biogas. The transformation can be divided in four steps:

1. Hydrolysis: solubilization of particulate fraction and transformation of organic polymers into monomers (amino-acids, simple carbohydrates and fatty acids). This process causes the release of hydrogen.
2. Acidogenesis: conversion of produced monomers into volatile fatty acids (VFA), ammonium, hydrogen, carbon dioxide and hydrogen sulfide with the help of fermentative bacteria.
3. Acetogenesis: transformation of VFA into acetic acid, carbon dioxide and hydrogen.
4. Methanogenesis: the last step of the process operated by methanogens Archea. Two pathways can be utilized: acetoclastic one converts acetic acid into methane and carbon dioxide, while hydrogenotrofic one uses hydrogen and carbon dioxide to generate methane and water.

Environmental conditions in the reactor are important both for allow growth of several microorganism groups both to manage the chemical equilibria in the medium. In fact methanogens and acid forming bacteria need different physiologic and nutrient requirements, moreover the sensitivity to pollutants changes on bacteria type basis (Pohland and Ghosh, 1971). Inhibition of a sole step of anaerobic digestion causes the failure of the whole process (Jain et al., 2015).

Temperature is a parameter that significantly affects the microbial community, consequently bacteria activity and kinetics. Three range could be defined: psicrophilic (<25°C), mesophilic (35-42°C) and thermophilic (49-57 °C). Thermophilic temperature improved process efficiency and increases pathogens reduction, but often it is more sensitive to perturbation and toxic compounds, has lower methanogenic diversity (Levén et al., 2007) and needs higher energy supply (Kim et al., 2002).

The temperature is not the sole operational condition that can affect the process, also organic loading rate (OLR) and the hydraulic retention time (HRT) have to be chosen with attention. Organic loading rate is the amount of organic matter (expressed as volatile solids or COD) that is fed per unit volume of the reactor per day. Generally the biogas production is related to ORL but too high organic load could inhibit the process due to accumulation of intermediate metabolites. HRT is the average time that substrate elapsed inside the reactor then longer HRT is associated with greater reactor volume. It depends on hydrolysis kinetic of fed substrates: slowly biodegradable substrates need longer HRT than easily biodegradable ones. On the other hand short HRT can wash out the microbial biomass affecting the whole process of biogas production (Boone et al., 1993).

Accumulation of volatile fatty acid is the first signal of process failure but it doesn't derive directly from substrate but from readily degradation of organic compound, microbial community unbalance or presence of inhibiting compounds.

Carbon to nitrogen ratio is one of the most important parameter, in fact high nitrogen content, such as in livestock effluents, causes high ammonium concentration in the reactor and consequently process inhibition (Weiland, 2010). Most toxic nitrogen form is free ammonia (NH_3), its concentration is proportional to total ammonium concentration, pH and temperature. Hence, thermophilic processes are more sensitive to ammonia disease. However, nitrogen is an essential nutrient for microorganism growth (Liu and Sung, 2002) then low content is beneficial for process. On the other hand high organic matter content without buffer capacity can cause VFA accumulation and pH decrease. Mixture of substrates balances nutrients ratio, dilutes toxicants and supplies missing nutrients, stabilizes the process and increases biogas yield (Angelidaki and Ellegaard, 2003).

Beside ammonia, other organic and inorganic compounds can inhibit anaerobic digestion. Sulfides derive from sulfate reduction and have two inhibiting mechanisms: i) sulfate reducing bacteria compete for organic compounds utilization therefore they suppress methane production (Harada, 1994), ii) H_2S is toxic because it diffuses into cell membrane and denatures native proteins interfering with enzymes activity (Colleran et al., 1995).

Waste activated sludge and industrial effluents can contain heavy metals including copper, zinc, nickel, chromium, iron, cobalt and cadmium (Peikang et al., 1998). Their effect depends on concentration and chemical forms (free form, precipitated as sulfide, hydroxides or carbonates, adsorbed to solid fraction, biomass or inert fraction, or metal complex) (Chen et al., 2008). Free forms bind to protein or substitute metals involved in enzyme structure, therefore they impede the correct enzymatic activity. As reported by Cecchi and Cavinato (2015), by the date the inhibiting values are not well defined both because they are expressed as total content and the real availability is not considered, both because the synergistic and antagonist effects can take place in the reactor. Many heavy metals are also micro-nutrients necessary for enzymes synthesis and their lack can limit some degradation pathways (Moestedt et al., 2016; Nordell et al., 2015). Toxicity of pollutants also depends on biomass concentration, environmental conditions, acclimation, and temperature (Chen et al., 2008). Anaerobic co-digestion limit heavy metals toxicity diluting the concentrated substrates and supply missing elements.

When residues from agricultural activity are fed to the digester, lignin derivatives can be present and inhibit methanogenesis. About them, polyphenols characterized winery wastes because they are synthesized by grapevine and are extracted during the wine-making process. Anaerobic microorganism are able to degrade most of the organic compound included polyphenols and they became toxic only at high concentration (Battista et al., 2014; Melamane et al., 2007). The removal efficiency depends on temperature, in fact the majority of the known phenol-degrading consortia and the isolated bacteria are mesophilic (Qiu et al., 2008); moreover some enzymes involved in the degradation of phenol to benzoate are temperature-sensitive and are denatured at 55 °C (Levén et al., 2012).

When anaerobic process works without stability problem, great amount of organic matter is transformed into biogas and a stabilized effluent with interesting agronomic characteristics is generated. Easily biodegradable organic constituents are mostly degraded, leading to an increase in the stability of the remaining organic matter and reduction of greenhouse gases emissions. The process is also able to partially remove toxic compounds reducing total environmental toxicity (Holm-Nielsen et al., 2009). Moreover, degradation of polymers causes nutrients leaching: organic nitrogen and phosphorus are released as ammonium and ortho-phosphate, easily up-taken chemical forms by crops. Finally, anaerobic treatment minimizes the survival of pathogens (Weiland, 2010) with efficiency varying in basis of type of substrate fed to the reactor, temperature range and hydraulic retention time (Sahlstrom, 2003). Hence, application of digestate on soil becomes an inexpensive way to limit the loss of organic matter typical of soils under agricultural exploitation (Pivato et al., 2016). Each European country regulates digestate application in terms of typology of input substrate, nutrients, metals and pathogens content. Recently the End-of-Waste criteria (Saveyn and Eder, 2014) compared the existing European threshold limits and proposed to harmonize them at European level (Cecchi and Cavinato, 2015). In order to correctly assess the agricultural quality of the digestate, also the residual phytotoxicity should be considered (Di Maria et al., 2014). Germination index is the most applied assay to evaluate toxicity because considers the synergistic effect of pollutants, in particular Alburquerque et al. (2012) reported that digestate that is not completely exhausted causes unfavorable impacts on the soil-plant system and limit its fertilizing potential (Abdullahi et al., 2008; Salminen and Rintala, 2002).

State of art of winery wastes anaerobic digestion

Anaerobic digestion it is already widely utilized for winery wastewater because of wastewater chemical-physical characteristics and low energy requirement (Ioannou et al., 2015; Lofrano and Meric, 2015; Moletta, 2005). Despite that, application of the same technology to semi-solid wastes are been taken in consideration recently because of presence of alternative uses. In fact the costs of the conventional technologies and wide diffusion of AD encouraged to look for alternative methods. Considering the high biodegradability of winery wastes and low levels of nitrogen and phosphorus, anaerobic digestion represents a particularly suitable technology for this type of substrates. Moreover, the process removes polyphenols, consumes labile organic matter reducing phytotoxicity and generates renewable energy.

Studies about AD of winery wastes were published mainly from 2003 (Table 4) but the information are partial and a deeper investigation is necessary. Most of the authors evaluated the potential methane production by mesophilic batch tests of different type of winery wastes (stalks, fresh and exhausted marc, seeds, lees and molasses), seldom applying pre-treatments (milling, separation of seeds, extraction). The results varied on basis of grape cultivar that originated the wastes, the wine-making process utilized, production region and their soil characteristics. Grape stalks have the lowest methane potential (0.098-0.180 m^3 CH_4/kgVS) due to their high lignin content that cannot degraded in anaerobic condition (Dinuccio et al., 2010). Marcs methane production ranges from 0.096 to 0.283 m^3 CH_4/kgVS and Failla and Restuccia (2014) demonstrated that seeds have a positive effect thank to their oil content and interesting potential (0.187 m^3 CH_4/kgVS) (Fabbri et al., 2015). Kinetic study of biogas production evidenced inhibition because of presence of phenol, alkyl-phenols and alcohols, compounds that require long time to be converted into VFA. Exhausted grape marc has minor sugar content because initial carbohydrates are transformed into alcohol and later subjected to distillation, therefore their methane potential is lower (0.089 m^3 CH_4/kgVS) (Danieli and Aldeovandi, 2011). Utilization of lees was less explored probably because of their commercial value, in fact ethyl alcohol and tartaric acid are recovered from them (Danieli and Aldeovandi, 2011). Methane production from lees change on basis of substrate origin and characteristics, in particular storage causes lose of volatile compounds: fresh lees reached 0.488 m^3 CH_4/kgVS while aged one had specific production of 0.367 m^3 CH_4/kgVS (Danieli and Aldeovandi, 2011; Lo and Liao, 1986).

Batch tests allow for determine potential biogas but the feasibility of the process has to be evaluated by continuous reactor. These type of process showed volatile fatty acid accumulation (Lo and Liao, 1986). The stability problems were limited by addition of a co-substrate such as livestock effluent (Lo and Liao, 1986; Riaño et al., 2011) and this approach allowed for improve methane production until 0.214 m^3 CH_4/kgCOD ((Riaño et al., 2011; Fountoulakis et al., 2008).

All the described data were obtained by batch tests under mesophilic condition, except for results reported by Fountoulakis et al. (2008) that operated at 55°C but obtained methane production comparable with mesophilic one. The same authors demonstrated that co-digestion in thermophilic temperature can increase methane production of 29% and 14% using olive mill wastewater and slaughterhouse wastewater as co-substrate, respectively.

Table 4 State of art of anaerobic digestion applied to winery wastes

	Co-substrate	Type of reactor	Temperature	Produzione specifica di biogas e metano			CH_4	Reference
Winery waste			°C	m^3biogas/kgTVS	m^3CH_4/kgTVS	m^3CH_4/kgCOD	%	
grape stalks	No	batch	40	0.225	0.098		44	Dinuccio et al., 2010
	No	batch	35		0.18			Gunaseelan, 2003
	No	batch	35-37	0.297	0.14		47	Fabbri et al., 2015
grape marc	No	batch	40	0.25	0.116		46	Dinuccio et al., 2010
	No	batch	35		0.283			Gunaseelan, 2003
	No	batch	40	0.120-0.159	0.096-0.128		80-81	Failla and Restuccia.,
	Yes	batch, attached biomass	35		0.15			Colussi et al., 2009
	Yes	batch	35			0.188-0.214		Fountolakis et al., 200
	Yes	batch	55			0.219-0.301		Fountolakis et al., 200
	No	CSTR	35			0.147	62-69	Fountolakis et al., 200
	Yes	CSTR	35-55			0.163-0.191	62-69	Fountolakis et al., 200
	No	batch	35-37	0.322-0.406	0.145-0.254		49-67	Fabbri et al., 2015
	Yes	batch	35		0.03			Makadia et al., 2016
milled grape marc	No	batch	40	0.104	0.124		84	Failla and Restuccia.,
grape marc without seeds	No	batch	40	0.093	0.076		82	Failla et Restuccia., 2
grape skin	No	batch	35-37	0.256-0.340	0.101-0.154		40-45	Fabbri et al., 2015
grape seed	No	batch	35-37	0.342	0.187		55	Fabbri et al., 2015
fresh lees	Yes	fixed-film reactor	35		1.048		65	Lo and Liao, 1986
	No	batch	36		0.488		60	Danieli and Aldeovan
aged lees	Yes	fixed-film reactor	35		0.367		64	Lo and Liao, 1986

	Yes	batch	35		0.08			Colussi et al., 2009
exhausted grape marc	No	batch	36		0.089		57	Danieli and Aldeovan
winery wastewater	Yes	batch	35			0.269	68	Riaño et al., 2011
winery wastewater	Yes	CSTR	35			0.087	59	Riaño et al., 2011
winery wastewater	Yes	batch	37					Rodriguez et al., 2007
molasses	Yes	CSTR	38	0.72	0.5		69	Soldano et al., 2009

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2. *Thesis Objectives*

Winery sector produced a great deal of organic wastes concentrated in space (Europe producea about the 60% of worldwide wine production) and time (few months around the harvest). These wastes have high biodegradability, consequently their direct disposal is environmentally hazardous. Literature review showed promising preliminary results about anaerobic digestion, a technology able to valorize winery wastes, but the evaluation of continuous process is missing and thermophilic range should be deeper investigates.

The overall research aim of the PhD project is to optimize the biogas production using winery wastes as main substrates considering mesophilic and thermophilic conditions. This purpose should be reached evaluating different operational conditions (organic loading rate and hydraulic retention time) in terms of process management, efficiencies and quality characteristics, and solving the critical situation observed during the experimentation.

The objectives of this study are:

- to evaluate thermophilic AD of winery by-products (grape marc and stalks, lees, waste activated sludge from winery waste treatment) by batch test in order to compare the methane potential with those from mesophilic process (§ 3.1).
- to investigate AD of the most abundant by-product, the grape marcs, alone and with other substrates by semi-continuous reactor(§ 3.1).
- to examine mesophilic and thermophilic AD considering three approaches to treat the by-product most available throughout the year, wine lees. The research focus on processes management and effluent characteristics.
    1. Local approach: centralize the winery waste treatment in the wastewater treatment plant using municipal waste activated sludge as co-substrate (§ 3.2);
    2. Wine sector-based approach: operate the anaerobic digestion inside the cellar borders using activated sludge from winery wastewater treatment as co-substrate (§ 3.3);
    3. Agricultural local approach: centralize the winery waste recovery in biogas plant treating livestock effluents, very diffused plant in Europe (§ 3.4).
- to maximize biogas production of biogas in mesophilic anaerobic digestion considering kinetic aspects and applying automatic feeding mode (§ 3.5).
- to understand the causes of winery wastes thermophilic AD failure with particular attention on trace elements requirement focus on process performances and stability (§ 3.6).
- to evaluate the feasibility of digestate spreading considering its chemical characteristics and phytotoxicity, the variation of digestate characteristics and plant sensibility. In order to estimate the

causes of germination stimulation/inhibition a deeper investigation of digestate characterizing chemical compounds should be kept in account (§ 3.7).

The thesis is organized in chapters that correspond to the investigated topics. The results are not organized in basis of the experimentation but instead considering the objectives of the thesis. Consequently, some experimentations were described considering different points of view. The Figure 3 summarized the topics of the project and showed the connection of the section of the thesis. In particular anaerobic digestion applied inside the cellar border was analyzed considering the different operational conditions (§ 3.3), the kinetics of mesophilic process (§ 3.5) and the role of trace elements in the thermophilic one, finally effluent from stable reactor operating at 37°C was evaluated in terms of phytotoxicity (§ 3.7).

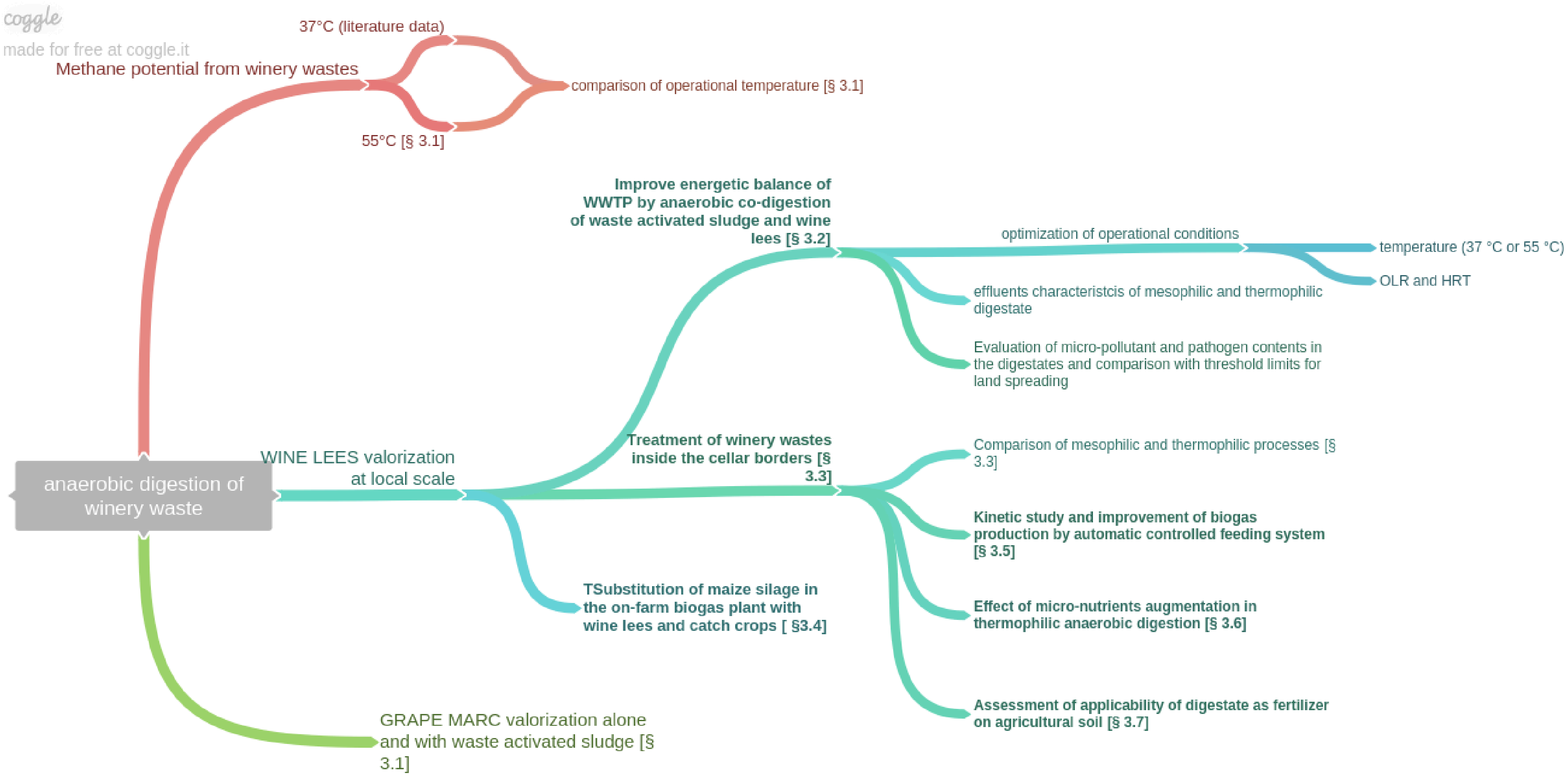

Figure 3 Scheme of topics/objective of the thesis

3. Results and discussion

3.1. Overview of methane production from thermophilic anaerobic digestion of winery wastes

Literature review revealed that there is a lack of knowledge about thermophilic anaerobic co-digestion of winery wastes. This section describes the first step in the investigation of the process working under thermophilic condition and considered the main residues from wine-making process (grape marcs, grape stalks, wine lees and waste activated sludge from winery wastewater). The section contains the research paper " Renewable energy from thermophilic anaerobic digestion of winery residue: Preliminary evidence from batch and continuous lab-scale trials" that derived from collaboration of researcher and professors. PhD Cavinato and professor Pavan helped me to develop the experimental design. The data were collected and elaborated by myself. All the co-authors co-worked in the paper writing as experts in the field.

The paper reports result by batch tests carried out using all different winery wastes and semi-continuous trials with grape marc alone and with a co-substrate. The BMP tests were not carried out until methane production was negligible. In fact utilization of kinetic models could forward potential data considering the behavior of the curve in the first 30 days. The Semi-continuous tests were carried out with grape marc, the winery by-product most abundant. In order to evaluate different operational conditions (HRT, OLR, flow rate), tap water was used to dilute the substrate. Dilution with clean water is not feasible at full-scale but use of wastewater would introduce a variable difficult to assess. The results obtained using CSTR reactor didn't consider the process in the long period but until the achievement of steady biogas production. Moreover, it supply some interesting data about the renewable energy that could be recovered from these substrates.

Research paper

Renewable energy from thermophilic anaerobic digestion of winery residue: Preliminary evidence from batch and continuous lab-scale trials

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Keywords: Anaerobic digestion; Winery waste; Renewable energy; BMP test; Grape marcs

Abstract. The wine-making process generates many by-products besides wastewater, mainly grape marcs, grape stalks, and wine lees. Anaerobic digestion is particularly suitable to treat winery waste because of its high content of nutrient-rich organic matter and for its noticeable energetic potential. To date, only results from mesophilic tests have been extensively reported. In this study, potential methane production and kinetic constants were determined by batch trials under thermophilic conditions and compared with mesophilic values already reported in literature. Grape marcs and wine lees appeared to be the most promising substrates with an estimated potential of 0.34 and 0.37 $Nm^3CH_4/kgVS_{fed}$, respectively, while grape stalks generated only 0.13 $Nm^3CH_4/kgVS_{fed}$. In order to assess the feasibility of a continuous anaerobic digestion process, a lab-scale semi-continuous reactor was constructed. Because of the consumption of buffer capacity, the biological process was difficult to control. On the other hand, biogas was produced when working with a hydraulic retention time of 40 d and with previously fermented grape marcs; a specific biogas production of 0.29 Nm^3/kgVSfed was observed. The results of the continuous tests were used to calculate the potential energy recovery from grape marcs produced in Italy (808 thousands of tonnes per year) in terms of heat and electricity; about 245 GWh of heat and 201 GWh of electricity per annum could be generated in Italian scenario.

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1. Introduction

The International Organization of Vine and Wine (OIV) estimated a global wine production of 278.6 Mhl in 2013 (OIV, 2014). Italy, France, and Spain had the highest production (131.6 Mhl combined), and together with other European countries accounted for about 59% of world production.

In Europe, wineries are steeped in tradition and have a high economic value to the agricultural sector. At the same time, through the processes of wine production, they also have a considerable environmental footprint, including intensive use of soil, introduction of pesticides and heavy metals, significant water consumption, and production of high quantities of by-products and waste (Bolzonella and Rosso, 2009).

In the early 2000s, the Italian Agency for Environmental Protection (ANPA, 2000) evaluated the amount of winery waste (WW) for each liter of wine produced, considering a variety of cellars in terms of dimensions and location. The wastewater generated is typically 2 L for each liter of wine produced, but it depends on the technology used and can go as high as 6 L per liter of wine (ANPA, 2000).

Grape stalks (GS) and marcs (GM), wine lees (WL), exhausted grape marcs, and vinasses are the main by-products generated by the wine and alcohol industries (Bustamante et al., 2007; Oliveira et al., 2014). The production of solid by-products and waste have been estimated to be 40 kg of grape stalks, 180 kg of grape marcs, and 60 kg of WL per m^3 of wine produced (Bustamante et al., 2007; Oliveira et al., 2014). The European Council Regulation (EC) 1493/1999 (European Council, 1999) on the common organization of the wine market introduced compulsory distillation of wine by-products, including GM and WL, to produce spirits, alcohol, tartaric acid, exhausted grape marcs, and liquid waste (vinasses). Recently, market-protective legislation (European Council, 2008a, 2008b) has allowed for alternative uses of grape marcs in wineries (Krzywoszynska, 2012) and has granted more autonomy for marcs management strategies. In Italy, since 2011 (MIPAAF, 2008), marcs could be distilled or otherwise utilized by producers (under the supervision of environmental protection agencies) including anaerobic digestion, pyrolysis or incineration, spreading on agricultural land (raw or composted) according to local legal frameworks, or as a raw material for cosmetics and pharmaceuticals production. Alternative technologies to reuse winery waste include the extraction and recovery of polyphenolic compounds, the production of grape seed oil, and fermentation to produce lactic acid and laccase enzymes (Arvanitoyannis, 2010).

The use of raw, solid winery waste for agricultural purposes can be detrimental because of the oxygen consumption in the soil determined by the high organic content of these wastes. Moraes et al. (2014) have also suggested the following possible impacts: potential greenhouse gas (GHG) emissions resulting from organic matter degradation in soil, generation of unpleasant odors, and attraction of harmful insects. The presence of polyphenols, compounds exhibiting phytotoxic and antimicrobial effects, and low pH due to a high concentration of organic acids such as tartaric, citric, and malic acids, make the treatment and disposal of the waste indispensable and its management highly difficult. Biological treatment increases the biological stability and improves the chemical oxygen demand: nitrogen: phosphorous (COD:N:P) ratio, which makes it an attractive solution to the environmental problem. Among available technologies, composting of winery solid organic waste can produce a good quality fertilizer (Bertran et al., 2004), but the cost of energy required for pile handling and venting must be taken into account (Bonifazi et al., 2013). Moreover, winery waste has unbalanced nutrient ratios that make it unsuitable for aerobic degradation (low nitrogen and phosphorus contents).

Anaerobic digestion (AD) is a particularly suitable biological treatment that stabilizes winery waste while producing biogas (Moletta, 2015), even with low levels of nitrogen and phosphorus. This process can also remove organic pollutants such as polyphenols. AD is considered an important future contributor to the energy supply of Europe (Appels et al., 2008) and WW represents a consistent substrate for this technological application. The appropriate use of biogas for energy generation may also reduce GHG emissions as it would avoid the release of biogenic methane (CH_4) into the atmosphere (Moraes et al., 2014).

Semi-dry and dry technologies have been the preferred in treating semi-solid waste, such as grape marcs, grape stalks, and wine lees. Although Luning et al. (2003) have demonstrated the similarity in performances of the two configuration processes, the use of wet completed stirred tank reactors (CSTR), a more diffused technology, guarantees better mixing and performances. Many examples of AD of WW have been reported in literature, but all trials were conducted in mesophilic conditions, with temperatures ranging from 35 C to 42 C. In most cases, the batch mode was applied and the potential methane production under best operational conditions was determined. There was a wide range in results due to several factors, such as inoculum characteristics and the substrate to inoculum ratio (VS basis). These parameters affect the microorganisms' acclimation to new substrates (Rebecchi et al., 2013) and, in fact, winery waste can contain substances such as lignin, ethanol, and polyphenols. The variability of substrate characteristics also caused different biogas productions (Da Ros et al., 2014; Lo and Liao, 1986). In particular, the type of grape, its origin, and the wine-making technologies generating winery waste were the main factors affecting substrate variability. For example, Fabbri et al. (2015) have reported that the best bio-methane potential was achieved using a white grape marc (0.273 $m^3CH_4/kgVS_{fed}$), while the red grape marc reached only 0.101 $m^3CH_4/kgVS_{fed}$. Anaerobic digestion of GM has been the more studied process, and the specific production has ranged from 0.076 to 0.283 $m^3CH_4/kgVS_{fed}$ (Failla et al., 2009; Dinuccio et al., 2010; Gunaseelan, 2004). Specific methane production from grape stalks (0.098-0.180 $m^3CH_4/kgVS_{fed}$) is lower than other agro-industrial biomasses, most likely due to a high lignin content (23.3% of solids), which cannot be degraded in anaerobic conditions (Angelidaki and. Ahring, 2000; Ward et al., 2008). The lignin also affects the availability of hemicellulose and cellulose because it preserves them from most chemical and biological attacks. Few researchers have studied WL, although the potential methane is very high in terms of biogas produced and methane content (Da Ros et al., 2014; Lo and Liao, 1986), probably because of the low quantity of WL that could be recovered (60 kg per m^3 of wine produced). Moreover, there are many types of lees depending on the kind of fining agent, such as bentonite, gelatine, albumin, or activated carbon, that was added to settle suspended solid and colloidal particles. Sometimes wineries recover must or wine in the lees by centrifugation or filtration through a filter consisting of diatomaceous earth or perlite (Setti et al., 2009). In such cases, lees at the end of the recovery operation are composed of the filter medium as well as the lees, thus the final lees have a high concentration of inert material.

Generally, the greatest difficulty in AD of WW was controlling pH in the reactor; in fact, the abundance of organic acids caused the rapid formation of volatile fatty acids (VFA) and the drop in pH (Lo and Liao, 1986). Limited buffer capacity was partially due to a low nitrogen content in substrates and it was usually supplied by adding sodium hydroxide (NaOH) or lime solutions (Sheli et al., 2014; Melamane et al., 2007), but the use of chemicals reduces the economic revenue potential of the final product. An alternative to increase the ammonium content and alkalinity in the anaerobic reactors is to introduce organic waste from other agro-industrial sectors. Anaerobic co-digestion (AcoD) with manure or waste activated sludge allows for a more favorable C/N ratio, and consequently the biogas production and removal efficiency increase (Riaño et al., 2011; Rodríguez et al., 2007; Soldano et al., 2009).

The results of cited studies were mainly obtained through a mesophilic process, while a thermophilic condition was rarely applied (Rebecchi et al., 2013; Da Ros et al., 2014). Increasing the temperature up to 55 °C offers benefits including an improved solubility of the organic compounds and pathogen depletion (Sahlstrom et al., 2004) and enhanced biological and chemical reaction rates (Toscano et al., 2013; Bustamante et al., 2008). However, the application of high temperatures also has some disadvantages, including a greater presence of free ammonia, which plays an inhibiting role for the microorganisms, and a change in the pK_a of the VFAs. Both of these temperature effects make the process more susceptible to inhibition (Appels et al., 2008). In both of the studies on thermophilic anaerobic digestion of winery waste, process instability was observed with VFAs accumulation and pH drop. In particular, Rebecchi et al. (2013) found results lower than under mesophilic conditions, and a continuous process was not investigated. On the other hand, some studies (Da Ros et al., 2014; Riaño et al., 2011) have described trials carried out using a co-substrate and hence did not evaluate potential biogas production of a single winery waste under optimal conditions.

The aim of this work was to evaluate the performance of thermophilic anaerobic digestion of winery solid waste, both in batch and continuous modes. Batch tests were carried out, according to guidelines established by Angelidaki et al. (2009), using varied wastes, and preliminary results on biogas production were compared with mesophilic methane production and limited existing data at 55°C. Empirical regression models were developed to evaluate digestion performance and to compare mesophilic and thermophilic processes in terms of kinetics parameters. However, as it is well established that biogas yields under semi-continuous conditions can vary from those observed under batch conditions, lab-scale semi-continuous trials were carried out to determine the best operational conditions and to highlight shortcomings in treating grape marcs. Economic and energy aspects were then evaluated considering a potential full-scale application of the technology.

2. Materials and methods

2.1. Winery waste

The winery by-products considered in this study were GM, GS, and WL. All samples were collected from a cellar in northeast Italy. The stalks were removed from crushed grape, just before the fermentation, by the process called destalking (Toscano et al., 2013). Two types of GM were considered, fresh grape marc and naturally fermented grape marc. Fresh GM was derived from white grapes while fermented GM came from red ones. Red GMs were separated from wine during alcoholic fermentation and stored in a closed tank for three months at room temperature; yeast present on marcs surface hydrolyzed organic matter and transformed sugars into ethanol. Wine lees derived from wine clarification before the bottling using bentonite: fine clay that allows for aggregate and settle the suspended solids in the raw wine. All samples were labeled and stored in a freezer at 20°C until needed. These substrates were analyzed at least in triplicate for total and volatile solids, chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), and total phosphorus, according to the Standard Methods (1999).

2.2. BMP tests

The biogas production was determined using biochemical methane potential (BMP) tests at 55 C according to the guidelines provided by Angelidaki et al. (2009). The sludge used as inoculum originated from a mesophilic full-scale digester co-treating the organic fraction of municipal solid waste and waste activated sludge. This sludge was then acclimatized at 55 °C for a prolonged period and was used as inoculum for BMP tests. Inoculum had an average solid concentration of 43 gTS/kg_{ww}, with 67% volatile solids. The particulate COD was 928 mg/g_{dw}, while nitrogen and phosphorus were present at 16.1 and 7.8 mg/g_{dw}, respectively. BMP tests with substrates and with inoculum alone (blanks) were conducted. The volume of produced biogas was determined by water displacement and the composition by a gas chromatograph equipped with an HP-Molesieve column (30 m × 0.3 mm × 0.25 mm film thickness) and a thermal conductivity detector (TCD). Argon was used as carrier gas and the operating temperature of oven was 40 °C. The results were elaborated and expressed at standard temperature and pressure (0 °C and 1 atm), thus the unit of specific methane production (SMP) was $Nm^3CH_4/kgVS_{fed}$. The average methane yield from the blanks was subtracted from the yield of tested samples to accurately assess the BMP yields.

The COD content of winery waste and the theoretical methane production on a COD basis (0.350 $Nm^3CH_4/kgCOD_{fed}$ by stoichiometric conversion reaction) were used to calculate the theoretical maximum methane potential from each substrate, and the biodegradability index was calculated considering the ratio between the measured and the theoretical methane production (Eq. (1)). Here COD was considered completely available for degradation and generation of new biomass was not accounted for

$$Biodegradability\ index = BMP_{measured} / (0.350 \times COD_{fed}) \qquad (1)$$

Two kinetic models were used to fit the cumulative methane production data from the BMP tests. Assuming first-order kinetics for the hydrolysis of particulate organic matter, the cumulative methane production can be described by the kinetic Eq.(2):

$$BMP= BMP_{\infty} (1 - exp (-kt)) \quad (2)$$

where BMP is the cumulative methane yield at digestion time *t* days (mL CH_4/gVS_{fed}), BMP_{∞} is the methane potential of the substrate (mL CH_4/gVS_{fed}), *k* is the methane production rate constant (first order disintegration rate constant) (d^{-1}), and *t* is the time (d). The duration of the lag phase is also an important factor in determining the efficiency of anaerobic digestion. The lag phase can be calculated with the modified Gompertz model as described in Eq. (3):

$$BMP = BMP_{\infty} exp \{-exp [(R_{max} exp/ BMP_{\infty}) (\lambda - t) + 1]\} \quad (3)$$

where R_{max} is the maximum methane production rate (L $CH_4/kgVS_{fed}$ d), e = 2.7183, and λ is the lag phase until methane production begins (d).

Following a suggestion by Browne et al. (2014), a nonlinear least-square regression analysis was performed using Excel™ to determine the equations parameters (k, R_{max}, λ) and the predicted methane yield. The predicted methane yield obtained from the regression analysis was plotted with the measured methane yield. The statistical indicators, correlation coefficient (R^2), and root mean square error (RMSE) were calculated to assess the goodness-of-fit and to evaluate the best kinetic equation to describe WW biogas production.

2.3. Continuous AD trials

The BMP tests estimated potential methane production while semi-continuous tests allowed an overall evaluation of the feasibility of the process. Continuous trials were carried out in a lab-scale CSTR. The reactor had 5 L of working volume and temperature was maintained at 55 °C in a controlled oven. The system was inoculated with the same mesophilic digestate sludge used in BMP tests. The reactor temperature was then increased in one single step, transforming in a brief period from mesophilic to thermophilic conditions as suggested by Cecchi et al. (1993). Biogas flow was measured using a MilliGascounter® (Ritter) and the gas was collected in a Tedlar gas bag for composition (CH_4, CO_2) analysis as reported in paragraph 2.2.

The GMs were used as the main substrate because of availability and BMP results. The reactor was fed once a day with substrate and tap water to reach the correct solid content and flow rate to enable a comparison of results from continuous trials with literature (Fountoulakis et al., 2008; Colussi et al., 2009). Clearly, in a full-scale application, winery wastewater and wine lees would be used instead of freshwater to dilute substrates. Different operational conditions were tested in terms of feeding substrate, hydraulic retention time (HRT), and organic loading rate (OLR), dividing the tests into four RUNs, as outlined in Table 1. RUN1 and RUN2 operated with the same HRT and C/N ratio, of 53, the ORL tested were 1 and 2 $kgVS/m^3d$, respectively. On the basis of the results of the first RUNs, two different conditions were then tested to improve the process stability and biogas production while reducing tap water utilization: the co-treatment of GM with another substrate (RUN3) and application of longer HRT (RUN4) were tested. RUN3 operated with an HRT of 20 d, the same than previous RUNs, but the organic load increased to 4 $kgVS/m^3d$ because water was substituted by waste activated sludge. The feed was composed of 66% fresh GM (in terms of volatile solids) and the rest derived from sludge. This trial was an example of water substitution with diluted substrates, which increases the process sustainability due to higher alkalinity in the feeding mixture and C/N decreases to 40, moreover it reduces management costs. Another method to reduce water consumption is a reduction in the flow rate by application of a longer HRT, so in the last trials (RUN4) an HRT of 40 d was applied. This operational condition and utilization of pre-treated GM caused an C/N of 68, improved the hydrolysis rate and ammonification, but the biogas production should be considered comparable in basis of BMP tests.

Table 1. Operational conditions of semi-continuous trials

	Feed	Organic loading rate ($kgVS/m^3d$)	HRT (d)
RUN1	Fresh GM	1	20
RUN 2	Fresh GM	2	20
RUN 3	Fresh GM + Activated Sludge	4	20
RUN 4	Fermented GM	1	40

Partial and total alkalinity (PA and TA), pH, VFA, and ammonium nitrogen were measured twice a week to monitor process stability, while characteristics of the effluent in terms of total and volatile solids, COD, TKN, and total phosphorus (P_{tot}) were analyzed weekly. All analyses, except for VFA, were carried out in accordance with the Standard Methods (1999). Volatile fatty acids content was monitored using a gas chromatograph (Carlo Erba Instruments) with hydrogen as the gas carrier, equipped with a Fused Silica Capillary Column (Supelco NUKOL™, 15 m 15 m × 0.53 mm × 0.5 μm film thickness) and a flame ionization detector (200 °C). The temperature during the analysis started at 80 °C and reached 200 °C through increments of 140 °C and 160 °C, with a rate of 10 °C per minute. The analyzed samples were centrifuged and filtrated on a 0.45 μm membrane.

3. Results and discussion

3.1. Characterization of winery waste samples

The characteristics of substrates tested in trials are reported in Table 2. The results reflected typical characteristics of WW: total solids ranging from 227 to 451 gTS/kg_{ww}, of which about 90% were volatile solids. Fresh and fermented GMs were comparable in terms of volatile to total solids ratio, COD and nitrogen content although they derived from different cultivars. WL showed a high content of organic matter but had a relatively low volatile solids content because during the wine-making process, bentonite, an inert material, was added to remove suspended solids and colloidal particles. A remarkable difference among the substrates is the organic matter composition, in fact GM and GS has COD concentration higher than 1000 mg/kg, but part of the organic matter was lignin and cellulose (Dinuccio et al., 2010), compounds difficult to degrade. On the other hand a major part of COD in WL was soluble: organic acids, sugars and alcohol, all readily biodegradable compounds, were the main components. Nitrogen and phosphorus content changed according to the different substrates considered (Table 2). In general the GS showed the lowest nutrients concentration (as reported by Bustamante et al. (2008)) while WL has the highest likely due to extraction during the wine-making process. All the considered substrates had COD:N:P ratio limiting for the biological process, in fact the ratio ranged from 196:4:1 to 697:5:1.

During the experimentation, waste activated sludge (WAS) that originated from wastewater treatment plant internal to the cellar and dedicated to the treatment of winery wastewater was also used. The most significant operational conditions of the wastewater treatment plant were a solid retention time of 15 d and a food-to-microorganism ratio of 0.15 kgCOD/kgMLVSS per day. The WAS contained 45.4 gTS/kg_{ww} with 60% volatile solids. The concentrations of COD, TKN, and P_{tot} were 921 mgCOD/g_{dw}, 31.4 mgN/g_{dw}, and 7.0 mgP/g_{dw}, respectively, corresponding to a COD:N:P ratio of 132:4:1 on a dry mass basis.

Table 2. Chemical-Physical characteristics of analyzed winery wastes

Parameters	Unit	GS	Fresh GM	Fermented GM	WL
TS	g/kg_{ww}	227± 41	371 ± 51	451 ± 21	62 ±28
VS	g/kg_{ww}	202 ± 55	334 ± 30	425 ± 38	34 ±13
COD	mg O_2/ kg_{ww}	1306 ± 30	1078 ± 32	1138 ± 68	268,000 ± 50,000
TKN	mgN/g_{dw}	6.1 ± 1.0	20.4 ± 0.8	16.8 ± 4.5	30.3 ± 12.1
P_{tot}	mgP/g_{dw}	3.6 ± 1.8	5.5 ± 4.1	1.2 ± 0.3	6.2 ± 2.6

3.2. Methane production in batch trials

The specific methane production (SMP) values were measured for each substrate (WL, GS, fresh GM, and fermented GM) using the BMP tests in thermophilic conditions. Figure 1 shows the cumulative average SMP from the batch experiments and the corresponding 90% confidence interval. Tests were carried out for more than 30 d, which was adequate time to convert the organic matter under anaerobic conditions, and, in fact, within this time frame the daily variation of methane was lower than 5% of the cumulative production. Specific methane production of GS was 0.133 ± 0.003 m^3/$kgVS_{fed}$. This value differed greatly from the theoretical methane production calculated on COD concentration (0.514 m^3/$kgVS_{fed}$); only 26% of organic matter was converted into methane. The low biodegradability index (Table 3) was validated by data reported by Dinuccio et al. (2010); a high content of lignin (23% TS), which was not biodegradable in anaerobic conditions, was present. Moreover, cellulose and hemicellulose (15.9% TS and 23.5% TS, respectively), which were partially degraded, were the main constituents of GS. Significant differences were not observed between fresh and fermented GM. The average SMP of fresh GM was 0.347 ± 0.012 Nm^3/$kgVS_{fed}$, with 82% of COD conversion into methane. The production value was higher than values reported in literature (Table 3). The methane production from fermented GM reached 0.360 ± 0.018 Nm^3/$kgVS_{fed}$, corresponding to an equal biodegradability index of fresh GM The natural fermentation of GM was a pre-treatment that allowed for the acquisition of rapidly biodegradable compounds, such as ethanol, from complex molecules but can't significantly increase the biodegradability. The lag phase observed during this test could be due to the presence of a high content of ethanol or polyphenols that caused methanogenic inhibition at the beginning of the trial. The WL had SMP reaching 0.370 ± 0.014 $Nm^3/kgVS_{fed}$ and conversion efficiency was significant (80%) due to high concentrations of soluble COD.

Biogas production of winery waste reported in literature has shown a wide variability in terms of specific methane production (Table 3) likely due to different operational conditions (Raposo et al., 2011). In particular, methane production from GS differed significantly from values obtained by marcs and lees. As well as considering the single type of substrate, the methane production at mesophilic temperatures differed in the literature: SMP of GM at mesophilic temperatures ranged from 0.096 to 0.283 $m^3/kgVS_{fed}$ while SMP of GS ranged from 0.098 to 0.180 $m^3/kgVS_{fed}$ (Table 3). Cause of the presence of contradictory SMP values a comparison of mesophilic and thermophilic potential proved difficult, and in order to compare the yields, the following average values were determined: 0.196 $m^3/kgVS_{fed}$ and 0.139 $m^3/kgVS_{fed}$ for GM and GS, respectively.

Table 3. Comparison of biogas and methane production reported in literature and in this study (* indicates value obtained by continuous process, bolt data refer to thermophilic process)

Substrate	Temperature	Specific production			CH_4	References
	°C	m^3biogas/ $kgVS_{fed}$	m^3CH_4/ $kgVS_{fed}$	m^3CH_4/ $kgCOD_{fed}$	%	
GS	35-37	0.297	0.140			Fabbri et al., 2015
	40	0.225	0.098		44	Dinuccio et al., 2010
	35		0.180			Gunaseelan, 2004
	55		**0.133**			**This study**
GM	35-37	0.406	0.273		67.32	Fabbri et al., 2015
	35-37	0.322	0.157		48.71	Fabbri et al., 2015
	40	0.120-0.159	0.096-0.128		80-81	Failla et al., 2009
	40	0.250	0.116		46	Dinuccio et al., 2010
	35		0.283			Gunaseelan, 2004
	35		0.15			Colussi et al. 2009*
	35			0.147		Fountoulakis et al., 2008
	55	**0.39**	**0.04**			**Rebecchi et al., 2013**
	55		**0.347-0.360**			**This study**
WL	35		0.367-1.048			Lo and Liao, 1986
	36		0.488		60	Danieli and Aldeovandi, 2011

55	**0.370**	**This study**

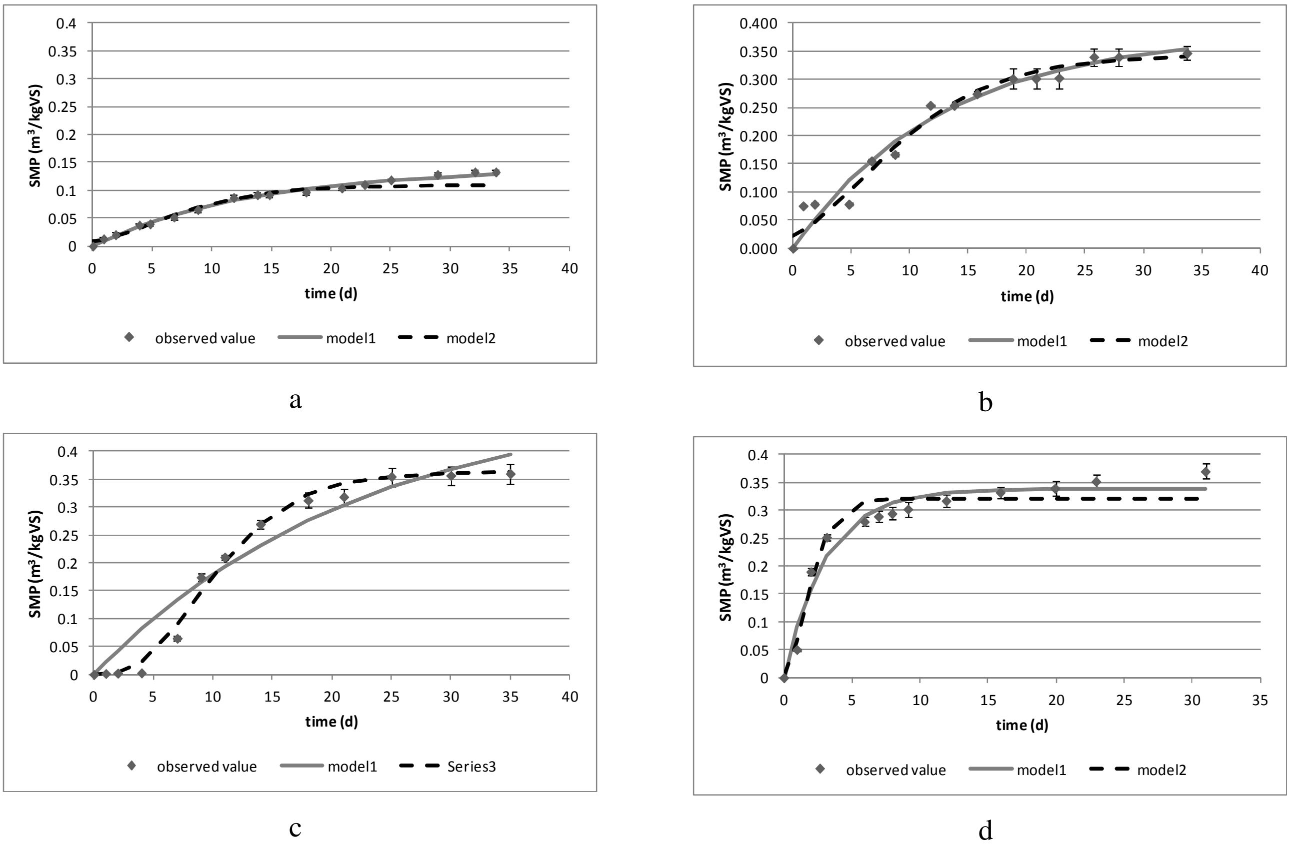
Methane potential observed for GS in thermophilic conditions was consistent with mesophilic conditions; a temperature increase did not show an improvement in degradation of recalcitrant substrates. The biogas potential observed for WL fell within the range of values reported in literature (Lo and Liao, 1986, Danieli and Aldeovandi, 2011), but characteristics of this type of substrate strongly depend on wine-making activity and comparison is difficult. Results of thermophilic digestion trials for GM (Rebecchi et al., 2013) reported in literature were discouraging: 0.04 m^3CH_4/ $kgVS_{fed}$. However, in this study we determined methane yields greater than average values reported for mesophilic trials (Table 3).

Both types of marcs were characterized by methane production higher than 0.3 $m^3CH_4/kgVS_{fed}$, and considering a confidence interval of 90%, the values did not significantly differ. In tests fed with fermented GM, a lag phase was observed, most likely due to the presence of ethanol, a compound easily degradable after acclimation of microorganisms.

Overall, the thermophilic AD appeared more promising for the treatment of GM, both fresh and fermented.

3.3. Kinetic study results

The results of the application of the first order model and the modified Gompertz model are represented in Figure 1 and kinetics parameters are summarized in Table 4.



a

b

c

d

Figure 1. Cumulative specific methane production of grape stalks (a), fresh and fermented GM (b and c), and WL (d), at thermophilic temperatures. Continuous lines indicate first order model predictions (model 1), while dotted lines indicate modified Gompertz model predictions (model 2). Error bars express a 90% confidence interval.

The first order model resulted in similar values for the hydrolysis constant, *k*, for both GS and GM, while WL were characterized by a hydrolysis constant of 0.326 d^{-1}. The fermentation of GM was affected by an initial lag phase during which microorganisms acclimated to the substrates. This aspect is more easily described by the modified Gompertz model using the lag phase parameter (λ), as reported in Table 4. Both models exhibited a good fit when plotted against the measured data, with correlation coefficient (R^2) ranging from 0.97 to 1.00 for the first order model and from 0.98 to 1.00 for the Gompertz model (data not shown). A correlation equal to 1 was derived from an approximation of error lower than 1%. The RMSE ranged from 4 to 37 ml CH_4/gVS_{fed} for the first order model while the Gompertz model resulted in lower values, ranging from 3 to 24 ml CH_4/gVS_{fed}. The RMSE demonstrated that the modified Gompertz model generally fit better than the first order model for the trials characterized by a lag phase. The Gompertz model gave a more accurate predicted maximum methane yield than the first order equation, especially when lag time was observed. On the other hand, the model best describing the production of GS was the first order kinetic model. The modified Gompertz model resulted in slightly lower predicted BMP yields than the measured data, ranging from 5% to 19%, while the first order model generally resulted in higher predicted methane yields.

Few authors (Fabbri et al., 2015; Gunaseelan, 2004) investigated the kinetics of winery wastes degradation. Looking at grape stalks, both Fabbri et al. (2015) and Gunaseelan (2004) reported an absence of a lag phase and similar methane production; however kinetic constants were different, in fact, they reported hydrolysis rates of 0.104 and 0.016 d^{-1}, respectively. In this study, the thermophilic digestion of the lignocellulosic substrate demonstrated a similar behavior than in mesophilic digestion and the differences observed were likely due to inoculum and operational conditions applied.

Table 4. Results of the kinetics analysis using the first order kinetic model and the modified Gompertz model. [a] Error less than 1%.

	Unit	**GS**	**Fresh GM**	**Fermented GM**	**WL**
Substrate					
BMP	$m^3CH_4/kgVS_{fed}$	0.133±0.003	0.347±0.012	0.360±0.018	0.370±0.014
Biodegradabilty index	%	26	82	82	80
First order kinetic model					
BMP predicted	$m^3CH_4/kgVS_{fed}$	0.128	0.354	0.394	0.338
Δ BMP	%	-4%	+2%	+13%	9%
k	d^{-1}	0.074	0.078	0.045	0.326
RMSE	$m^3CH_4/kgVS_{fed}$	0.004	0.021	0.037	0.022
R^2		1.00*	0.98	0.97	0.98
Modified Gompertz kinetic model					

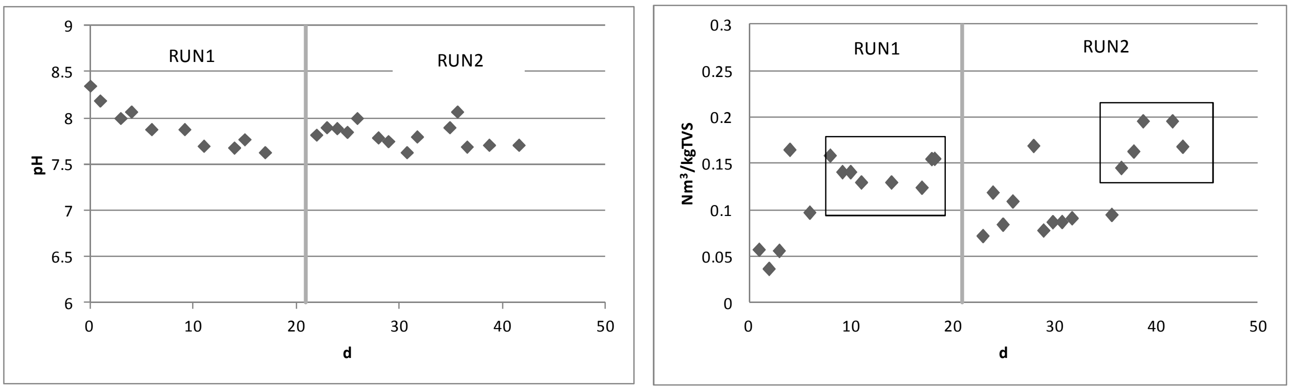
BMP predicted	m^{3}CH$_4$/kgVS$_{fed}$	0.108	0.343	0.363	0.321
Δ BMP	%	-19%	-1%	+5%	-13%
λ	d	-	-	4.091	0.401
R$_{max}$	m^{3}CH$_4$/kgVS$_{fed}$d	0.008	0.019	0.030	0.110
RMSE	m^{3}CH$_4$/kgVS$_{fed}$	0.003	0.021	0.014	0.024
R^2		0.99	0.99	1.00^a	0.98

GM showed a broader range in methane potential production both in mesophilic and thermophilic studies, and also the kinetics parameters varied. The application of first order model for mesophilic test determined lower hydrolysis constants than in thermophilic one. In particular Gunaseelan et al. (2004) did not detect lag-phase and the kinetic constant was 0.091 d^{-1}, significantly lower than at 55 °C. Fabbri et al. (2015) applied the same models to GM digestion performance and observed lag phases due to the presence of phenol, alkyl-phenols, and alcohols, which are common in winery waste. Consequently, the best fitting results were obtained by application of the Gompertz model. When a long lag phase (5.5 d) was detected later the methane production occurred with slow rate (19 ml /kgVS$_{fed}$), while absence of inhibition was associated with maximum rate of 33 ml /kgVS$_{fed}$. Conversely, in this study, the maximum methane production rate (30 ml /kgVS$_{fed}$) was observed in tests characterized by the presence of an initial lag phase, indicating that the slow initiation of the methanogenic process did not affect methane production.

Based on these considerations, thermophilic digestion in a batch reactor appeared quite similar to mesophilic digestion, and no inhibition effects or metabolite accumulation was observed.

3.4. Biogas production in lab-scale CSTR

GM appeared to be the most promising by-product of the wine-making process in terms of biogas production. Hence, semi-continuous tests were carried out using this substrate to feed the continuous reactor to evaluate the long term stability of the biological process. Two trials were conducted using fresh GM as unique substrates, testing an OLR of 1 and 2 kgVS/m^{3}d and an HRT of 20 d, respectively (RUN1 and RUN2). Considering the low OLR applied, the process did not require a real start-up and acclimatization period. By the end of the first HRT, the system had reached a steady methane production. The biogas production was 0.114 and 0.145 Nm^3/kgVS$_{fed}$ in RUN1 and RUN2, respectively, with a 60% methane content in both cases. Based on the mass balance around the system it was found that the volatile solid removal rates were 12% and 15%, respectively, for an OLR of 1 and 2 kgVS/m^{3}d. Both partial and total alkalinity decreased during experimentation, indicating a net reduction of system buffer capacity. This reduction could result in biological process failure although the pH value was above 7.5 (Figure 2a). The low level of VFAs (lower than 100 mgCOD/L), the satisfactory percentage of methane, and the stability parameters suggested that methanogenesis was not inhibited and that hydrolysis was the limiting step in the process. The low hydrolysis rate was most likely due to an unbalanced substrate COD:N:P ratio that limited the biomass growth and, consequently, its activity.

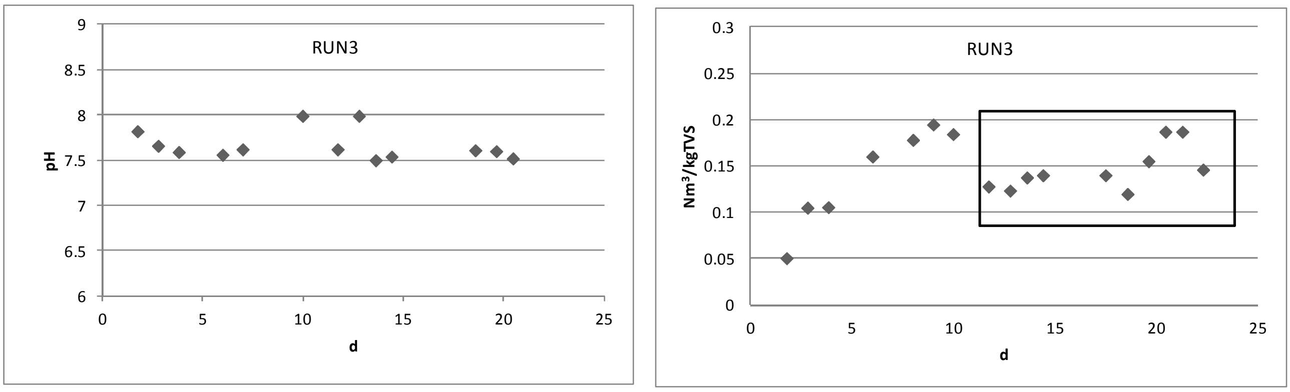


a) b)

Figure 2. Trends of pH a) and SGP b), at OLR 1 kgVS/m3d (RUN1) and 2 kgVS/m3d (RUN2)

Considering the low biogas yields in RUN2, the test was suspended and waste activated sludge from winery wastewater treatment (largely available at wine making facilities (Brucculeri et al., 2005) was added as a co-substrate (RUN3) to increase the buffer capacity of the system. The feed was composed of 66% fresh GM (on a volatile solids basis) and the remaining percentage was derived from sludge. The total OLR applied was 4 kgVS/m^{3}d. The addition of a co-substrate had the following effects on the process: it supplied missing nutrients, improved the stability of the process, and increased the active microbial population in the reactor. Moreover, the addition of this diluted substrate allowed for a reduced water requirement.

As illustrated in Figure 3b, the average biogas production was 0.146 Nm^3/kgVS$_{fed}$, with 50% methane. On a mass balance basis, the volatile solids removed by biogas conversion corresponded to 20%.

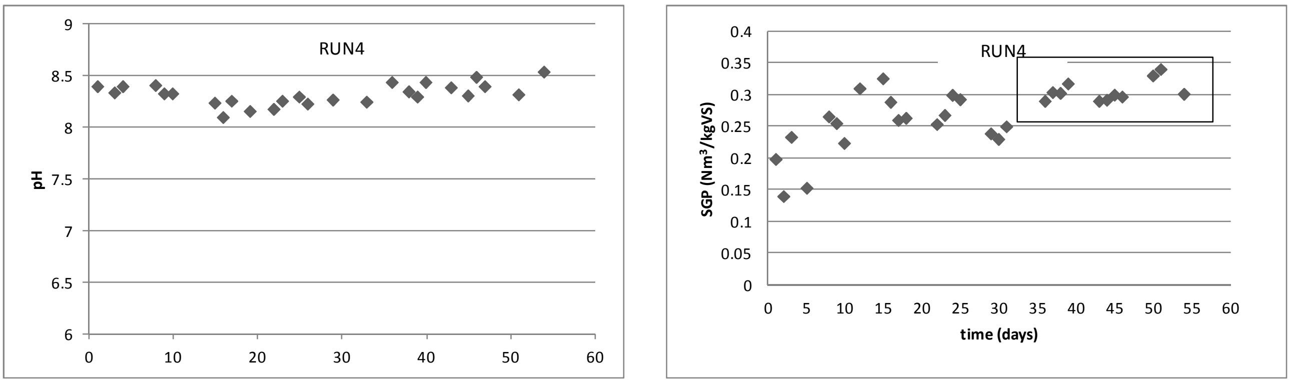


a) b)

Figure 3. Trends of pH a), SGP b), at OLR 4 kgVS/m3d (RUN3).

The pH value ranged from 7.5 to 8, without noticeable variations during testing, while alkalinity increased slightly and reached 1.95 gCaCO$_3$/L in the final period. The ammonia concentration was constant at 143 mgN/L. The volatile fatty acids were completely consumed by methanogens and the concentration in the effluent was lower than 100 mgCOD/L. As confirmed by previous studies (Riaño et al., 2010), the utilization of a co-substrate improved the stability of the process (pH, alkalinity, and ammonium concentration) and increased the volatile solids removal. However, the specific biogas production remained lower than the value obtained by BMP tests. The other proposal to improve the biogas production and the stability increased the degradation efficiency. For this aim, a longer HRT (40 d) was applied and fermented GM was used as a substrate (RUN4). Increasing the HRT, the flow rate was reduced from 250 ml/d to 125 ml/d. In order to guarantee mixing in the reactor, the solid content was reduced and, consequently, the organic loading was comparable to RUN2 (1 kgVS/(m^3d)). The process was monitored for about 60 d and reached a semi-steady state after 35 d (Figure 4). The average SGP was 0.290 $Nm^3/kgVS_{fed}$ with 61% methane. Based on mass balance around the system, the corresponding VS conversion into biogas was 35%, higher than previous continuous trials because the long retention time and previous fermentation promoted the hydrolysis of organic matter; however, it was less than half of the biodegradability index obtained by batch tests (82%). Beside a greater biogas production, more stability in the process was also observed. pH remained higher than 8 during the trial due to the higher concentration of ammonium in the digestate. In fact, the concentration at a steady state was 734 mgN/L, significantly higher than in other examined digestates, because of a higher protein degradation. At the beginning, alkalinity reduced similarly to tests with GM alone, but it stabilized at 1.99 $gCaCO_3/L$ at a semi-steady state. Table 5 summarizes the operational conditions, effluent characteristics, and process yields. The effluent solids concentrations were affected by operational conditions; in particular, the TS concentration increased with an increasing OLR. The VS/TS ratio and COD concentration reflected the substrates stabilization; the reactor working with a long HRT was characterized by the lowest VS/TS percentage and a lower COD content, due to higher organic matter conversion.

Comparing the SGP recorded during the continuous trials with GM as a unique substrate or together with WAS, the biogas production was similar, but the percentage of methane was significantly higher when only one substrate was used. The different biogas composition could be due to organic matter composition in the feed; in particular, GM contained oil, therefore compounds with higher theoretical methane production.



a) **b)**

Figure 4. Trends of pH a), ammonia b), total and partial alkalinity c) and SGP d), at OLR 1.2 kgVS/m3d (RUN4).

The specific yields calculated on removed COD showed different solubilization efficiencies of particulate COD. In fact, anaerobic digestion of winery waste alone resulted in yields near the theoretical value with errors ranging between 1% and 3% (RUN1 and RUN2) up to 15% (RUN4), while the anaerobic co-digestion trial was characterized by a yield of 0.136 $Nm^3/kgCOD_{removed}$. As reported by Lu et al. (2015), the total amount of COD that outflows from the reactor also consists of soluble COD that was not considered in this experiment. In fact, in the liquid phase, there should also be present organic compounds such as proteins and carbohydrates deriving from waste activated sludge.

Table 5. Comparison of operational conditions, effluents characteristics and specific yields of continuous trials

		RUN1	**RUN2**	**RUN3**	**RUN4**
Operational conditions					
V_r	L	5	5	5	5
HRT	d	20	20	20	40
OLR	kgVS/(m^3 d)	1	2	4	1.2
Q	L/d	0.25	0.25	0.25	0.125
Effluent characteristics					
TS	gTS/kg_{ww}	21.8	37.1	55.1	55.0
VS	gVS/kg_{ww}	15.5	32.1	47.8	37.6
VS/TS	%	71	86	87	68
COD	mg/g_{dw}	920.7	1031.0	981.2	889
pH	-	7.7	7.8	7.5	8.4
$N\text{-}NH_4^+$	mgN/ L	367.5	258.6	142.5	734
PA	$gCaCO_3/L$	1.56	1.09	1.22	1.52
TA	$gCaCO_3/$ L	2.44	1.95	1.92	1.99
Yields					
SGP	$Nm^3/kgVS_{fed}$	0.114	0.145	0.146	0.290
CH_4	%	75%	76%	50%	61%
VS_{rem}	%	12%	15%	20%	35%

Few data are available on anaerobic digestion of winery waste in continuous reactors. Fountoulakis et al. (2008) have reported biogas yields obtained by anaerobic digestion of GM alone or with a co-substrate, operating in a draw-and-fill mode (on a daily basis) with a retention time of 20 d at a temperature of 35 C. Comparing their results with this study, it is evident that a thermophilic temperature did not improve biogas production, yet it did not seem to be more sensitive than a mesophilic process, and, in fact, no inhibition effects were detected. Instead, an increase of HRT favored hydrolysis and SGP reached 0.290 $Nm^3/kgVS$.

3.5. Potential energy recovery from winery waste production

The Italian, European, and global production of winery waste were calculated considering the reported wine production of 2013 and the specific production factor reported by Italian Environmental Protection Agency (ANPA, 2000). GM represented 65% of the total organic waste production by the winery sector (Table 6) and recent legislation encourages its use for energy production.

Table 6. Production of GM, GS and WL in 2011

	Wine	GM	GS	WL
	10^6 m^3	Thousands of tonnes		
Italy	4.5	808.2	179.6	269.4
Europe	16.4	2955.6	656.8	985.2
World	27.9	5014.8	1114.4	1671.6

Table 7. Production of GM and corresponding heat and electricity production (source of specific emission: (Danieli and Aldeovandi, 2011)).

	GM	Thermal Energy	Electricity	CO_2 emission avoided
	10^3 tonnes	GWh/y	GWh/y	10^3 tonnesCO_2/y
Italy	808.2	245	201	155
Europe	2,955.6	896	734	566
World	5,014.8	1,520	1,245	1462

Considering this biogas yield and the production of GM in Italy, Europe, and around the world, the evaluation of potential energy from this by-product was calculated. If the biogas produced is exploited using combined heat and power (CHP) units, with a thermal efficiency of 50% and an electrical efficiency of 35%, the energy produced would be substantial. The heat energy reported in Table 7 is a net value, and by subtracting the thermal energy used for biomass and digester heating, the process could be self-sustaining. Assuming the same biogas yields operational conditions at 37 C, the available heat would increase by 8%.

The economic gain obtained from selling the electricity depends on the incentives applied, but even considering an average plant sized smaller than 1 MWh, the minimum price for 2014 was 174.4 €/MWh (DM July 6, 2012). Hence, it would be possible to obtain about 35,054,400 €/y for Italy alone. Using this kind of renewable energy would also reduce CO2 emissions from fossil fuel use. In fact, considering a specific emission of 406 gCO_2/kWh, in Italy alone 181 thousand tonnes of CO_2 could be avoided.

4. Conclusions

Thermophilic batch trials were conducted using various winery waste. Results showed specific methane production of 0.347 and 0.360 Nm^3CH_4/kgVS$_{fed}$ for fresh and fermented GM, 0.133 Nm^3/ kgVS$_{fed}$ for GS, and 0.370 Nm^3CH_4/kgVS$_{fed}$ for WL. Comparing values obtained at 55° C with those reported in literature under mesophilic conditions, potential methane production for GM increased to 84%. The kinetic study demonstrated that a modified Gompertz model well fit (R^2 0.98-1.00) the methane production cumulative curve in cases with a lag phase at the beginning of the test, while the anaerobic degradation of GS was better described by the first order equation (R2 1.00). The determined specific yields and kinetic parameters were comparable with those at 37 °C. Anaerobic digestion semi-continuous trials of GM treatment were characterized by low biogas production with yields ranging from 0.114 to 0.145 Nm^3CH_4/kgVS$_{fed}$. The addition of waste activated sludge improved process stability but did not increase volatile solids removal. The biogas production increased in a test with fermented GM as a unique substrate and a longer HRT (40 d), obtaining 0.290 $Nm^3CH_4/kgVS_{fed}$; the effluent had a lower COD content due to higher organic matter degradation (35% of volatile solids). Considering the best biogas yields, potential electricity and heat were calculated using a typical CHP unit efficiency. The combustion of biogas generated by GM could generate, globally, 1520 GWh/y of heat and 1245 GWh/y of electricity that could be used by wine cellar facilities.

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3.2. Integration of wine lees treatment in municipal wastewater treatment plant

Wine lees are available during all the year and are characterized by high biodegradability, thus they could be used as co-substrate to improve energy production in the municipal wastewater treatment plant equipped with anaerobic digestion reactor. This approach has some theoretical advantages such as to increase biogas production as well as energy generation, then to improve economical balance of the whole plant. This option was tested in pilot scale applying different operational conditions (temperature, OLR and HRT) as described in paper "Anaerobic co-digestion of winery waste and waste activated sludge: assessment of process feasibility". The process yields were expresses in terms of biogas production instead of methane on because biogas composition is not continuously monitored, then specific methane productions would not be truthful results. As soon as the feasible organic loading rate was detected (OLR 2.8 $kgCOD/m^3d$) and the effluent characteristics were analyzed considering different aspects:

- stability parameters and macro-nutrient contents were reported in paper "Treatment of waste activated sludge together with agro-waste by anaerobic digestion: focus on effluent quality"
- micro-nutrients (PCBs, dioxins, heavy metals) and pathogens in the effluents were instead described in paper " Winery waste recycling through anaerobic co-digestion with waste activated sludge". The aim of the study was to compare level of contaminants in the digestates with threshold limits of amendment.

The experimentation was supported by the FP7 EU project ROUTES (Contract No 265156, FP7 2007-2013, THEME [ENV.2010.3.1.1-2] Innovative system solutions for municipal sludge treatment and management) then the ideas of coordinators Cecchi F. and Bolzonella D. were fundamental for the development of the study. Pavan P. and Cavinato C. collaborate on data elaboration and manuscript writing.

Research paper

Anaerobic co-digestion of winery waste and waste activated sludge: assessment of process feasibility

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Keywords: anaerobic digestion, co-digestion, waste activated sludge, wine lees, winery waste.

Abstract

In this study the anaerobic co-digestion of wine lees together with waste activated sludge in mesophilic and thermophilic conditions was tested at pilot scale. Three organic loading rates (OLRs 2.8, 3.3 and 4.5 kgCOD/m^{3}d) and hydraulic retention times (HRTs 21, 19 and 16 days) were applied to the reactors, in order to evaluate the best operational conditions for the maximization of the biogas yields. The addition of lee to sludge determined a higher biogas production: the best yield obtained was 0.40 Nm3biogas/kgCODfed. Because of the high presence of soluble chemical oxygen demand (COD) and polyphenols in wine lees, the best results in terms of yields and process stability were obtained when applying the lowest of the three organic loading rates tested together with mesophilic conditions.

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1. Introduction

Wine production is one of the most important agricultural activities throughout the world; it has a great economic relevance in the producing countries like France, Italy and Spain. At the same time the wine making generates large amounts of solid and liquid by-products which need a proper disposal. Lees are one of the main by-products of processing and originate from the separation of solids from wine. It consists mainly of fine fruit pulp, tartrate salts, spent yeast, bacteria and a certain amount of other debris carried on the grape. There are two main types of lees: solids remaining in the unfermented juice or 'must lees', and sediment remaining after fermentation or 'wine clarifier lees'. They are obtained by settlement, sometimes adding chemical fining agents like bentonite, gelatine, albumin, or by filtration using activated carbon or diatomaceous earth as filter medium (Setti et al. 2009). Typically, any cubic meter of wine produced generates 60 kg of lees (ANPA 2001): this material has to be properly treated or disposed of. Lees are usually destined to distillery industry to recover ethanol and tartaric acid while a small part of them are disposed directly on fields but this can generate an over-supply of nitrogen, or sodium and potassium, thus salinity. Lees, like other winery residues, are characterized by low pH, high content of organic acids, low concentration of nitrogen with regards to carbon content, and presence of potentially toxic compounds like polyphenols.

Moletta (2005) and others authors reported that anaerobic digestion (AD) is particularly suitable for winery and distillery waste and wastewater. AD reduces the organic content of the waste (stabilization) and produces biogas, which could eventually cover part of the energy requests of a wine-making facility. Furthermore, the digester effluent can be used in agriculture as a secondary fertilizer. In spite of the advantages of anaerobic digestion, the anaerobic treatment units for winery wastes are not so common. The factors limiting their diffusion are probably related to the high capital costs and the need for skilled personnel for the process management.

Examples of anaerobic digestion with wine lees (WL) alone were not reported in the literature. Instead, many cases of anaerobic co-digestion (AcoD) of winery waste together with other substrates were described. Lo and Liao (1986) reported about the AcoD of WL with manure, while Rodríguez et al. (2007) with waste activated sludge (WAS), both worked in mesophilic condition. They obtained high biogas production, mixing different substrates, because AcoD supplies the system with missing nutrients and stabilized the whole process (Fountoulakis et al. 2008; Mata-Alvarez et al. 2011; Riaño et al. 2011). With specific reference to the anaerobic co-digestion of winery wastes and waste activated sludge, the implementation of the AcoD in wastewater treatment plants can also be considered a proper solution to improve the energy balance of the anaerobic reactor (Bolzonella et al. 2005, 2006). Moreover, all the residual streams (solid and liquid) can be properly managed on site. In the present work, the anaerobic co-digestion of wine lees and waste activated sludge was studied. Different organic loading rates (OLRs) and hydraulic retention times (HRTs) were tested in mesophilic and thermophilic conditions at pilot scale to examine the effect of temperature, to evaluate process stability and any critical aspects, to determine kinetic constants of the anaerobic digestion process.

2. Materials and Methods

2.1 Pilot plants

Four pilot scale continuous stirred reactors (CSTRs) of 230 liters working volume were employed. The substrates mixtures (WAS and WL) were prepared every morning and were loaded into the reactors once a day. The reactors were heated by a hot water recirculation system and were maintained at 37 WC and at 55 WC. Trials with high (4.5 kgCOD/m^{3}d), medium (3.3 kgCOD/m^{3}d) and low (2.8 kgCOD/m^{3}d) organic loading rates were carried out, corresponding to hydraulic retention times of 16, 19 and 21 days, respectively. Table 1 resumes the applied operational conditions along the experimentation. The reactors were working in parallel based on loading conditions (two reactors, same OLR tested at two different temperature).

Table 1.5 Operational condition of trials at steady state.

Reactors	**LowOLR 55°C**	**LowOLR 37°C**	**Medium OLR 37°C**	**Medium OLR 55°C**	**High OLR 37°C**	**HighOLR 55°C**
Volume (m^3)	0.230	0.230	0.230	0.230	0.230	0.230
Temperature (°C)	55	37	37	55	37	55
HRT (d)	20.6	20.6	19.1	19.1	15.8	15.8
Wine lees in the feed (% v/v)	22	22	27	27	36	36
OLR (kgCOD/(m^{3}d))	2.8	2.8	3.3	3.3	4.5	4.5

2.2 Analytical methods

The substrates and the digesters effluents were monitored once a week in terms of total and volatile solids content (TS and VS), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and total phosphorus. The process stability parameters, pH, volatile fatty acids (VFAs) content and composition, total and partial alkalinity, and ammonia concentration, were checked two or three times per week. All the analyses, except for VFAs, were carried out in accordance with Standard Methods (APHA 1998). VFA content was monitored using a gas chromatograph (Carlo Erba instruments) with hydrogen as gas carrier, equipped with a Fused Silica Capillary Column (Supelco NUKOL™, 15 × 0.53 ×0.5 μm film thickness) and with a flame ionization detector (200 °C). The temperature during the analysis started from 80 °C and reaches 200 °C trough two other steps at 140 and 160 °C, with a rate of 10 WC/min. The analyzed samples were centrifuged and filtered on a 0.45 μm membrane. At steady state the total concentration of polyphenols in individual samples were measured using a modified version of the Folin-Ciocalteau reaction as reported in Laftka et al. (2007). The total concentration of polyphenols was then determined by measuring the absorbance of the sample at 700 nm, wavelength of maximum absorbance, and converting it to Gallic acid equivalents (mg/L).

The biogas production was monitored continuously by four drum-type gas flow meters (Ritter, Germany), while biogas composition (CO_2, CH_4, O_2 and N_2) was determined by a gas chromatograph (GC Agilent Technology 6890 N) equipped with the column HP-PLOT MOLESIEVE, 30 × 0.53 mm ID × 25 μm film, using a thermal conductivity detector and argon as gas carrier. The volume of biogas production was converted in standard temperature and pressure conditions (0 °C and 1atm) to compare the yields at different operating temperatures.

2.3 Inoculum

The seed sludge used as inoculum for the reactors was collected in the wastewater treatment plant (WWTP) located in Treviso (northern Italy) where a 2,000 m^3 anaerobic digester treats the source collected organic fraction of municipal solid waste (OFMSW) and WAS at a working temperature of 37 WC. The inoculum was than acclimatized to the operational temperatures (37 and 55 °C) without feeding for 1 month. Then, only activated sludge was fed for 1 week and later the two substrates were used as feeding.
The characteristics of the inoculum in terms of TS, total volatile solids (TVS), pH, nutrients and alkalinity are shown in Table 2.

Table 2. Inoculum characteristics COD: chemical oxygen demand; TKN: total Kjeldahl nitrogen. Avg: average, CV: coefficient of variation , Min: minimum value, Max: maximum value.

Parameter	Units	Avg	VC %	Min	Max	Samples
Total Solids (TS)	gTS/kg_{ww}	19.2	4.5	18.1	20.2	4
Total Volatile Solids (TVS)	gVS/kg_{ww}	13.0	18.3	11.5	16.5	4
VS/TS %	%	60.5	2.3	58.5	61.8	4
pH		8.2	6.5	7.6	8.9	4
COD	mg/g_{dw}	759	6.3	697	813	4
TKN	mg/g_{dw}	36.2	17.9	30.4	44.6	4
$P\text{-}PO_4^{3-}$	mg/g_{dw}	25.7	13.5	23.3	30.8	4
Total alkalinity	$mgCaCO_3/L$	2531	21.1	1975	3125	4

2.4 Kinetics study

The biogas production after a single-shot feeding was monitored continuously to obtain kinetic information relating to substrates degradation. The study was based on the Step-Diffusional Model elaborated by Cecchi et al. (1990). The approach adopted in construction of the step diffusional model is based on three considerations as follows: the observed changes in gas production rate between one feed and the next one, the nature and chemical characteristics of compounds present in the substrates, the metabolic characteristics of the different bacterial groups responsible for the degradation of the various compounds present in the feed. The organic compounds in the substrates could be divided into different groups: acetate and the compound utilized by methanogenic microorganisms, VFAs and ethanol, free organic matter, complex organic matter, not biodegradable organic matter. The degradation rate of each group is described by a specific equation that considers the concentration of compounds, the degradation rate and the diffusion of extracellular enzymes which must be excreted in order to hydrolyze the non-soluble compounds.

If the biogas production, expressed as degradation rate, is plotted against time, different utilization rates could be identified each describing the degradation of a specific group of compounds. Degradation rate decreases passing from easily biodegradable fraction to slowly degradable compounds. The biodegradation rate outline allows determination of the kinetic model constant: maximum degradation rates (v_1, v_2, v_3, v_4) and the slopes of the degradation rate versus time (4a, 4b, 4c) represent diffusional velocity of the substrates through microorganism membranes.

3. Results and Discussion

3.1 Substrates characteristics

The waste activated sludge used in this experimentation originated from a 70,000 people equivalent WWTP adopting a biological nutrient removal (BNR) process (Johannesburg scheme) located in Treviso. The solid content of thickened WAS was around 3%, while the total volatile solids and COD content were 73 and 78% respectively, and nitrogen and phosphorus presented typical concentrations of 48 and 18 mg/g_{dw}, respectively.

The wine lee was originated from a winery producing sparkling white wine with a natural process, and it was used as it is in this experimental test, without any pre-treatment. The WL presented a TS concentration of 85 g/kg with 91% TVS on TS basis, and very high levels of COD concentrations, around 143 gCOD/kg_{ww}, with 70% of soluble COD. The soluble COD is mainly composed by VFAs (9 gCOD/L), other organic acids (11 gCOD/L) deriving from grape and fermentation process, and usually about 10% v/v of ethanol and more than 1 g/L of reducing sugars (Setti et al. 2009). The N and P contents were similar to those of WAS: the COD/N_{tot} was therefore higher than the one of WAS. The range variation of ammonium nitrogen was very wide: ammonium concentration increased to more than 2,000 mg N/L, while in other period was lower than 500 mg N/L. In agreement with Bustamante et al.(2008) that used a similar type of wine lee, pH was around 4. The large variability of wine lees characteristics was due to several factors like the kind of grapes or wine treated, and the different biological fermentation used in the wine-making process (Table 3).

The reactors working at lower (2.8 kgCOD/(m^3d)) and higher (4.5 kgCOD/(m^3d)) OLRs were characterized by two start-up phases: in the first one the organic load changed too fast while in the second one the OLR was increased step by step at a rate of 0.3 kgCOD/(m^3d) per week. As a consequence, VFAs accumulated in the system, while pH and alkalinity dropped down. The original process instability confirmed the necessity to acclimate the inoculum to readily biodegradable substrates as wine lees. When the reactors were restarted, the feed was increased step by step maintaining a constant WAS contribute (0.7 kgCOD/(m^3d)) and increasing the volume of wine lees in the inlet. During transient conditions ammonia concentration, alkalinity and biogas production increased according to OLR. The steady state conditions were reached at day 92, 51 and 87 for high, medium and low OLR reactors, respectively. Later on, the HRTs were constant along the experimentation, but the OLR changed because of the variability of wine lees organic content, especially considering the soluble part.

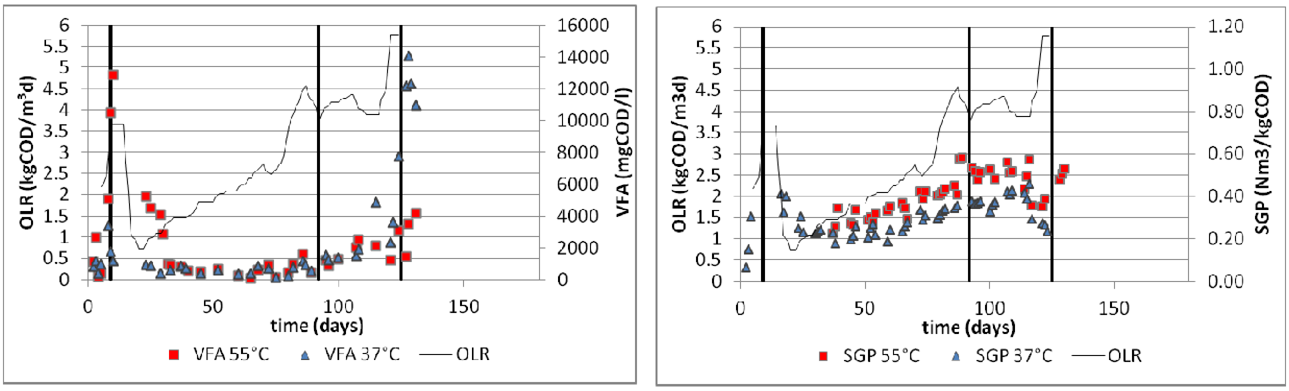
Table 3. Waste activated sludge and wine lees characteristics, TS: total solids; VS: volatile solids; EC: electrical conductivity; COD: total COD: sCOD soluble COD; CODpart: particulate COD; TKN: total Kjeldahl nitrogen; N_{tot}: sum of particulate and soluble nitrogen; CV: coefficient of variation.

Parameter	Units	SLUDGE					WINE LEES				
		Avg	CV %	Min	Max	samples	Avg	CV %	min	Max	sample
TS	gTS/kg_{ww}	29.5	15	17.4	39.4	73	85.2	28	23.5	163.7	86
VS	gVS/kg_{ww}	21.7	15	15.9	29.2	35	77.5	29	20.2	142.6	39
VS/TS	%	73.1	6	61.5	88.2	34	90.7	2	84.5	94.6	39
pH		6.5	2	6.3	6.7	8	4.6	21	3.1	6.2	28
Conductivity	mS/cm	0.9	11	0.7	1.0	9	5.2	64	2.2	10.9	19
$N-NH_4^+$	mg/L	21	36	7	32	15	743	88	100	2025	23
COD	g/kg_{ww}	24.3	19	15.2	32.8	24	214.3	30	72.0	322.8	45
Soluble COD (sCOD)	g/kg_{ww}	0.5	89	0.1	1.3	19	153.0	32	18.7	247.7	26
Particulate COD (CODpart)	mg/g_{dw}	769	10	595	998	34	952	11.1	683	1124	37
TKN	mg/g_{dw}	48	9	40	57	23	70	8	58	80	20
P_{tot}	mg/g_{dw}	18	7	16	22	24	14	29	10	28	24
COD/N_{tot}	mgCOD/mgN	16	14	13	22	22	35	36	17	59	16
COD/ P_{tot}	mgCOD/mgP	43	14	34	62	24	200	28	102	312	22
Polyphenols	mg/L	17	39	10	27	7	1803	25	1162	2556	14
Total Copper	$\mu g/g_{dw}$	259	19	203	297	3	801	15	662	859	3

3.2 OLR 4.5 kgCOD/(m^3d) – HRT 16 days

The reactor working at higher OLR maintained a stable condition for two HRTs after the startup phase (Figure 1). During this period, a continuous increase of VFA concentration was observed despite the average biogas production was high:0.36 and 0.47 $Nm^3/kgCOD_{fed}$ at 37 and 55 °C, respectively.

Around day 130 the VFA concentration suddenly increased and reached a concentration level in the range 12–14 gCOD/L. As a consequence, the biogas production dropped down to levels lower than 0.2 $Nm^3/kgCOD_{fed}$. The reactor did not work properly due to system overloading ascribed to excess of wine lees in the inlet. (Rétfalvi et al. 2011). This problem was associated with the variability of the wine lees characteristics: in fact, in those days the COD concentration ranged between 140 and 230 mg/L and the OLR passed from 3.9 to 5.8 kgCOD/(m^3d) in 2 days eventually determining the failure of the process. VFA concentration also led to a drop in pH to 5.8 in the mesophilic reactor while in the thermophilic one it remained at levels higher than 8 because of the higher level of ammonia and buffer capacity. In both cases the VFA-to-alkalinity ratio passed from 0.2, a typical value for a stable digester, to values up to 0.8, indicating a clear loss of stability. The processes with average OLR 4.5 kgCOD/(m^{3}d) were characterized by significant problems of instability linked with high biodegradability of wine lees, so the two reactors were stopped at day 124.

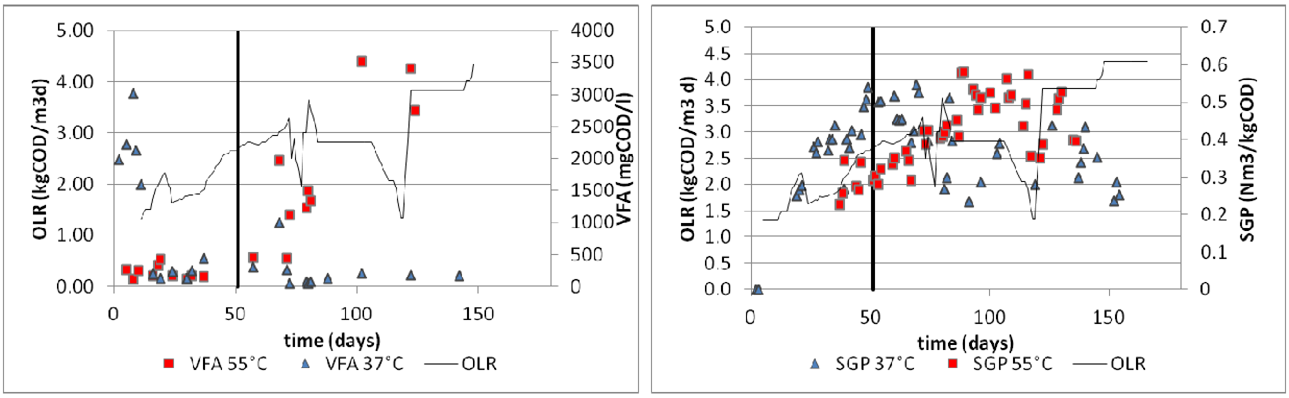


a) b)

Figure 1 High organic loading rate (OLR) trials: a) trend of OLR and volatile fatty acids (VFA) concentration, b) specific gas production (SGP).

3.3 OLR 3.3 kgCOD/(m^{3}d) – HRT 19 days

The reactors working at OLR of 3.3 kgCOD/(m^{3}d) and HRT of 19 days reached a steady state condition in some 50 days. The process in these conditions need strict monitoring in order to maintain stable conditions. It is the opinion of the authors that it was feasible but some stability parameter like VFA concentration and biogas production were not constant. In the mesophilic reactor, the VFA concentration was high in the first days, then it was generally lower than 1,000 mgCOD/L. On the other hand, in the thermophilic digester, VFAs accumulated up to concentrations higher than 3,000 mgCOD/L. The average specific biogas production (SGP) was 0.38 Nm^3/kgCOD$_{fed}$ at 37 °C, while at 55 °C the SGP reached values 0.36 Nm^3/kgCOD$_{fed}$ around day 100 but it then decreased to 0.25–0.30 Nm^3/kgCOD$_{fed}$ showing instability problems similar to those detected for high loaded reactors. The sustainable organic load at 55 °C appeared lower than at 37 °C (Figure 2).

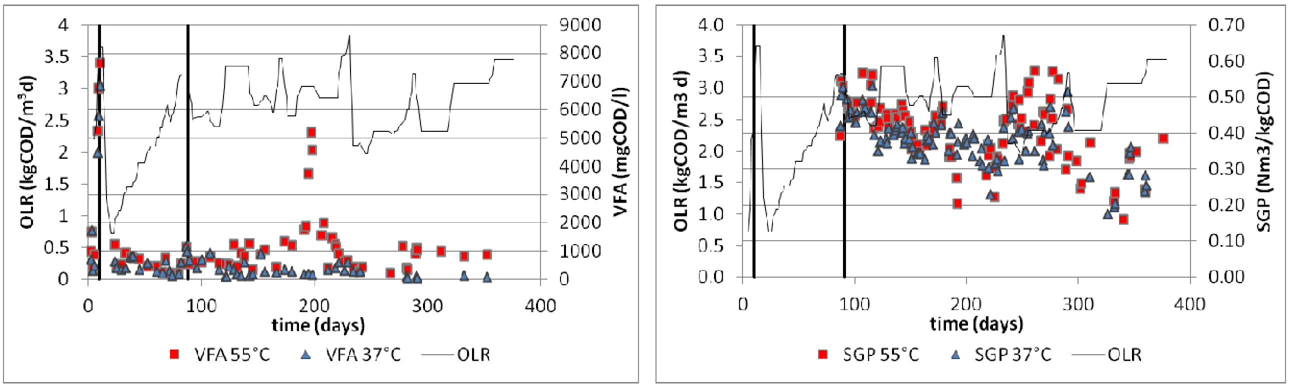


a) b)

Figure 2 Medium organic loading rate (OLR) trials: a) trend of OLR and volatile fatty acids (VFA) concentration, b) specific gas production (SGP).

3.4 OLR 2.8 kg COD/(m^{3}d) – HRT 21 days

The process was stable in both the reactors, with VFA concentrations lower than 1,000 and 1,500 mgCOD/L at 37 and 55 °C, respectively (Figure 3). The VFA accumulated in the thermophilic reactor at day 200 because of a sudden increase in ammonia concentration (near 2,000 mg N/L) due to a variation in cellar activity. The feed of this reactor was suspended and the VFAs were readily consumed.



a) b)

Figure 3 Low organic loading rate (OLR) trials: a) trend of OLR and volatile fatty acids (VFA) concentration, b)specific gas production (SGP)

The average SGP at steady state were 0.38 and 0.40 Nm^3/kgCOD$_{fed}$ in mesophilic and thermophilic conditions respectively. The composition of biogas was constant with 65 and 64% of methane at 37 and 55 °C. The SGP exhibited a downward trend: the maximum production was reached at the end of the start-up phase and then decreased continuously. Probably bacterial activity varied because of accumulation of inhibiting factors like polyphenols and heavy metals. The polyphenolic compounds showed low concentrations: 51 and 265 mg/L at 37 and 55 °C respectively. These levels cannot inhibit the process. Heavy metals could be potential inhibitors for anaerobic bacteria and can be present in the system as salts (Lawrence & McCarty 1965; Mosey et al. 1971), or are directly adsorbed onto the solid fraction, either biomass or inert particulate matter (Shen et al. 1993). Copper (Cu) is the most toxic metal (Lin 1993) and it was present in WL with average concentrations of 0.8 mg/g_{dw}. During the trials Cu concentration in the effluent increased, passing from 0.82 mg/gVS at day 150 to 1.83 mg/gVS at day 300. Reported concentrations that inhibit 50% of acidogenesis was 0.9 mg/gVS while for methanogenesis was 2.3 mg/gVS (Lin 1993). Probably, Cu inhibited the VFA formation but not the methanogenesis, so the biogas production decreased while methane content remained at good levels. In Table 4 are reported the average characteristics of the effluents and the yields at steady state conditions of best performed trials.

Table 4. Average effluents characteristics and process yields

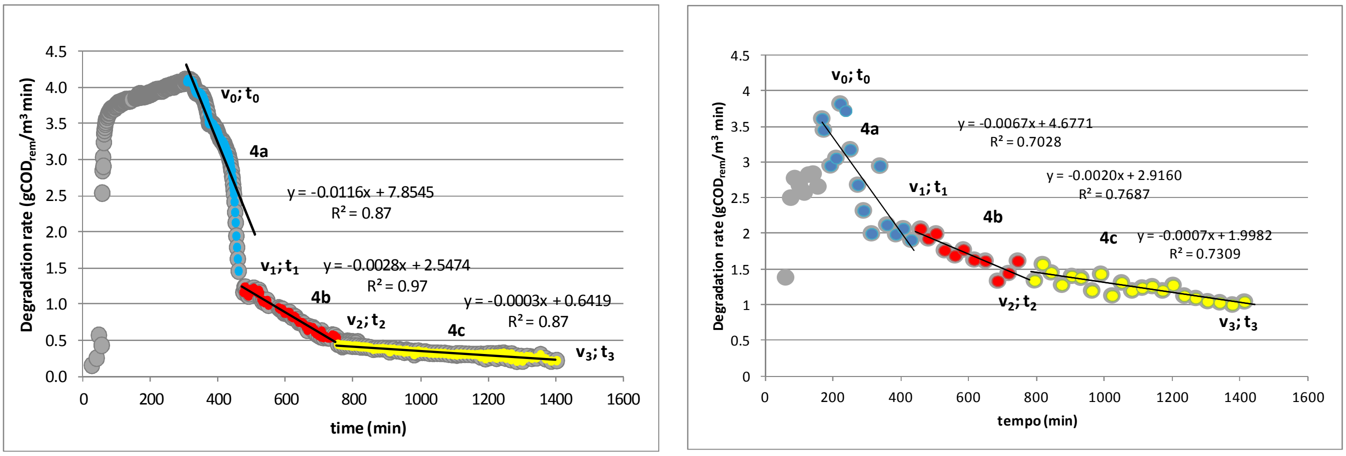
OLR	**kgCOD/(m^3d)**	**2.8**		**3.3**		**4.5**	
Temperature	**°C**	**37**	**55**	**37**	**55**	**37**	**55**
Effluents characteristics							
TS	gTS/kg_{ww}	23.0	19.6	20.5	20.1	16.9	18.4
VS	g VS/kg_{ww}	16.1	16.6	13.5	13.9	11.0	12.4
VS/TS	%	68.6	70.7	64.5	65.3	67.5	66.7
pH		7.83	8.13	7.73	8.04	7.52	8.24
Total alkalinity	mg $CaCO_3$/L	5,277	5,464	5,020	6,561	5,002	5,203
EC	mS/cm	9.2	9.7	10.5	11.5	10.0	9.3
N-NH_4^+	mg/L	1,291	1,347	1,389	1,613	1,446	1,425
VFA	mgCOD/L	318	1,125	244	1,768	5,244	4,260
COD	g/kg_{ww}	17.8	15.3	12.8	14.8	14.3	15.8
sCOD	g/kg_{ww}	1.2	2.3	1.6	2.1	3.4	3.2
CODpart	mg/g_{dw}	700	704	643	1052	701	720
TKN	mg/g_{dw}	48.7	42.1	48.3	41.7	49.7	45
P_{tot}	mgN/g_{dw}	25.5	27.1	28.2	30.9	28.5	28.9
COD/TKN	mgCOD/mgN	14.7	16.5	14.4	26.6	13.9	16.2
COD/ P_{tot}^{-}	mgCOD/mgP	18.1	26.1	20.1	39.7	25.3	26.0
Polyphenols	mg/L	51	265	75	120	159	230
Process yields							
SGP	$Nm^3/kgCOD_{fed}$	0.38	0.40	0.38	---	---	---
CH_4	%	64.8	64.0	61.4	---	---	---
TS removal	%	59	63	68	---	---	---
VS removal	%	72	77	81	---	---	---
COD removal	%	70	73	67	---	---	---
Polyphenols removal	%	88	35	85	---	---	---

The concentration of the polyphenolic compounds were higher in thermophilic condition, while at 37°C they were generally lower than 100 mg/L. Comparison of degradation at different conditions revealed a strong influence of temperature, with more efficient phenol degradation in anaerobic organic waste digestion processes at the mesophilic working temperature (Levén & Schnürer 2005; Levén et al. 2006). This fact is related to the different microbial population in the two temperature environments. Temperature not only influences the microbial community structure in general, but also the methanogenic consortia degrading phenol (Chouari et al. 2005; Fang et al. 2006; Hernon et al. 2006; Ariesyady et al. 2007; Weiss et al. 2008). Another possible explanation for higher removal in mesophilic temperatures could be that some enzymes involved in phenol to benzoate degradation are temperature-sensitive. Results from nuclear magnetic resonance (NMR) analyses performed with washed, dense cell cultures supported the hypothesis that the inability of the thermophilic community to degrade phenol above 48 °C was due to temperature inactivation of one of the initial enzymes in the degradation pathway (Levén & Schnürer 2005).

3.5 Anaerobic degradation kinetics

The kinetics of substrate degradation were determined in those reactors operating at the lowest OLRs, following the methodology given by Cecchi et al. (1990). In order to calculate the degradation rate, the biogas production was converted into COD removed. In both reactors the biogas productions started to be significant about one hour after feeding. This is actually the time the system took to return to the correct pressure and working volume. The thermophilic reactor reached maximum degradation rate, linked with acetate, earlier than mesophilic one due to temperature effect on bacterial activity. In particular at 55 °C it was reached after about 3 hours from feeding, while at 37 °C after 5 hours.

Readily biodegradable fraction was completely consumed at the seventh hour and later the degradation speed decreased significantly (Figure 4).



a) b)

Figure 4. Daily average degradation rate between a feed and the next one in mesophilic (a) and thermophilic (b) process

The outline of the degradation rate has been divided into three sections related with slopes obtained by linear regression of the curves (4a, 4b and 4c) reported in Table 5. They had the same order of magnitude of values obtained by Cecchi et al. (1990), and in thermophilic conditions they are slightly higher. They represent the diffusional velocity of the substrates through bacterial membranes and are less dependent from compounds concentration.

The maximum rates of degradation for methanogenesis, acidogenesis and hydrolysis of free and complex organic matter (v_0, v_1, v_2 and v_3) were calculated. The initial velocity v_0 of this study is about three times greater than the value obtained by Cecchi et al. (1990), this difference is due to high concentration of easily biodegradable substrates that determined a shift in the reactor population with advantage for methanogens. Consequently there is a v_0 greater than the ones obtained with substrates less rich in acetate, as the bio-waste. Instead degradation rates v_1 and v_2 are quite similar to values reported in previous works, especially at 37 °C. These degradation rates appeared more affected by temperature effect than by substrate composition.

Kinetic results emphasized the role of wine lees: it contributed to increase readily biodegradable substrate in the feeding mixture, in fact the biogas production in the first six hours derived mainly from acetate and simple compounds in winery waste. Also the microbiological community was affected by fed substrates promoting methanogenic microorganisms and increasing methanogenic velocity v_0 in both reactors.

4. Conclusions

The anaerobic co-digestion of waste activated sludge and winery waste (lees) was tested at pilot scale, both in mesophilic and thermophilic conditions. It was demonstrated that the co-digestion process can be considered stable for an OLR of 2.8 kgCOD/(m^{3}d) where winery lees represented 22% of the feeding volume (but 70% of the inlet COD). The observed biogas yields were 0.38 Nm^3/kgCOD$_{fed}$ in the mesophilic reactor and 0.40 Nm^3/kgCOD$_{fed}$ in the thermophilic one. Higher OLRs tested in this experimentation failed except for the mesophilic trial at an OLR of 3.3 kgCOD/m^{3}d. All the thermophilic digestate had VFAs and ammonia concentrations higher than mesophilic ones, because of higher degradation rates.

The polyphenols, potential inhibiting compounds, were not present in high concentrations in the effluent (always lower than 270 mg/L) and were removed during the process, especially at 37 °C (removal efficiency of 88%). The copper concentration in the reactors increased from day 150 to day 300: the high content of Cu could be the cause of a reduction in bacterial activity and biogas production. More detailed investigation will be carried out to determine the real inhibitors in wine lee.

Step-diffusional model revealed great importance of the diffusional velocity of the substrates through bacterial membranes in anaerobic process, but the fraction division of COD had major effect on kinetic constant.

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Research article

Treatment of waste activated sludge together with agro-waste by anaerobic digestion: focus on effluent quality

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Abstract

Waste activated sludge production and management plays an important role in wastewater treatment plants (WWTPs), especially from an economic point of view. One possible approach is the anaerobic co-digestion of waste activated sludge with others organic substrates in mesophilic and thermophilic conditions in order to exploit the spare volume of existing reactors, recover energy from biogas production, and obtain a fertilizer as final product. The anaerobic trials were carried out at pilot scale, applying two organic loading rates (2.8 and 4.5 kg chemical oxygen demand (COD)/($m^{3\cdot}$d)) with a hydraulic retention time of 16 and 21 days. Among agro-wastes, wine lees were chosen because of their continuous availability throughout the year, and their high COD content (up to 200–300 g/L, 70% soluble, on average). The addition of wine lees to activated sludge determined a higher biogas production (the best yield was 0.40 $Nm^3/kgCOD_{fed}$) improving the energetic balance of the sludge line of the WWTP. The characterization of both substrates fed and digester effluents was carried out in terms of heavy metals; comparison with EC proposed limits showed that, due to high content of Cu in wine lees, the loading rate of this agro-waste should be limited to maintain good characteristics of final biosolids.

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1. Introduction

The need to fulfill stringent effluent standards for chemical oxygen demand (COD) (or biochemical oxygen demand), nitrogen and phosphorus in wastewater treatments has determined in recent years the adoption of advanced activated sludge processes for nutrient removal. The biological nutrient removal (BNR) processes, either for nitrogen or both nitrogen and phosphorus removal, can be performed only when the necessary amount of carbon in the treated wastewater is available. A typical solution for preserving COD in the wastewater is avoiding the primary settling tanks. Moreover, in BNR wastewater treatment plants (WWTPs), in order to support the nitrification capability of the activated sludge, high solid retention times (SRTs) are applied to the activated sludge (>10 days). As a consequence, a partial sludge stabilization occurs in the activated sludge process itself, and the following anaerobic stabilization of waste activated sludge (WAS) can result in low efficiency both from a processing and an economic standpoint since this substrate shows a low bio-methanization potential (Bolzonella et al. 2005). This fact results in a decrease in biogas production so that the energetic balance of the anaerobic digester is often negative if sludge is not properly thickened, especially in winter.

In these WWTPs anaerobic digesters are typically low loaded (OLR <1 kg volatile solids (VS)/($m^3 \cdot d$)) and considerable volumes are unexploited in the reactors. In such a context, the implementation of the anaerobic co-digestion process can be a solution to improve the energy balance of these plants while finding a proper disposal to certain classes of bio-waste as well as the exploitation of the existing reactors. A typical co-digestion process is the one where WAS and the organic fraction of municipal solid waste (OFMSW) coming from source or separate collection systems are co-treated. Several studies reported the effectiveness of this approach and noticeable results were widely reported in the literature (Rintala & Jarvinen (1996), Edelmann et al. 2000, Krupp et al., 2005, Bolzonella et al. 2006, Cavinato et al. 2013 among the others).

However, beside OFMSW, also agro-waste should be considered with interest because of their peculiarities: these wastes are present in large amounts in given regions, and are characterized by low levels of contaminants and inert material (Mata et al. 2011, Athanasoulia et al. 2012). Moreover, these residues are typically characterized by high COD levels and biodegradability and low N and P contents. On the other hand, the production of these waste is seasonal. In some regions, however, like the Mediterranean basin, these streams are largely available throughout the year.

Winery wastes, produced during the wine-making process, are a particular type of agro-waste with some interesting characteristics. In fact, wine production is one of the leading sectors in the food processing industry and accounted for some 5.5 million tonnes in 2009; 64% of the production originated from European countries. Italy, France and Spain generated 50% of the worldwide production. Unfortunately, the wine-making process has some important environmental drawbacks: the intensive use of land, the application of pesticides, and the production of large amounts of waste and wastewater which need proper treatments to be disposed of. A part from wastewaters also semi-solid (lees, vinasses) wastes are produced; these are typically characterized by exceptionally high levels of COD, both particulate and soluble, and biodegradability (Bolzonella & Rosso 2009).

The Veneto region is one of the major wine producers in Italy and, in particular, the province of Treviso in 2011 counted 26,284 hectares of vineyard and 3100 million of liter of wine produced. According to the Italian Environmental Protection Agency (ANPA-ONR 2002), the average lees production in Italy is some 6 kg per hl of wine produced; therefore the province of Treviso generated about 18,600 tonnes of lees to treat.

Semi-solid wastes are normally treated in anaerobic stirred reactors (Melamane et al., 2007, Dinuccio et al., 2010, Fountoulakis et al., 2008). In these studies the applied organic loading rates (OLRs) were between 3 and 8 kg COD/(m^3d) and the hydraulic retention time (HRT) was higher than 20 days. The COD removal yields were in the range 65 to 95% and the biogas production was between 0.4 and 0.6 m^3/kg COD removed. The methane content in biogas ranged between 60 and 80%. If the substrates fed contain considerable levels of lignin, like the grape marc and stalks, the biogas production was lower than 0.3 m^3/kg volatile solids (VS).

This paper considers the feasibility of sludge and winery waste management in Treviso province. In fact the Treviso WWTP was equipped with a BNR process that produced about 90 tonnes per day of thickened activated sludge that was anaerobically digested. The anaerobic co-digestion with the wine lees could be a solution to optimizing the energy recovery and reducing the costs of winery waste disposal. The anaerobic co-treatment of WAS and lees both at mesophilic and thermophilic conditions was tested at pilot scale in continuously stirred reactors. Major attention was paid to the effect of feed composition on effluent quality in terms of heavy metals, in order to assess its utilization as fertilizer.

2. Materials and methods

The characteristics of WAS and lees used in the experimentation are shown in Table 1. Waste activated sludge used in this experimentation originated from a 70,000 people equivalent WWTP adopting a BNR process (Johannesburg scheme). The daily dry flow is some 19,000 m^3/d municipal wastewater while the SRT and food to microorganism ratio applied in the activated sludge process were 15 days and 0.15 kg COD/kg mixed liquor volatile suspended solids per day, respectively.

The solid content of WAS is around 3%, while the volatile and COD content is relatively low: 73 and 77%, respectively, and the COD/VS is about 1.1 rather than a typical 1.4. This clearly indicated that this substrate is already stabilized in the activated sludge process due to the high SRT and oxygen applied in the process. In fact, the anaerobic digestion of this material typically generates specific biogas productions lower than 0.2 $m^3/kgVS_{fed}$. Also nitrogen and phosphorus typical concentrations: 48 and 18 mg/g_{ww} total solids (TS), respectively. The ratio of COD to total Kjeldahl nitrogen (TKN) is relatively low, around 16.

The wine lees used in the anaerobic trials originated from a winery producing sparkling white wine with a natural process. The wine lees presented a total solid concentration of 85 gTS/kg, 91% VS/TS ratio, and very high levels of COD: around 214 gCOD/kg, 71% soluble COD. Similarly to WAS, the N and P contents were 70 and 14 mg/g_{dw} respectively, while the COD/TKN ratio was around 32.

Table 1 - WAS and wine lees characteristics. TS: total solids; VS: volatile solids; EC: electrical conductivity; COD: total COD: sCOD soluble COD; CODpart: particulate COD; TKN: total Kjeldahl nitrogen; CV: coefficient of variation.

Parameter	**Units**	**Waste Activated Sludge**				
		Avg	CV %	min	max	No. of samples
TS	g TS/kg_{ww}	30	16	17	39	58
VS	g VS/kg_{ww}	22	16	16	29	25
VS/TS	%	73	6	62	88	25
pH		6.5	2	6.3	6.7	5
EC	mS/cm	1.0	14	0.7	1.0	4
$N\text{-}NH_4^+$	mg/L	19	27	10	22	5
COD	g/kg_{ww}	25.1	18	17.5	32.8	19
sCOD	g/kg_{ww}	0.6	92	0.2	1.9	15
CODpart	mg O_2/g_{dw}	771	9	595	869	26
TKN	mgN/g_{dw}	50	8	43	57	16
P_{tot}	mgP/g_{dw}	18	4	17	19	17
COD/TKN	mg COD/mg N	15	12	11	17	13
COD/ P_{tot}^{-}	mg COD/ mgP	44	9	35	50	17
Polyphenols	mg/L	17.9	36,4	10,6	26,7	6
		Wine Lees				
		Avg	CV %	min	Max	No. of samples
TS	g TS/kg_{ww}	79	16	52	129	53
VS	g VS/kg_{ww}	72	15	47	103	26
VS/TS	%	92	1	90	95	26
pH		4.3	22	3.1	6.0	19
EC	mS/cm	3.9	71	2.2	10.1	14
$N\text{-}NH_4^+$	mg/L	512	115	61	1621	13
COD	g/kg_{ww}	183.5	17	133.9	238.9	15
sCOD	g/kg_{ww}	103.6	20	71.2	164.7	22
TKN	mg/g_{dw}	71	7	63	80	13
P_{tot}	mg/g_{dw}	13	20	10	20	19
Polyphenols	mg GAE/L	1765	26	1162	2565	12

The reactors were inoculated with sludge from the full-scale anaerobic reactor that treats WAS and OFMSW in mesophilic conditions. The reactors were maintained for a month at operational temperature without feeding and, as reported by Cecchi et al. (1993), this short transient period allows the shift from mesophilic condition to thermophilic one without using a specific inoculum.

The experimental protocol was designed to examine the effect of temperature on the production of biogas by co-digesting wine lees and WAS. The OLR was gradually increased from 0.7 kg COD/(m^3d) (100% WAS) to 2.8 kg COD/(m^3 d), always maintaining the same load of WAS, in the low-loaded systems, while it was increased up to 4.5 COD/(m^3·d) in the high-loaded systems. The corresponding HRTs were 21 and 16 days, respectively. The reactors were monitored for gas production and composition, TS and VS, pH, alkalinity, ammonia concentration, COD, TKN, total phosphorus (P_{tot}) and heavy metals, according to Standard Methods (APHA et al. 1998).

3. Results and discussion

At the beginning the reactors were fed with a load of 0.7 kg COD/(m^3·d) and the load was then increased gradually by adding wine lees. After about 100 days all reactors reached the OLR assigned (high loaded, 4.5 kgVS/(m^3·d); low loaded, 2.8 kgVS/(m^3·d)). After 50 days of constant biogas production (about 0.36 and 0.47 $Nm^3/kgCOD_{fed}$ at 37 and 55 °C), both the mesophilic and thermophilic high-loaded reactors showed some process instabilities: volatile fatty acids (VFA) accumulated up to 8 g/L and pH decreased, respectively, and both gas production and methane content decreased. The ammonia content was higher in the thermophilic reactor (2 g N/L) compared with the mesophilic one (1.6 g N/L) and also the VFA composition was different: in the mesophilic condition acetic acid represents 64% of total volatile acids, while in the thermophilic one acetic and propionic acids content were similar, about 35% of total volatile acids. The VFA accumulation was confirmed also by alkalinity; in fact the gap between partial and total alkalinity was 4 and 3 g $CaCO_3$/L in mesophilic and thermophilic reactors respectively. Consequently, reactor feeding was stopped in the highly loaded reactors and only the two low-loaded systems were kept in operation and monitored. The two low-loaded reactors did not show any instability signal, and the steady-state conditions were verified for more than five HRTs. Figure 1 shows the OLR and specific gas production (SGP) trends for the low-loaded mesophilic and thermophilic reactors.

After 180 days the low-loaded reactor working in thermophilic condition showed a decreased biogas production, with a sudden increase of VFA (at about 6 g/L) (Figure 2). For this reason the feeding was stopped in order to recover the system stability. The average results of the steady-state conditions and related yields for the mesophilic and thermophilic low-loaded reactors (R2 and R3, respectively) are reported in Table 2.

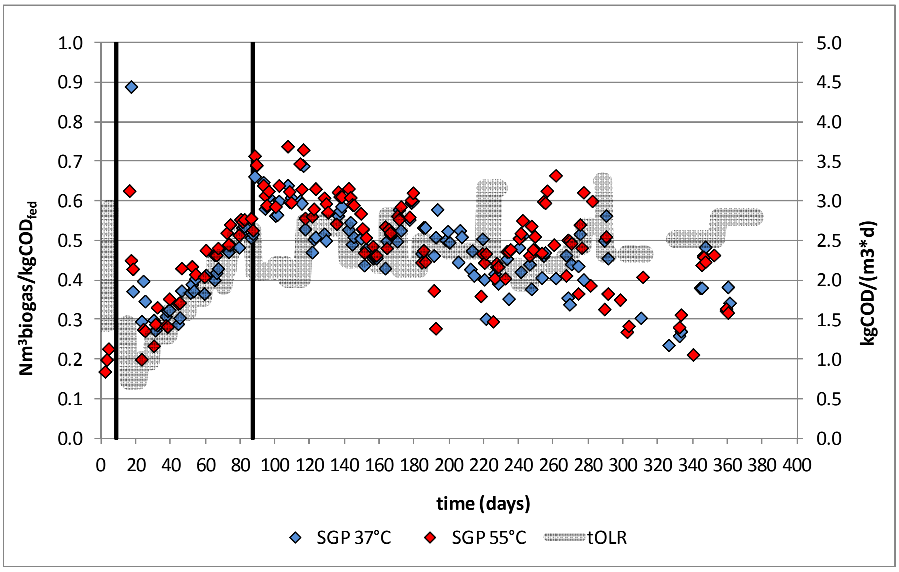

Figure 1 - Trends of OLR and Specific Gas Production (SGP) for low loaded mesophilic and thermophilic reactors.

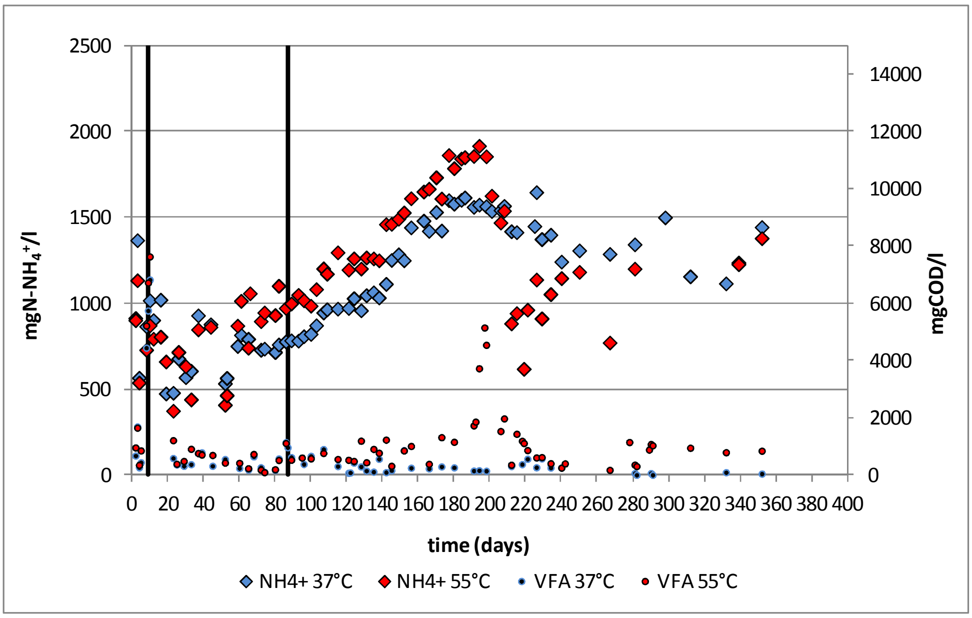

Figure 2 - Trends volatile fatty acids (VFA) and ammonia in low loaded mesophilic and thermophilic reactors.

Comparing the mesophilic and thermophilic trial results, it is evident that performances did not improve in thermophilic conditions. The SGPs were similar, 0.38 $Nm^3/kgCOD_{fed}$ in mesophilic conditions to 0.40 $Nm^3/kg\ COD_{fed}$ in thermophilic conditions, and this was associated with a similar COD removal that was 70 and 73%, respectively, in the two reactors. Higher temperature affected also the hydrolysis rate that determined lower volatile solids content and different distribution of COD between particulate and soluble fractions. In both cases, the process was particularly stable: pH was 7.8 and 8.1 in the mesophilic and thermophilic reactors respectively, the total alkalinity increased with load and reached values greater than 5 $gCaCO_3/L$, while VFA were about 318 mg/L in mesophilic and 1125 mg/L in thermophilic reactors.

Table 2 - Effluent characteristics, stability parameters, and yields for the two experimental conditions

Parameter	**Units**	**R2**		**R3**	
		Avg	CV%	Avg	CV%
Operation condition					
OLR	kg COD fed/(m^3 d)	2.4	11.9	2.4	11.9
HRT	d	20.6	-	20.6	-
Temperature	°C	37	-	55	-
Effluent characteristics					
TS	g TS/kg_{ww}	23.1	16	17.7	17
VS	g VS/kg_{ww}	16.1	20	12.4	16
VS/TS	%	68.6	5	69.7	11
pH		7.8	8	8.1	2
EC	mS/cm	9.2	15	9.7	14
$N\text{-}NH_4^+$	g/L	1.29	20	1.36	24
Partial alkalinity	g $CaCO_3$/L	3.42	17	3.32	19
Total alkalinity	g $CaCO_3$/L	5.28	16	5.38	14
COD	g/kg_{ww}	17.8	7.7	17.6	8
SCOD	g/kg_{ww}	1.2	28	2.3	28
VFA	mg O_2/kg_{ww}	302	77	1125	91.6
COD particulate	mg/g_{dw}	700	9	703	8
TKN	mg/g_{dw}	48.7	11	42.1	16
P_{tot}	mg/g_{dw}	25.5	4	27.1	10
COD particulate/TKN	mg COD/mg N	14.7	9	16.5	17
COD/ P_{tot}	mg COD/mg P	28.1	9	26.1	12
Polyphenols (Gallic Acid)	mg/L	51	16	265	69
Yields					
SGP	Nm^3/kg COD_{fed}	0.48	18.3	0.50	26
CH_4 content	%	65	6	64	7
TS removal	%	53	-	60	-
COD removal	%	74	-	71	-
Polyphenols removal	%	88	-	35	-

The concentrations of the polyphenolic compounds were 265 mg/L in thermophilic condition against 51 mg/L in mesophilic one. Comparison of degradation at different conditions revealed a strong influence of temperature, with more efficient phenol degradation in anaerobic organic waste digestion processes at the mesophilic working temperature (Levén & Schnürer 2005 Levén et al. 2006). This fact is related to the different microbial population in the two working temperatures. Actually, temperature influences not only the microbial community structure in general, but also the methanogenic consortia degrading phenol (Sekiguchi et al. 1998, Dollhopf et al. 2001; Chouari et al. 2005; Fang et al. 2004, 2006; Hernon et al. 2006; Ariesyady et al. 2007; Levén et al. 2007; Weiss et al. 2008). Another possible explanation for higher removal in mesophilic temperature could be that some enzymes involved in phenol-to-benzoate degradation are temperature-sensitive. Results from nuclear magnetic resonance analyses performed with washed, dense cell cultures supported the hypothesis that the inability of the thermophilic community to degrade phenol above 48 WC was due to temperature inactivation of one of the initial enzymes in the degradation pathway (Levén & Schnürer 2005).

Effluents of anaerobic digestion were analyzed to assess their use as fertilizer; in particular heavy metals were determined. The heavy metals content of inlet substrates (WAS and wine lees) and of mesophilic and thermophilic digestates were measured and reported in Table 3. Comparing the results obtained with the limits preliminarily proposed by the European Community on sludge management (third draft working document on sludge, 2000), only Cu was very close to the limit. This was a consequence of the high amount of copper in wine lees, which contributed 71% of copper in the inlet feeding. The amount of substrate that could be co-treated together with WAS depends on its copper concentration. Also considering the amount of heavy metals per kg of phosphorus, all metals were lower than those proposed, Cu excepted.

Table 3: heavy metals content in inlet substrate and low loaded reactors.

Metal	u.m.	WAS	Wine lee	Mesophilic reactor	Thermophilic reactor	Directive 86/278/EEC	EC 3rd draft working document on sludge 2000 PROPOSED
Cd	mg/kg $_{dw}$	1.4	0.1	1.6	1.4	20-40	10
Cr	mg/kg $_{dw}$	49.6	3.2	43.9	42.7	-	1000
Cu	mg/kg $_{dw}$	259	801	1103	927	1000-1750	1000
Ni	mg/kg $_{dw}$	25.8	1.7	24.0	24.2	300-400	300
Pb	mg/kg $_{dw}$	124.2	6.2	110.8	99.4	750-1200	750
Zn	mg/kg $_{dw}$	1035	131	1186	1120	2500-4000	2500
Hg	mg/kg $_{dw}$	0.40	0.01	0.30	0.30	16-25	10

Some considerations about the economic advantage of a full-scale implementation could be done using data obtained in this experimentation. The thickened WAS treated by anaerobic digestion in Treviso WWTP is approximately 32,700 tonnes/yr; applying the same OLR used in these trials, the amount of wine lees is 6,552 tonnes/yr (out of 18,600 tonnes/yr). Using the biogas yield of the mesophilic trial, the obtained electric energy (190 kW combined heat and power unit) is about 1.8 GWh/yr. The gain obtained from selling the electric energy depends on the incentives applied, but even considering the electric energy market price (in

Italy the minimum guaranteed price for 2013 is 80.6€/MWh; www.gse.it), it is possible to obtain about €142,000/yr. This gain must be considered in a contest where the winery wastes are often sent to distillation processes in order to recover ethanol and tartaric acid.

4. Conclusions

Waste activated sludge and agro-waste (lees) co-digestion could be an economic advantageous approach in areas with a large wine production in terms of energy recovery, final product recovery and sludge management. The experimental work demonstrated the process stability for an OLR of 2.8 kgCOD/(m^3·d) (70% lees on a COD basis) with biogas yields of 0.38 $Nm^3/kgCOD_{fed}$ in mesophilic temperature and 0.40 $Nm^3/kgCOD_{fed}$ in thermophilic ones. Nevertheless the COD removal was similar in both systems. The thermophilic reactor showed higher VFA and polyphenols than the mesophilic reactor, and also the stability of the 55 °C process could be compromised with this kind of substrate. The quality of digested sludges was evaluated in terms of heavy metals and organic micro-pollutant content and the analysis showed a pollution level lower than those proposed by the European Community third draft on sludge management for the use on land, except for copper, which exceeded the limit in the case of wine lees. The economic aspects were evaluated and shown to be beneficial.

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Research article

Winery waste recycling through anaerobic co-digestion with waste activated sludge

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Abstract. In this study biogas and high quality digestate were recovered from winery waste (wine lees) through anaerobic co-digestion with waste activated sludge both in mesophilic and thermophilic conditions. The two conditions studied showed similar yields (0.40 $m^3/kgCOD_{fed}$) but different biological process stability: in fact the mesophilic process was clearly more stable than the thermophilic one in terms of bio-process parameters. The resulting digestates showed good characteristics for both the tested conditions: heavy metals, dioxins (PCDD/F), and dioxin like bi-phenyls (PCBs) were concentrated in the effluent if compared with the inlet because of the important reduction of the solid dry matter, but remained at levels acceptable for agricultural reuse. Pathogens in digestate decreased. Best reductions were observed in thermophilic condition, while at 37 °C the concentration of *Escherichia coli* was at concentrations level as high as 1000 UFC/g. Dewatering properties of digestates were evaluated by means of the capillary suction time (CST) and specific resistance to filtration (SRF) tests and it was found that a good dewatering level was achievable only when high doses of polymer (more than 25 g per kg dry solids) were added to sludge.

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1. Introduction

Wine production is one of the leading sector in food processing industry. The world production accounted for 281 million tonnes in 2013: Italy, France and Spain generated 46% of the worldwide production (OIV, 2013). Winery process generates different kind of waste as grape stalks, grape marc, exhausted yeast, wine lee and high loaded wastewater (Bustamante et al., 2008). The winery wastes, although prevalent during the vintage period, are distributed along the year and could be considered hazardous materials if they are not properly disposed of (Devesa-Rey et al., 2011). These wastes are typically characterized by exceptionally high levels of COD, both particulate and soluble, and high biodegradability.

Semi-solid wastes like lees and vinasses are often treated in anaerobic stirred reactors (Moletta, 2005) to recovery renewable energy: applied organic loading rates (OLRs) are between 3 and 8 kgCOD/m^3 d and the corresponding hydraulic retention time (HRT) is higher than 20 d. The COD removal yields are in the range 65–95% and the corresponding biogas production is between 0.4 and 0.6 m^3/kgCOD$_{removed}$, with a methane content around 60% (Moletta, 2005). If fed substrates contain considerable levels of lignin, like the grape marc and stalks, then, biogas production is lower than 0.3 m^3/kgVS (Dinuccio et al., 2010). The most common observed problems when digesting winery wastes are related to process instability due to missing nutrients and to the presence of recalcitrant compounds like polyphenols and copper (Melamane et al., 2007).

The anaerobic co-digestion with another substrate has some advantages in process management, environmental impact and economic sustainability. The effluents of anaerobic digestion can be used as amendment, in this way organic matter and the nutrients were recycled, while pollutants emissions are reduced. The treatment of winery waste in the digesters of wastewater treatment plants (WWTPs) together with waste activated sludge (WAS) represents an interesting disposal option for at least part of these materials at a local level.

At the same time the implementation of this process into WWTPs would improve the economical and energetic balance of the sludge line and will result in a resolution of final disposal problems for such streams (Bolzonella et al., 2006).

Clearly, the implementation of a co-digestion process should consider the final disposal of digestate: this issue needs to be properly addressed case by case. The beneficial properties of digestate as bio-fertiliser are well known, but the characteristics of digestate produced by a co-digestion treatment need to be considered against law limits. At the moment European Community rules governing the application of digestate in agriculture do not exist. The sludge application limits are defined by Directive 86/278/EEC, but more stringent limits were proposed by 3rd draft on sludge (EEC, 2000) and from End of Waste criteria (Saveyn and Eder, 2014). The aim of legislation is to prevent harmful effects on soil, vegetation, animals and human beings. Heavy metals content typically increases during anaerobic digestion (AD) because of the reduction of the organic content of sludge. Recently, more interest has been dedicated to organo-chlorine micro-pollutants (poly-chlorinated biphenyls – PCBs – and dioxin/furans – PCDDs/Fs–) and some limits are proposed by Saveyn and Eder (2014).

These compounds are recalcitrant and accumulate along the trophic chain but in anaerobic conditions could be partially degraded by reductive dehalogenation (Bertin et al., 2007). The same regulation defines limits for pathogens contents. In this case anaerobic treatment depletes pathogens in the WAS with variable efficiency depending on nature of pathogens, moreover process temperature (especially thermophilic) and hydraulic retention times (HRT) could inactivate pathogens within a few days of treatment (Sahlström, 2004).

Biosecurity is not the only aspect to influence sludge use: dewatering properties have consequences on logistic and management costs and should be considered in feasibility studies. Several factors affect dewaterability characteristics of sludge and experimental tests are necessary for their determination.

According to the above scenario, this study considered the anaerobic co-treatment of waste activated sludge and wine lees both at mesophilic and thermophilic conditions in pilot scale stirred reactors. Different organic loading rates were tested and the process could be considered stable, for both temperatures, only with OLR lower than 3 kgCOD/(m^3 d) (Da Ros et al., 2014). Stability parameters and biogas production in these operational conditions were monitored to determine the real benefits from anaerobic cotreatment. Moreover, digesters effluents characterization in terms of nutrients content, concentration of pollutants and pathogens was carried out, to evaluate the characteristics of digestates and their potential use as fertilisers or amendment for productive soils. This study is the first one, to authors knowledge, that provides a comprehensive evaluation of this process option: critical aspects and performances were determined and digestates were fully characterized to define their potential use as fertilisers.

2. Materials and methods

2.1. Experimental setup

AD tests were carried out at pilot scale, in mesophilic and ther mophilic conditions. Two identical continuous stirred reactors (CSTRs) of 230 L of working volume each were employed. The operational temperatures (37 °C and 55 °C) were monitored by PT100 probes that controlled hot water recirculation system in the external jackets of the reactors.

The seed sludge used as inoculum for the reactors was collected in the wastewater treatment plant located in Treviso (northern Italy) where a 2200 m^3 anaerobic digester treats waste activated sludge produced by wastewater treatment and separately collected biowaste at a working temperature of 35 °C.

In the thermophilic reactor the temperature was increased in one step, from mesophilic to thermophilic condition as suggested by Cecchi et al. (1993). Inoculum sludge, maintained without feeding, was then acclimatized for one month to the higher operational temperature and no instability conditions were observed in the reactors. The reactor feed was prepared daily, mixing thickened WAS and winery waste. The mixture of two substrates was pumped into reactors once a day to obtain hydraulic retention time of some 21 d and an organic loading rate of 2.8 $kgCOD_{fed}/(m^3$ d): 22% of the load (COD basis) was wine lee. The HRT was chosen to guarantee sufficient time for growth of anaerobic micro-organisms, for organic matter degradation and for hygienisation of the input materials. Pilot trials lasted one year to evaluate the long-term process stability.

2.2. Analytical methods

Substrates and effluents were monitored once a week in terms of total and volatile solids content (TS and VS), chemical oxygen demand (COD), TKN and total phosphorus. The process stability parameters, namely pH, volatile fatty acids (VFAs) content and speciation, total and partial alkalinity and ammonia, were checked daily. All the analyses, except for VFAs, were carried out in accordance with the Standard Methods (APHA–AWWA–WEF, 1998). Volatile fatty acids content was monitored using a gas chromatograph (Carlo Erba instruments) with hydrogen as gas carrier, equipped with a Fused Silica Capillary Column (Supelco NUKOLTM, 15 m x 0.53 mm x 0.5 μm film thickness) and with a flame ionization detector (200 °C). The temperature during the analysis started from 80 °C and reached 200 °C trough two steps at 140 and 160 °C, with a rate of 10 °C/min. The analyzed samples were centrifuged and filtrated on a 0.45 μm membrane. Gas production was monitored continuously by two gas flow meters (Ritter Company, drum-type wet-test volumetric gas meters), and its composition (CH_4–H_2–O_2–N_2) was defined by gas chromatography equipped with HP-Molesieve column (30 m x 0.3 mm x 0.25 μm film thickness) employing thermal conductivity detection (TCD).

Once steady state conditions were reached in the AD process, pollutants and heavy metals were determined in triplicate. Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/F), polychlorinated biphenyls (PCB) were analyzed according with US EPA 1613/B, 1994 and US EPA 1668/B, 2008 methods. The analytical methods provide determination of these compounds in sludge sample by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). The detection limits and quantization levels were estimated by internal standard. The heavy metals content were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The concentration of total polyphenols was measured using a modified version of the Folin–Ciocoltau reaction as reported in Laftka et al. (2007) and converted into Gallic acid equivalent (mg/L).

Pathogens analyses on wastes and on mesophilic/thermophilic digestate were carried out in order to evaluate both the pathogens content in the treated wastes and the quality of digested sludge compared with the limits requested for agricultural use (End of Waste criteria). Total coliforms, Escherichia Coli and Salmonellae spp. were analyzed considering IRSA-CNR methods (2006) for sludge sample.

The anaerobic process leads to modification of the structural matrix of sludge flocs and particles, affecting consequently particle size distribution, and dewaterability (Yan et al., 1987). Filterability characteristics of the raw and conditioned effluents were determined by capillary suction time (CST) tests using a CST instrument (Triton, A304M model), according with APHA–AWWA–WEF (1998) and specific resistance to filtration (SRF) according with IRSA-CNR (2006). These parameters are not comprehensive in its description of dewatering behavior, but they supply preliminary information and allow comparative considerations (Smollen, 1986).

Digestates produced were characterized against criteria defined in the EEC Directive 86/278, the 3rd draft of the new Directive on sludge (EEC, 2000) and the criteria defined in the document on End of Waste of digestate and composts proposed by the Joint Research Center of Sevilia.

2.3. Substrates origin

Waste activated sludge (WAS) used to fed the reactors was originated from a 70,000 people equivalent WWTP adopting a BNR process (Johannesburg scheme). The daily dry flow is some 19,000 m3/d municipal wastewater while the solid retention time and food to microorganism ratio applied in the activated sludge process were 15 d and 0.15 kg COD/kg MLVSS d respectively.

Wine lees were collected in a wine making facility producing sparkling white wines with a natural process. The cellar is located in north-east of Italy and produces more than 300 thousands of hectoliter of wine per year. The winery waste was collected weekly and it was used without any pre-treatment.

3. Results and discussion

3.1. Seed anaerobic sludge and substrates characteristics

Seed anaerobic sludge was analyzed at the beginning of the experimentation. Characteristics of the anaerobic digestion inoculum are shown in Table 1: pH was 8.2, ammonia content was 815 mg/L, VFAs concentration was lower than 1 g per liter (avg. 898 mg COD/L) while total alkalinity showed a sufficient buffering capacity (2531 mg $CaCO_3$/L at pH 4).

As for WAS, the solid content was around 3%, while the volatile fraction and COD content was relatively low: 73% and 77%, respectively, while the COD/VS was about 1.1 rather than a typical 1.4. This clearly indicated that this substrate was already stabilized in the activated sludge process due to the high SRT applied in the process. In fact, the anaerobic digestion of this material typically generated specific biogas productions lower than 0.2 $m^3/kgVS_{fed}$ (Bolzonella et al., 2005). Also nitrogen and phosphorus presented typical concentrations: 48 and 18 mg/gdw, respectively. The COD/N was relatively low, around 15.

The parameters that describe the organic matter content of WL (TS, VS and COD) showed high standard deviations because of the variability of this substrate: in fact they depended on the kind of wine produced in the cellar and on the alcoholic fermentation process adopted. The wine lees presented a total solid concentration of 85 g/kg, 91% volatile, and very high levels of COD: about 214 g/kg, 70% soluble. The soluble COD was mainly composed by VFAs (9 g COD/L on average), other organic acids (11 g COD/L) deriving from grape and fermentation process, the fermentation reactions generated also ethanol (8.4% v/v) and the residual sugar usually was more than 1 g/L (Setti et al., 2009). The polyphenols derived from grape and ranged from 1162 to 2556 mg/L, they were biodegradable compounds but, because of their antioxidant proprieties, they could be also considered as inhibitors for anaerobic digestion at high concentration. Similarly to WAS, the N and P contents were 70 and 14 mg/g_{dw} respectively, but nutrients content appeared unbalanced for bacterial growth if compared with high concentration of total COD (Table 1).

The inlet substrates and seed sludge were analyzed also in terms of heavy metals, polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/F), polychlorinated biphenyl (PCB), pathogens indicators.

Table 1. Characteristics of seed sludge, waste activated sludge and wine lees.

Parameter	Units	Seed sludge	Waste Activated Sludge	Wine lees
TS	g TS/kg$_{ww}$	19 ± 1.0	30 ± 4.5	85 ± 23.8
VS	g VS/kg$_{ww}$	12 ± 0.2	22 ± 3.3	78 ± 22.6
VS/TS	%	61 ± 1.2	73 ± 4.4	91 ± 1.8
pH		8.2 ± 0.6	6.5 + 0.2	4.6 ± 1.0
Conductivity	mS/cm	5.7 ± 0.8	0.9 ± 0.1	5.2 ± 3.3
$N\text{-}NH_4^+$	mg/L	815 ± 204	21 ± 8	743 ± 654
Volatile fatty acid	mg O_2/kg $_{ww}$	898 ± 180	< 200	9177 ± 435-
Total alkalinity	mg $CaCO_3$/L	2531 ± 532	376 ± 32	-
Soluble COD	mg/ kg $_{ww}$	-	-	153 ± 49
Particulate COD	mg/g$_{dw}$	759 ± 46	769 ± 77	952 ± 105
TKN	mg/g$_{dw}$	36 ± 6.5	48 ± 1.6	70 ± 5.6
P_{tot}	mg/g$_{dw}$	24 ± 1.2	18 ± 1.2	14 ± 4.1

3.1.1. Organo-chlorinated compounds

Distributions of PCDD/F congeners in WAS and seed sludge were similar and consistent with data reported in literature (Eljarrat and Barcelo, 2003). The results obtained for WAS were lower than those reported for sewage sludge contaminated by heavy polluted wastewater (Patureau and Trably, 2006): this is because biological sludge like waste activated sludge is typically cleaner than primary sludge (Mininni et al., 2004). The molecules with a higher number of chlorine atoms were the most abundant in both substrates. The reason of OCDD and HpCDD abundance is their high value of the octanol/water partition coefficient kow, corresponding to low solubility in water. Their chemical-physical properties and general abundance of highly chlorinated dioxins in incoming wastewater (Bolzonella et al., 2010a) had synergistic effect on accumulation in solid fraction of sludge. Their concentrations increased after the AD process: Disse et al.(1995) found that the increase of the PCDDs/Fs concentrations during AD is caused mainly by the accumulation of these compounds: this can be ascribed to the degradation of organic matter. However, PCDDs/Fs concentrations in terms of toxicity of sludge and digestate were one order lower than suggested values of 100 and 30 ng I-TE/kg$_{dw}$ reported in the 3rd draft of the sludge directive (EEC, 2000). Also PCBs were present at low concentrations if compared with proposed limits: 0.8 mg/kg$_{dw}$ (EEC, 2000) and 0.2 mg/kg$_{dw}$ (Saveyn and Eder, 2014).

Wine lees showed a more homogeneous presence of PCDDs/Fs (Figure 1), with a lower content of dioxins and furans, except for TCDD. Considering the toxicity of these compounds, different compounds concentrations were associated with very similar total toxicity. PCBs showed higher concentrations than PCDDs/Fs but their global value was lower than the proposed limit of 0.8 mg/kg$_{dw}$ reported in the 3rd draft of the sludge directive (EEC, 2000) and 0.2 mg/kgdw reported in the EoW document for composts and digestates (Saveyn and Eder, 2014). The concentration of PCB in wine lees was clearly lower than in WAS.

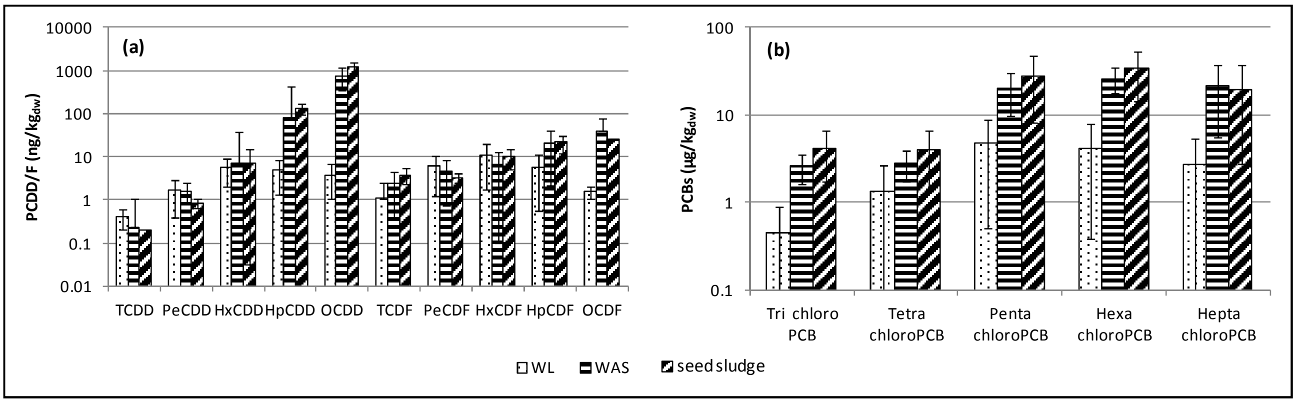

Figure 1. PCDDs/Fs (a) and PCBs (b)congeners profiles in wine lee, waste activated sludge and seed sludge.

3.1.2. Heavy metals content

The total concentrations of heavy metals determined in the substrates and in seed sludge are shown in Table 2. The relative abundance of metals in the waste and digested sludge followed the general order: Zn > Cu > Pb > Cr > Ni > Cd > Hg. Zn and Cu clearly were the most abundant compounds in both WAS and seed sludge. These concentrations depended by inlet wastewater characteristics (Bolzonella et al., 2010b). Moreover, it was observed that AD caused an increase in the heavy metal concentrations on a dry matter basis. In fact, since the determination of heavy metal contents was based on dry weight, the reduction of the organic matter during the anaerobic process caused the observed increase in heavy metal contents in the digested sludge. When considering wine lees, Cu dominated the total metals content (85%), because it is widely used in the vineyard and during wine-making process. The other most abundant metal was Zn with 14%. These results are consistent with those reported in Bustamante et al. (2008) except for Cd and Ni that were present at higher concentrations in WL in their study.

3.1.3. Pathogenic indicators

Comparing hygienic quality of substrate with law limits (EEC, 1986) for land disposal (*E. coli* lower than 500 UFC/g) it turns out clear that the sludge could not be used without a hygienisation step. The results about *E. coli* were similar to those reported by Payment et al. (2001) while *Salmonellae spp*. were present in one sample out of 6 examined, a value lower than the typical literature data (Sahlström et al., 2004). The WL did not show the presence of pathogenic agents. Considering the high biodegradability and the contents in pathogens of raw substrates, it is clear that they could not be disposed directly on agricultural soils. The anaerobic process could improve agronomic characteristics and biosecurity of the effluents.

Table 2. Concentrations of organic micro-pollutants, heavy metals and pathogens in seed sludge, waste activated sludge and wine lees.

Parameter	**Unit**	**Seed sludge**	**Waste activated sludge**	**Wine Lees**
PCDD/F	ng I-TE/kg_{dw}	5.643 ± 0.558	5.030 ± 0.508	5.240 ± 7.552
PCB	µg/kg_{dw}	48.389 ± 28.651	38.845 ± 15.247	0.833 ± 0.458
Cd	mg/kg_{dw}	1.9 ± 0.1	1.4 ± 0.3	0.1 ± 0.1
Ni	mg/kg_{dw}	31.9 ± 1.3	25.8 ± 3.9	1.7 ± 0.2
Pb	mg/kg_{dw}	154.2 ± 23.1	124.2 ± 60.9	6.2 ± 3.5
Cu	mg/kg_{dw}	355.8 ± 64.0	258.9 ± 30.2	801.0 ± 120.2
Zn	mg/kg_{dw}	1320.1 ± 79.2	1035.9 ± 62.2	131.9 ± 13.2
Cr	mg/kg_{dw}	63.7 ± 2.5	49.6 ± 7.4	3.2 ± 0.4
Hg	mg/kg_{dw}	0.6 ± 0.2	0.4 ±0.1	0.1 ± 0.1
Total coliforms	UFC/g_{ww}	$5.30\ 10^5 \pm 6.65\ 10^5$	$2.08\ 10^6 \pm 2.82\ 10^6$	$1.77\ 10^2 \pm 4.04\ 10^2$
E.coli	UFC/g_{ww}	$4.00\ 10^4 \pm 2.84\ 10^4$	$2.63\ 10^4 \pm 2.84\ 10^4$	6.67 ± 16.33
Salmonellae spp.	UFC/g_{ww}	Absent	Present in 1 sample out of 6	Absent

3.2. Anaerobic digestion yields

Different operational conditions, in terms of HRT and OLR, were tested. Trials with HRT of 19 and 16 d and OLR of 3.3 and 4.5 COD/m^3 d determined not steady processes, in particular VFA accumulated and biogas production reduced. The best performances were achieved with 2.8 kgCOD/m^3 d and 21 d of HRT (Table 3). The stability parameters (pH, alkalinity, ammonium concentration and volatile fatty acids) were monitored and were in the optimal range for AD process. Average biogas yields were 0.38 and 0.40 $Nm^3/kgCOD_{fed}$ at 37 °C and 55 °C, respectively, with about 65% methane. The higher biogas production in thermophilic conditions was coupled with greater hydrolysis and ammonification rates. The content of polyphenolic compounds were not as high to inhibit the process, while the increasing concentration of copper and ammonia in the reactors probably affected bacteria activity and caused a slight reduction in biogas production in the long term. More details about the process and the critical aspects found during the experimentation were reported in Da Ros et al. (2014).

3.3. Effluents characteristics

The average solid content in the effluents were 23 and 20 g TS per kg at 37 °C and 55 °C respectively with similar levels of volatile solids (about 69% of total solids). The polyphenols concentrations reduced of 88%

in mesophilic condition, but only 35% at 55 °C. Comparison of degradation of polyphenols at different conditions revealed a strong influence of temperature as reported in Da Ros et al. (2014). Ammonium concentrations were 1.29 and 1.35 $gN–NH_4^+/L$ in the mesophilic and thermophilic digestates, respectively, due to major ammonification rate increasing with temperature. Consequently, the total alkalinity and pH were higher in the effluent of the thermophilic reactor. Total COD content was 17.8 gCOD/kg in the mesophilic effluent, only 7% soluble, while the mesophilic one showed lower content of total COD (15.3 gCOD/kg) but 15% was soluble, half due to VFAs. Concentrations of particulate nitrogen and phosphorus were quite similar in the two effluents (Table 4).

Table 3. Operational conditions applied

Parameter	Unit	Average
Temperature	°C	37-55
Reactor Volume	m^3	0.230
HRT	days	20.6
Lee Flow	% Total flow	22
OLR	$kgCOD/(m^3d)$	2.8
Lee OLR	% OLR	75
Feed TS	$g\ TS/kg_{ww}$	43

3.3.1. Organo-chlorinated compounds

The concentrations of PCDDs/Fs found in the effluents of the two reactors were very low and analytical errors could be important: in fact, the coefficient of variation calculated for three replicates reached 50% of the average value in some samples. Therefore, little differences of concentration were not appreciable. Typical concentrations of PCDDs/Fs found in anaerobic digestion effluents were 1488 ng/kg_{dw} and 1155 ng/kg_{dw}: these values were consistent with those reported by Oleszek-Kudlak et al. (2005) and Fuentes et al. (2007). The ''apparent'' increase of PCDD concentrations after digestion was affected by the concentration of the persistent contaminants due to the degradation of the dry matter as reported by Disse et al. (1995). As a consequence, removal of PCDD/F was evident only if concentrations in terms of wet weight were considered. For example the most toxic dioxin, 2, 3, 7, 8 TCCD, was present at concentrations lower than 0.5 ng/kg_{dw}, both in the inlet and the effluents but considering the reduction of solids the mass of this congener reduced of 50%. On the contrary, the highly chlorinated dioxins (HpCDD and OCDD) were the most abundant: they represented 99% of the total dioxins in both digestates. In the mesophilic reactor an increase of HpCDD and OCDD, 26% and 69%, respectively, was observed as reported by some authors (Oleszek-Kudlak et al., 2005; Klimm et al., 1998): the formation of these compounds could be due to degradation of their precursor compounds as pentachlorophenol (Disse et al., 1995). As for other congeners, those showed similar levels in the inlet and effluent flows.

Table 4. Effluent characteristics and stability parameters for the two experimental conditions (avg: average, st.dev: standard deviation)

Parameter	**Units**	**37°C**	**55°C**
		Avg ± st.dev	Avg ± st.dev
TS	g TS/kg$_{ww}$	23.0 ± 3.7	19.6 ± 3.3
VS	g VS/kg$_{ww}$	16.1 ± 3.2	12.4 ± 2.0
VS/TS	%	68.6 ± 3.4	69.7 ± 7.7
pH		7.8 ±0.2	8.1 ± 0.2
Conductivity	mS/cm	9.2 ± 1.4	9.7 ± 1.4
$N\text{-}NH_4^+$	mg/L	1291 ± 255	1360 ± 323
Partial alkalinity (pH6)	g $CaCO_3$/L	3.42 ± 0.58	3.27 ± 0.49
Total alkalinity (pH4)	g $CaCO_3$/L	5.28 ± 0.84	5.38 ± 0.75
Total COD	g/kg $_{ww}$	17.8 ± 2.5	15.3 ± 0.2
Soluble COD	mg/kg $_{ww}$	1167 ± 841	2339 ± 656
VFA	mg O_2/kg $_{ww}$	302 ± 230	1125 ± 1031
Particulate COD	mg/g $_{dw}$	700 ± 252	704 ± 56
TKN	mgN/g $_{dw}$	48.7 ± 7.3	42.1 ± 6.7
P_{tot}	mgP/g $_{dw}$	25.5 ± 3.1	27.1 ± 2.7
Particulate COD/TKN	mg COD/mg N	14.7 ± 3.1	16.5 ± 2.8
Particulate COD/ P_{tot}	mg COD/mg P	28.1 ± 4.5	26.1 ± 3.1
Polyphenols (Gallic Acid)	mgGAE/L	51 ± 8.2	265 ± 183

The total concentrations of furans were 70 and 89 ng/kg$_{dw}$, at 37 °C and 55 °C respectively. The most abundant compounds were the highly chlorinated ones: in particular OCDF and 1, 2, 3, 4, 6, 7, 8-HpCDF contributed for 40% and 34% of the presence of total furans in mesophilic condition, and for 46% and 24% respectively in the thermophilic one. The other species ranged between 0.7 and 5.2 ng/kg$_{dw}$.

The distributions of PCDDs/Fs were similar to those reported by de Souza Pereira and Kuch (2005) where highly chlorinated congeners contributed for more than 95%. This outline indicated that the main source of PCDDs/Fs were thermal processes.

In terms of toxicity, PCDDs/Fs were 5.997 and 9.220 ngI-TE/kg$_{dw}$ for mesophilic and thermophilic digestates. The global toxicity showed similar levels in inlet and effluent streams, both in mesophilic and thermophilic conditions. The increase of toxicity in the digestates was not associated with formation of ‘‘new’’ toxic compounds but with the reduction of solids: in fact considering the mass balance on a wet weight basis the removal of PCDDs/Fs was observed in both the reactors. At the moment limits about PCDDs/Fs are not defined at European level but these values could be compare with limit suggested by Saveyn and Eder (2014) of 30 ng/kg$_{dw}$, much higher than levels found in digestates.

PCBs data in literature are generally expressed as sum of 7 congeners (28, 52, 101,118, 138, 153 and 180) on dry weight basis. In this study the concentrations in mesophilic and thermophilic digestates were similar, with 46.4 and 41.9 μg/kg$_{dw}$. Considering the mass balance, the total concentrations in the effluents could be considered equal to inlet (observed differences were lower than 20%), probably due to low bacterial capability of degrading these compounds. In fact, low concentrations of pollutants were generally insufficient for the stimulation of degradative enzymes or to support growth of competent organisms or maybe the mean cellular retention time was inadequate for the growth of degrading bacteria (Borja et al., 2005). These concentrations are consistent with values reported in literature (Dąbrowska and Rosińska, 2012). The concentrations of PCBs expressed on wet weight basis, considering the differences in terms of solid content, become 1.07 and 0.82 ng/kg, at 37 °C and 55 °C, respectively. The higher removal efficiency in thermophilic conditions was strongly related to high solids reduction (Patureau and Trably, 2006). The lower content of solid caused minor adsorption of PCBs on particulate fraction and increased the solubility and availability of pollutants for microorganisms. The affinity of PCBs to particle matter increased with the number of chlorine atoms, then, considering the lower content of solids in thermophilic digestate, the organic pollutants became more available and degradation of Hexa- and Hepta-PCB was higher than in mesophilic conditions at 37 °C (Figure 2). The degradation of PCB occurs by reductive dehalogenation and consequently low chlorinated compound can be generated (Dąbrowska and Rosińska, 2012). Although the increase of relative content of low chlorinated PCB, the distribution of congeners remained similar to the one of WAS and contributes for 87% of total PCB in the inlet mixture. Some European Countries imposed PCB limits of 0.2 mg/kg$_{dw}$ for use of digestate as ammendant and this value was suggested also by Saveyn and Eder (2014). The concentrations of PCB in digestate observed in this study were far below the reported threshold limits.

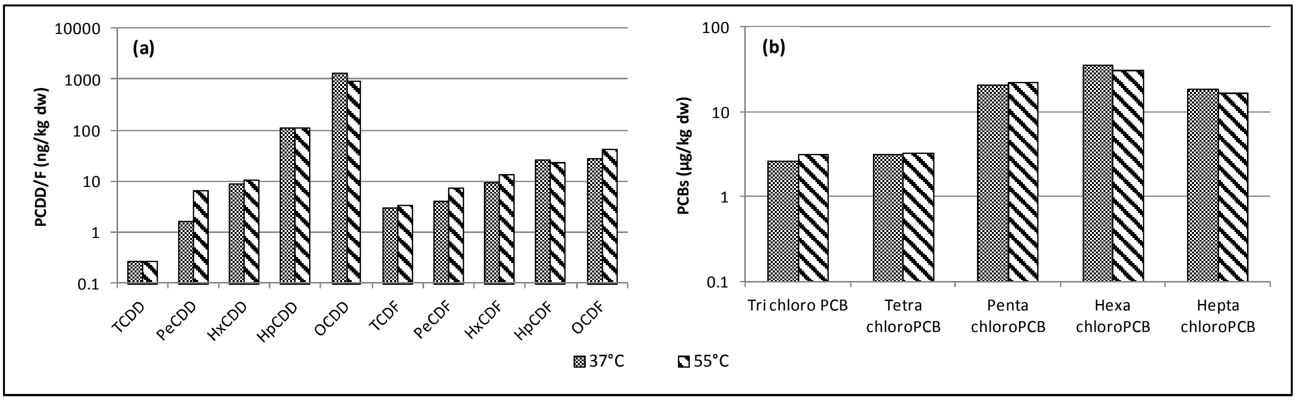

Figure 2. PCDDs/Fs (a) and PCBs (b) congeners profiles in effluent of mesophilic and thermophilic anaerobic digestion.

3.3.2. Heavy metals content

Concentrations of heavy metals found in digestates in this study are reported in Table 5. The most abundant metals in the effluents were Zinc (1199 and 1121 mg/kg$_{dw}$), then Copper (929 and 927 mg/kg$_{dw}$), Lead (115 and 99 mg/kg$_{dw}$), Chrome (48 and 43 mg/kg$_{dw}$), Nickel (26 and 24 mg/kg$_{dw}$), Cadmium (1.6 and 1.4

mg/kg$_{dw}$), and finally Mercury (0.4 and 0.3 mg/kg$_{dw}$). The difference between the mesophilic and thermophilic effluents were negligible.

Table 5. Content of heavy metals, organic micro-pollutants and pathogens in the effluents (avg: average, st.dev: standard deviation)

Parameter	Units	37°C	55°C
		Avg ± st.dev	Avg ± st.dev
PCDD/F	ng I-TE/ kg $_{dw}$	5.997 ± 2.339	9.220 ± 3.965
PCB*	µg/ kg $_{dw}$	46.41 ± 6.033	41.93 ± 20.126
Cd	mg/kg$_{dw}$	1.6 ± 0.3	1.4 ± 0.2
Cr	mg/kg$_{dw}$	48.4 ± 1.6	42.7 ± 5.1
Cu	mg/kg$_{dw}$	929.0 ± 319.6	927.1 ± 345.3
Ni	mg/kg$_{dw}$	25.8 ± 1.4	24.2 ± 1.9
Pb	mg/kg$_{dw}$	114.6 ± 32.2	99.4 ± 21.7
Zn	mg/kg$_{dw}$	1198.9 ± 76.9	1120.7 ± 128.9
Hg	mg/kg$_{dw}$	0.4 ± 0.1	0.3 ± 0.1
Total coliforms	UFC/g	3.63 10^4 ± 2.82 10^4	3.50 10^3 ± 4.36 10^3
E.coli	UFC/g	2750 ± 2400	333 ± 516
Salmonella spp.		Absent	Absent

The concentrations of heavy metals could be compared with digestate derived by AD of municipal sludge (Dąbrowska and Rosińska, 2012). Contents of Cr, Ni, Pb and Hg were consistent with those reported by Alvarez et al. (2002), while Cd was present at low concentration. Both Cu and Zn showed relatively high values. Winery waste contributed for more than 70% in terms of Copper mass in the inlet mixture.

Comparing the inlet and effluent concentration, their relative contents, on dry weight basis, were respected but they increased during biological treatment. The increase in heavy metal contents in digestate was expected because biodegradable organic matter is decomposed to end products by anaerobic microorganisms and the inert elements concentrated. Most increasing concentrations were observed for Cd, Ni and Cu (more than 60%), while Hg showed the lowest variations.

Distribution of heavy metals, after anaerobic process, was studied by some authors (Bin et al., 2012; Alvarez et al., 2002) who agreed in considering two main groups: the first includes mobile forms (metals in ionic form or bound to amorphous iron and manganese oxides and hydroxides) and the second considers the less available forms (metals bound with organic matter, sulfides and crystalline iron and manganese oxides). Cu, Cr, Pb and Cd are present in immobile fraction, then Cu, that in our study showed relatively high concentrations, was not available for its high affinity for the organic matter, in accordance with the high stability constant of the copper complexes with organic matter of this metal.

The distribution of Ni was not clear, usually 60–70% of metal in the digestate is immobilized but Bin et al. (2012) reported that bioavailability of Ni increased during the process. Also considering Zn, there were some discordances in terms of fractions distribution but it was evident a lower affinity with organic matter, thus higher bioavailability. It is then important to remember that Zn and Cu are considered micro-nutrients, therefore, their presence in digestate is considered an added value for corps growing (Saveyn and Eder, 2014). Comparing the heavy metal concentrationa of effluents with the limits of Directive 86/278/EEC the limits were respected, while considering more stringent values proposed by 3rd draft and end-of-waste criteria (Saveyn and Eder, 2014) Cu exceeded the limits. Cu, which presence is determined by winery waste, is the factor limiting the amount of wine lees which can be co-digested with waste activated sludge to preserve digestate quality.

3.3.3. Pathogenic indicators

The results of pathogens determination highlighted the sanitation effect of AD in both temperature conditions. Clearly, as expected, the thermophilic condition showed the best performances in terms of sanitation. *Salmonellae spp.* were never detected in both the digestates: it was totally eliminated during the experimentation as reported by Gantzer et al. (2001). More differences, between the two operational conditions, were observed for Coliforms presence. The Total Coliforms concentrations were $3.63 \cdot 10^4$ and $3.50 \cdot 10^3$ at 37 °C and 55 °C on average, respectively. The corresponding reductions were 1.4Log and 1.8Log. Total Coliforms concentration was used as indicator of presence of pathogens, it includes both fecal coliforms and E. coli. These values enabled the development of a rough outlook of the degree of disinfection taking place inside the digesters and was confirmed by *E. coli* presence. *E. coli* had concentration of $2.7 \cdot 10^3$ and $3.3 \cdot 10^2$, then AD reduced the content of 1.3 Log and 1.7 Log, at 37 °C and 55 °C. High operation temperature, with retention time of 21 d, ensured total sanitation of sludge and met well the requirements established by Directive 86/278/EEC and proposal of IPTS in terms of concentration, but not for *E. coli* reduction. Sahlström et al. (2004) reported complete *E. coli* depletion during thermophilic digestion, while in this study coliforms were still present in the effluent, probably because of semi-continuous feeding mode. The mesophilic digestate would instead need a further process to reach the law limits.

3.4. Dewatering properties of the effluents

Dewatering properties of digestates and optimal dose of cationic polymer conditioning (Hidrofloc C 675-Hydrodepur) were evaluated by CST and SRF tests. These data are interesting considering the dewatering capability of digestate, an important parameter for reducing sludge volume and, consequently, the cost of transporting sludge to its final disposal site.

The digestates were difficult to filter without conditioning; in particular the SRF values were $4.42 \cdot 10^{13}$ and $9.09 \cdot 10^{13}$ m/kg_{dw} and CST values of 969 and 844 s for mesophilic and thermophilic digestates. The ratios between CST and solid concentration in the digestates were calculated to compare substrates with different characteristics. CST/TS ratios became practically equal in mesophilic and thermophilic condition, 48.4 and

48.0 s · kg/g_{dw} respectively. The AD had a negative effect on filterability of sludge as reported by varies authors (Yan et al., 1987) because the process determined reduction of particle size, modification of the structural matrix of sludge flocs and particles, degradation of EPS and increase of soluble proteins and polysaccharides. Consequently filtration properties got worse and the need for chemical conditioning increased. The operational temperature had no significantly effect on dewaterability of raw sludge. The optimal polymer dose was experimentally calculated considering different addition of polymer to sludge until it became mechanical filterable: when SRF and CST values were lower than $5 \cdot 10^{12}$ m/kg and 300 s. The optimal dose of polymer was higher than 25 g/kg_{dw}, typical value for zootechnical digestates and corresponding to more of double of dose used for waste activated sludge alone. Then the costs for dewaterability became a relevant aspect in the choice of the application of this process.

4. Conclusion

The anaerobic co-digestion of waste activated sludge and wine lees was studied at pilot scale: it was found that biogas yields were of 0.38 and 0.40 Nm^3/kgCOD, 65% methane, in mesophilic and thermophilic conditions respectively. Digestates produced were characterized against criteria defined in the EU Directive 86/278, the 3rd draft of the new Directive on sludge (EEC, 2000) and the criteria defined in the document on End of Waste of digestate and composts proposed by the Joint Research Center of Sevilia. It was observed a good level of stabilization, with a reduction of about 60% of total and volatile solids. With reference to pollutants it was found a removal of 50% of TCDD, while content of other PCDDs/Fs and PCB remained quite constant or increased slightly because of the total solids reduction. Concentrations of Cu and Zn in the digestates were about 930 and 1200 mg/kg_{dw} respectively, because of high content of these metals in raw substrates, especially wine lees: these two elements, with their concentrations, determine the real limit for the co-digestion process. The amount of wine lees to be co-digested is determined on a mass balance basis according to Cu and Zn presence. As for pathogens, *Salmonellae spp.* indicator was not present in the effluent and anaerobic digestion reached 1.3Log and 2.7Log reduction in mesophilic and thermophilic conditions, clearly showing the importance of the use of 55 °C instead of 37 °C in this specific aspect.

As a final remark, the anaerobic digestion, especially at thermophilic temperature, represents a promising treatment to generate renewable energy and effluents with good agronomic characteristics.

Acknowledgments

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3.3. Management of winery waste inside the cellar borders

Large cellars are often equipped with winery wastewater aerobic treatment plant. This process is easy to manage but it has high expenses due to energy and chemicals requirement, and to waste activated sludge disposal . Coupling conventional wastewater treatment with anaerobic co-digestion would allow for reduce final downstream to dispose of, and for improve energetic balance of the cellar. The paper " Mesophilic and thermophilic anaerobic co-digestion of winery wastewater sludge and wine lees: An integrated approach for sustainable wine production" described this approach simulated at pilot-scale and shows the criticism of process operating under thermophilic condition. The manuscript demonstrated also the benefit of metal augmentation in thermophilic process that is a topic dealt with in section 3.6. In this study the dewaterability of the digestates were analyzed but a further investigation about the effect of metals on dewaterability should be kept in consideration.

The experimental design was set-up with support of P. Pavan and C. Cavinato, while D. Bolzonella was involved into elaboration of the data and paper writing.

Research article

Mesophilic and thermophilic anaerobic co-digestion of winery wastewater sludge and wine lees: An integrated approach for sustainable wine production

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Keywords: Anaerobic digestion, Dewatering, Mesophilic, Thermophilic, Trace elements, Winery wastes

Abstract

In this work, winery wastes generated by a cellar producing approximately 30,000 m^3 of wine per year was monitored for a period of one year. On average, 1.96 m^3 of wastewater, 1 kg of waste activated sludge (dry matter) and 16 kg of wine lees were produced per cubic meter of wine produced. Different winery wastes, deriving from different production steps, namely waste activated sludge from wastewater treatment and wine lees, were co-treated using an anaerobic digestion process. Testing was conducted on a pilot scale for both mesophilic and thermophilic conditions. The process was stable for a long period at 37° C, with an average biogas production of 0.386 $m^3/kg\ COD_{fed}$. On the other hand, for thermophilic conditions, volatile fatty acids accumulated in the reactor and the process failed after one hydraulic retention time (23 days). In order to fix the biological process, trace elements (iron, cobalt and nickel) were added to the feed of the thermophilic reactor. Metals augmentation improved process stability and yields at 55° C. The pH ranged between 7.8 and 8.0, and specific gas production was 0.450 $m^3/kg\ COD_{fed}$, which corresponded to dry matter and COD removals of 34% and 88%, respectively. Although the observed performances in terms of biogas production were good, the thermophilic process exhibited some limitations related to both the necessity of metals addition and the worse dewaterability properties. In fact, while the mesophilic digestates reached a good dewatering quality via the addition of 6.5 g of polymer per kg of dry matter, the required dosage for the thermophilic sludge was greater than 10 g/kg of dry matter.

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1. Introduction

The wine-making process produces large volumes of waste streams, including solid organic waste, wastewater, greenhouse gases, and packaging waste (Lucas et al., 2010). Winery wastewater is a major waste stream resulting from a number of activities that include tank, floor and equipment washing; barrel cleaning; wine and product losses; bottling facilities; filtration units; and rainwater captured in the wastewater management system (Ioannou et al., 2014). The quantification of the produced wastewater is difficult, and it depends on the cellar dimensions and the technologies applied. In general, wastewater production ranges from 0.7 to 14 L per liter of wine produced (Andreottola et al., 2009), but specific studies conducted in different countries demonstrated that typical values are approximately 2-6 L of wastewater per liter of wine produced (a short review of winery wastewater in the main production countries is available in Supplementary Material).

This effluent generally presents a considerable level of COD, the major part of which is soluble (Beck et al., 2005) and highly biodegradable (Andreottola et al., 2005) due to the presence of ethanol, sugars, and organic acids (Malandra et al., 2003; Mosteo et al., 2008; Petruccioli et al., 2000; Vlyssides et al., 2005). Because of its characteristics, this stream is generally treated using either aerobic or anaerobic processes (Ioannou et al., 2014). Among biological processes, the activated sludge process is the most commonly employed because of its high efficiency and simplicity. It can remove 98% of COD and cope with large variations in the hydraulic and pollution load (Beck et al., 2005; Fumi et al., 1995; Petruccioli et al., 2000).

The removal of organic material generates considerable quantities of excess sludge, normally in the range 0.21-0.28 kg MLVSS (mixed liquor volatile suspended solids) per kg of COD removed (Brucculeri et al., 2005; Torrijos and Moletta, 1997). Ruggieri et al. (2009) estimated that dewatered wastewater sludge represents 12% of the total organic solid waste produced by wineries and that its management via external companies is expensive and often difficult. An alternative to valorize this waste stream could be the use of an anaerobic digestion process. Anaerobic digestion (AD) is a mature technology and it is applied to treat different types of organic wastes (municipal solid wastes, sewage and waste activated sludge, agro-industrial residues, livestock effluents, etc.) and to reduce their biodegradability while simultaneously recovering bio-energy. The combination of the conventional activated sludge process (CAS) and AD is a common practice in municipal wastewater treatment plants and limits the external management costs for sludge disposal thanks to a reduction in the sludge volume. Biogas is a renewable source of energy that is usable inside the same production process and/or wastewater treatment plant, which reduces the energy requirements (Shen et al., 2015). Moreover, digestate, the effluent from the anaerobic process, can be reused in agricultural fields because of the presence of nutrients such as N, P, and K together with stabilized C and humic substances. AD removes pathogens and polyphenolic compounds with different efficiencies based on the operating conditions used. Pathogen reduction is affected by temperature, retention time and fed substrates (Poudel et al., 2010; Sahlstrom et al., 2004), whereas the efficiency of polyphenols degradation is mainly determined by the operational temperature (Cavinato et al., 2014; Leven and Schnürer, 2005).

Once AD is implemented for winery wastewater WAS, other wine-making process residues (e.g., wine pomace, pressed cake, or lees) should be co-treated to increase the biogas production, to improve the reactor utilization and to make the anaerobic process more economically advantageous. Wine lees (WL) in particular are an interesting co-substrate because of their biodegradability and availability throughout the year. Like wastewater, WL contain a high organic content and their disposal requires the appropriate treatment. The composition of WL depends on the wine-making technology, although, according to de Bustamante and Temiño (1994), the main characteristics are an acidic pH (between 3 and 6), a COD greater than 30,000 mg/L, potassium in concentrations greater than 2500 mg/L, and phenolic components in quantities up to 1000 mg/L.

This paper considers the production of winery waste activated sludge and lees and their anaerobic co-digestion under both mesophilic and thermophilic conditions. The study assesses the process feasibility at pilot scale and evaluates the effluent quality in terms of pollutant removal and dewatering capacity. The suggested approach is schematically represented in Figure 1.

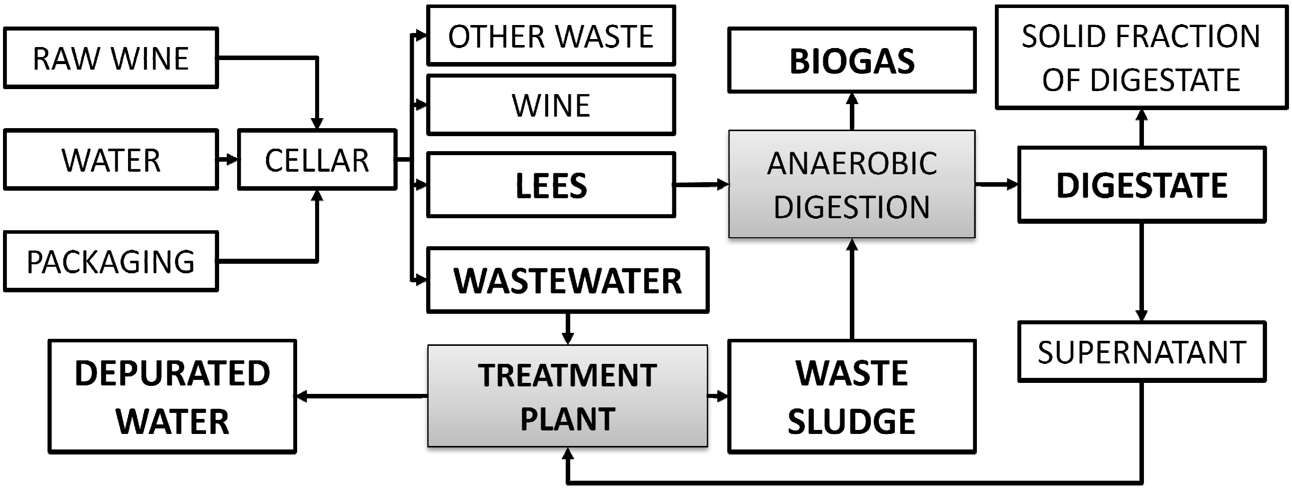


Figure 1. Integration of anaerobic digestion in the wine-making process

2. Materials and methods

2.1. Experimental set-up

2.1.1. Winery wastewater treatment plant

Waste activated sludge was collected in a cellar where a wastewater treatment plant was operating. The cellar was located in the northeast of Italy and produced approximately 30,000 m^3 of wine per year. It processed and bottled both self-produced and bought wines; therefore, the working period is not restricted to the grape harvest, but rather, it is distributed throughout the year. Therefore, there is no real seasonal variation in the output.

Considering the wine production and winery wastewater flow in the monitored cellar during a one-year period, the specific wastewater generation was calculated to be 1.96 L of wastewater per liter of wine produced. Winery wastewater was treated in the internal WWTP. After pre-treatment (screening and primary sedimentation), the wastewater is sent to a 1400 m^3 aerobic bioreactor. In order to balance the nutrients ratio and improve the activated sludge activity, urea and ammonium phosphate were added to the biological reactor. The activated sludge process operated with average hydraulic and sludge retention times (HRT and SRT) of 6.7 d and 35 d, respectively. Considering the HRT and SRT values, the volume of biological reactor is oversized in order to withstand the load picks. The MLVSS was 3010 mg/L and the corresponding food to microorganisms ratio was 0.26 kg COD/kg MLVSS per day. The treated water and waste activated sludge are separated in a secondary sedimentation tank. The treated water is eventually disinfected and filtrated using quartz sand before discharging. The sludge treatment process consists of a thickening section followed by a filter press. The sludge is not stabilized. On average, 3858 kg of wet sludge, with a dry mass content between 15 and 20%, was produced per week. This corresponded to some 6.13 tonnes of dry matter per week, or 1 kg of dried sludge per cubic meter of wine produced. The dewatered sludge is usually managed by composting, which has an average cost of approximately 110 €/tonnes of fresh matter.

Inlet and effluent streams for the wastewater treatment plant were monitored for one year to determine their characteristics (Table with winery wastewater and effluent characteristics is available as Supplementary Material). Additionally, dewatered sludge after filter pressing was collected and analyzed.

2.1.2. Pilot scale anaerobic reactors

Two parallel continuous stirred tank reactors (CSTRs) with working volumes of 230 L were employed for anaerobic co-digestion tests. Mesophilic (37 °C) and thermophilic (55 °C) conditions were maintained by externally jacketed hot water recirculation systems. PT100 probes monitored the process temperatures and managed the water recirculation pumps. Biogas production was continuously monitored using a drum-type gas flow meter (Ritter, Bochum, Germany). The pilot-scale reactors were filled with mesophilic and thermophilic inoculum digestates derived from previous experiments using municipal waste activated sludge and fermentation lees, maintained at the same temperature conditions.

The inocula were well stabilized, with solid contents lower than 10 g TS/kg_{ww} and stability parameters in the optimum ranges for AD (Table 1). The low content of COD in the inocula indicated the absence of organic matter that could affect the process, whereas the nitrogen and phosphorus concentrations were 41.6 and 33.1 mg N/g_{dw}, and 27.7 and 26.8 mg P/g_{dw} at 37 °C and 55 °C, respectively. The activities of seed sludges were evaluated during a start-up period, whereas the organic loading rate and wine lees content in the feed mixture increased stepwise. During this period, the anaerobic microorganisms acclimated to the substrates and to different readily biodegradable compounds present in the winery by-products, and the biogas production increased according to OLR. At the end of start-up, organic loading rate was 3.2 kg COD/(m^3d) and the HRT was 23 d. The distribution of the organic load between the two co-substrates considered the real waste flows: 80% was due to WL and the remaining 20% was due to waste activated sludge (WAS). Once the operational conditions were reached, the tests were conducted for several HRTs to obtain steady state biogas production, stability parameters, and digestate characteristics.

Table 1. Inocula characteristics. [a] pCOD: particulate COD, [b] sCOD: soluble COD

Parameter	Unit	37°C inoculum	55°C inoculum
TS	g TS/kg_{ww}	8.84	9.37
VS	g VS/kg_{ww}	5.92	4.69
VS/TS	%	67%	50%
pCOD [a]	g/kg_{ww}	4.9	7.0
sCOD [b]	g/kg_{ww}	0.9	1.1
pH	-	7.53	8.33
TKN	mg N/g_{dw}	41.63	33.09
Ammonium	mg N/L	193.4	539.4
P_{tot}	mg P/g_{dw}	27.7	26.8
Polyphenols	mg GAE/L	83.75	58.35

2.2. Analytical methods

The substrates and the digester effluents were collected and monitored once a week to determine the total and volatile solid content (TS and VS), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), and total phosphorus (P_{tot}) (American Public Health Association et al., 1999). The process stability parameters, pH, volatile fatty acids (VFAs) content and composition, total and partial alkalinity, and ammonia concentration were checked two or three times per week. VFAs content was monitored using a gas chromatograph, as reported by Cavinato et al. (2014). At steady state conditions, the total polyphenols were analyzed spectrophotometrically using the Folin Ciocalteu assay (Lafka et al., 2007). The concentration was reported in terms of Gallic acid equivalent per liter (mg GAE/L). Biogas composition (CO_2, CH_4, H_2, and O_2) was determined by a gas chromatograph (GC Agilent Technology 6890 N) equipped with a column HP-PLOT MOLESIEVE, 30 x 0.53 mm ID x 25 mm using a thermal conductivity detector and argon as the gas carrier.

The anaerobic process leads to changes in the structural matrix of the sludge flocs and particles, consequently affecting the particle size distribution and dewaterability (Yan et al., 1987). Filterability characteristics of the raw and conditioned effluents were determined by a capillary suction time (CST) test using a CST instrument (Triton, A304 M model) according to the standard method (American Public Health Association et al., 1999) and by specific resistance to filtration (SRF), as reported by IRSA - CNR (1985).

3. Results and discussion

3.1. Substrates characterization

The substrates fed to the reactors were waste activated sludge from secondary sedimentation tank of plant treating winery wastewater and wine lees, both of which originated from the same cellar.

The solids in the dewatered WAS generally ranged from 129.0 to 193.7 g TS/kg$_{ww}$. However, outliers were detected due to technical reasons (conditioner doses and filter press setting, Table 2). The volatile solids to total solids ratio (VS/TS) in the winery was higher (88%) than for typical sludge from municipal wastewater, probably due to the high biodegradability of the raw wastewater. The high amount of volatile solids and COD concentration (868 mg/g $_{dw}$) were indicative of the low biological stability of the WAS. However, the nutrients were well balanced for biological stabilization (Table 2) with a COD:N:P ratio of 124:7:1. A chemical analysis of the WAS showed limited contamination by metals (Cd < 0.5 mg/k $_{dw}$, Cr^{6+} <0.5 mg/kg$_{dw}$, Cr 46 mg/kg $_{dw}$, Hg < 0.1 mg/kg $_{dw}$, Ni 18 mg/kg $_{dw}$, Pb 7 mg/kg $_{dw}$, Cu 280 mg/kg$_{dw}$, and Zn 97 mg/kg $_{dw}$). Therefore, it is possible to apply the WAS on land as an amendment (Directive 86/278/CEE, Italian Decree 99/1992).

Wine lees were formed during the wine decanting step by adding bentonite (a fine clay used to remove suspended solids in the wine). Approximately 10 tonnes of lees were produced per week, corresponding to 1.6 kg/hl of wine produced. In this cellar, the typical wine lees production was lower than the reported average production from Italian wineries of 6 kg/hl (Laraia et al., 2001) because only part of the wine is processed within the cellar. WL were usually aimed at distillation and in any case need to be managed to avoid any soil contamination (Fernandez-Calviño et al., 2015). The use of wastes derived from wine-making processes as an amendment can generate different phytotoxic substances that may influence the germination of some plant species (Bustamante et al., 2008; Moldes et al., 2008). Wine lees from both red and white wine processing were collected during experimentation to evaluate substrate variability and how it affects the process. Approximately 90% of wine lees samples had solid concentrations between 37.9 and 77.2 g TS/kg$_{ww}$. However, extreme values were also detected (Table 2). Generally, these winery residues were characterized by a low content of volatile solids (57% of total solids) due to the presence of bentonite. The COD was mainly present in its soluble form (sCOD was the 83% of total COD), whereas the particulate fraction was typically between 48.3 and 205.1 g COD/kg The nitrogen and phosphorus levels were limiting factors for bacterial growth compared with the COD concentration. In fact, the COD:N:P ratio was 502:5:1, which is consistent with values reported by (Paradelo et al., 2013). Polyphenols were also detected in samples. Polyphenolic compounds are a large and complex family of substances characterized by the presence of large, multiple phenol structural units (Battista et al., 2015). They are originally synthesized by plants as a defense against pathogens and are extracted from the grapes during the wine-making process. The concentration of polyphenolic compounds in WL varies greatly from 260 to 3980 mg GAE/L.

Considering the typical concentrations of COD and nutrients in the waste activated sludge and wine lees, the anaerobic co-digestion of these two wastes may be considered an optimal option for their treatment. The process was tested at the pilot scale using continuous reactors.

Table 2 Waste activated sludge and wine lees characteristics. nd: not determined

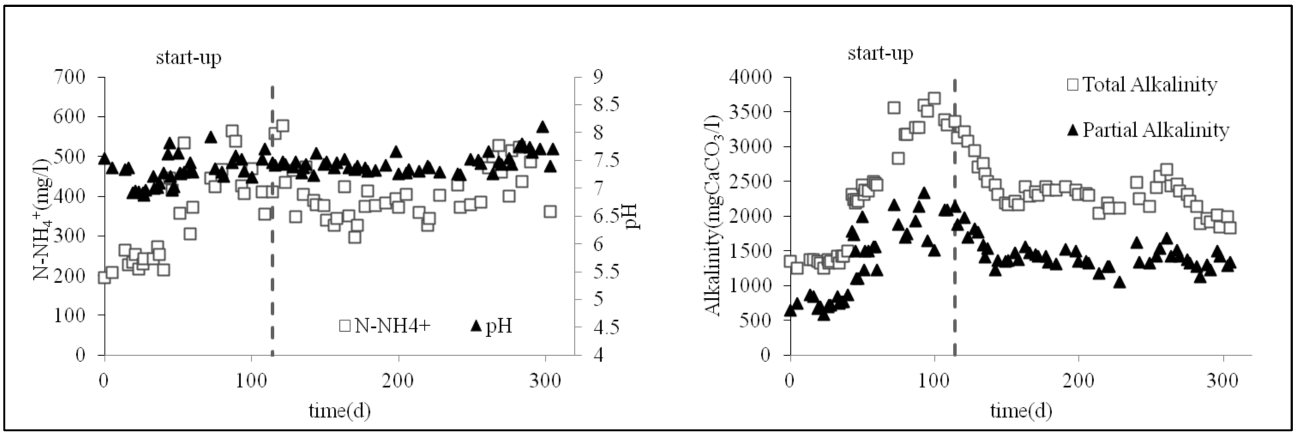
Parameter	Unit	Waste Activated Sludge			Wine Lees		
		average	St.dev	Range	average	St.dev	range
TS	**gTS/kg_{ww}**	158.9	49.3	22.7-267.8	62.0	27.9	12.3 -120.0
VS	**gVS/kg_{ww}**	143.5	41.6	20.7 – 237.3	33.6	15.1	10.3 -73.0
VS/TS	%	88%	3	79- 93%	57%	13%	29 - 86%
COD	**mg/g_{dw}**	868	69.4	749-1008	559	151	312 – 919
sCOD	**g/L**	nd	nd	nd	167	45	111 -204
TKN	**mg N/g_{dw}**	52.7	16.3	14.5 -80.3	30.3	12.7	9.7 -68.7
NH_4^+	**mg N/L**	nd	nd	nd	33.9	22.7	6.7 – 95.3
P_{tot}	**mg P/g_{dw}**	7.3	2.0	2.5 -10.7	6.2	2.9	2.6 - 14.3
Polyphenols	**mg GAE/L**	nd	nd	nd	1537	1189	260-3980

3.2. Performance of the mesophilic anaerobic co-digestion process

The organic load was gradually increased during the start-up phase, from day 1 to day 114. In particular, a constant quantity of sludge was used (equivalent OLR of 0.6 kg COD/(m^3d)), and the amount of wine lees fed to the reactor was increased gradually from 0 to 2.6 kg COD/(m^3d). A prolonged start-up phase and progressive increase in the OLR promoted the biomass adaptation. Within this transient period, the stability parameters of the mesophilic reactor improved. The total alkalinity reached 3690 mg $CaCO_3$/L because of the increase in ammonium, whereas the pH remained at 7.5. At the same time, the gas production rate rose from less than 0.1 $m^3/(m^3_{reactor}d)$ to 1.2 $m^3/(m^3_{reactor}d)$.

At the end of the start-up phase, the organic loading rate was 3.2 kg COD/(m^3d) and HRT 23 d. These conditions were determined as optimal for the process. Once the operational conditions were stable, the alkalinity decreased until reaching a stable value of 2248 mg$CaCO_3$/L. The pH did not change significantly and ranged between 7.2 and 7.8. The ammonium concentration was the most variable parameter (Figure 2) because of high degree of variability in the substrate characteristics. This fluctuation did not affect the process stability.

Based on the stability parameters, the process did not exhibit any particular difficulties, and over a long period, the performance improved. The total solid and COD concentrations decreased with time to average values of 24 gTS/kg_{ww}, 58% volatile, and 13.6 gCOD/kg, respectively. The particulate COD removal was coupled with a slight increase in the soluble COD, which was lower than 1 gCOD/kg. The 40-50% of the soluble COD was due to the presence of volatile fatty acids, with acetic acid being the dominant volatile fatty acid (52% of the total VFAs) and propionic acid being the second most abundant (12% of the total VFAs).



(a) (b)

Figure 2. Trend of pH and ammonium concentration (a), partial and total alkalinity (b) under mesophilic condition.

The nutrient concentrations generally decreased until reaching steady values of 37 mg N/g_{dw} and 9 mg P/g_{dw}. The average COD:N:P ratio in the effluent digestate was 70:4:1. Thus, the solid digestate had a good fertilizing quality and a high phosphorus content.

The amount of polyphenolic compounds were in the range from 20 to 80 mg GAE/L during the start-up phase. Their concentration decreased to lower than 40 mg GAE/L during steady state conditions. The anaerobic microorganisms adapted to these compounds and were eventually able to degrade 94% of the inlet polyphenols.

Biogas production varied significantly depending on the characteristics of the wine lees in the inlet. The average specific biogas production and gas production rates were 0.386 $m^3/kgCOD_{fed}$, and 1.2 $m^3/(m^3_{reactor}$ d), respectively. Values as low as 0.30 $m^3/kgCOD_{fed}$ were also observed (Figure 3), but they were not indicative of instability problems. The methane content ranged from 64 to 73% during the day due to degradation of the different types of compounds.

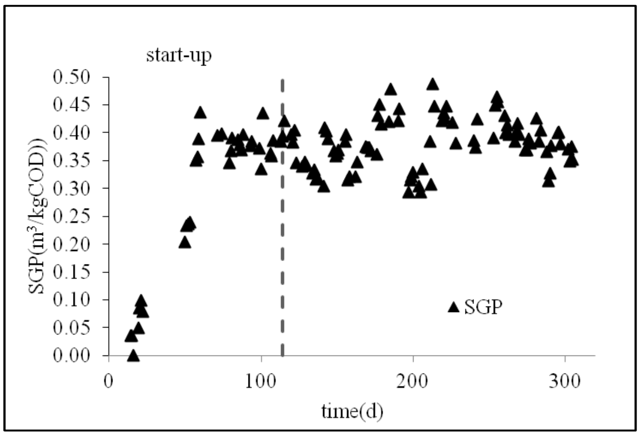


Figure 3. Trend of specific gas production under mesophilic condition.

Based on the mass balances around the system, the solid conversion efficiency of the organic feed into biogas was 28%, whereas 79% of the COD in the inlet was removed. Most of the biogas derived from the degradation of soluble COD (92%). Additionally, the ammonification of organic nitrogen, which represented approximately 99% of the total nitrogen in the inlet, was limited by the hydrolysis process, and ammonium nitrogen in the effluent accounted for 32% of the total nitrogen. This value was consistent with protein degradation efficiencies after WAS anaerobic digestion (Bougrier et al., 2007; Pinnekamp, 1989; Yang et al., 2015). Digestate supernatant may be capable of acting as a source of nutrients for the wastewater treatment and to reduce the required urea and ammonium phosphate addition.

3.3. Performance of the thermophilic anaerobic co-digestion process

For the thermophilic process, the start-up phase lasted 114 days, whereas the OLR was stepwise increased to obtain the defined conditions. During the start-up phase, the pH stabilized above 7.5 due to the high buffer capacity in the inoculum (defined by the total alkalinity). However, at the end of this period, VFAs started to accumulate. In fact, at the end of the first HRT operating under constant condition VFAs reached a concentration of 6 gCOD/kg_{ww}, the pH fell down to 5 (Figure 4a) and the specific biogas production decreased accordingly. The different behaviors observed for mesophilic and thermophilic conditions could be due to the temperature effect on the microbial community (Cavinato et al., 2014; Yu et al., 2014).Thermophilic microorganisms are more susceptible to toxic compounds such as the polyphenols that accumulated in the reactor and reached 160 mg GAE/L. It is well documented that polyphenolic compounds have antioxidant, nutritional and anticancer properties. However, they are reported to be toxic to microorganisms because they inhibit microbial growth (Ramos-Cormenzana et al., 1996). The first signal of instability is the accumulation of polyphenols, followed by the VFAs concentration increase. It is not clear whether the high content of polyphenols caused the inhibition of methanogenesis or whether other inhibitors affected both the polyphenols and VFA degradation. Often the difference in the performance of mesophilic and thermophilic processes is attributed to the high concentration of free ammonia (FA), in this case FA concentration is lower than 200 mg N-NH_3/L, far from inhibiting values.

VFAs in the bulk were composed mainly of 78% acetic and 10% propionic acids, whereas longer fatty acids accounted for less than 15%. This distribution indicated that the activity of the acetate-consuming microorganisms (acetoclastic methanogenesis or syntrophic acetate oxidizing microorganisms) was the rate-limiting step (Drake et al., 2013; Florencio et al., 1994; Nordell et al., 2015).

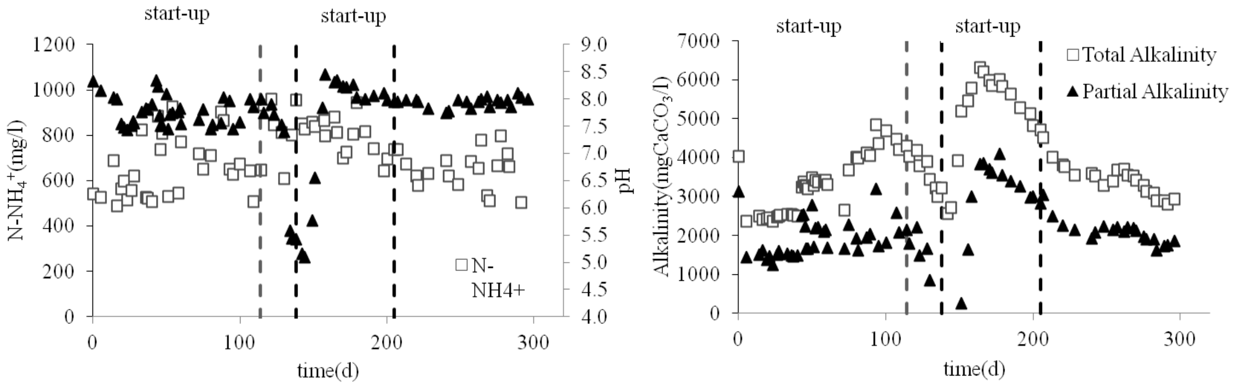


Figure 4. Trend of pH and ammonium concentration (a), partial and total alkalinity (b) under thermophilic condition.

As reported in previous studies (Takashima et al., 2011; Uemura, 2010), elevated concentrations of VFAs, particularly propionate, at the thermophilic temperature could be caused by trace nutrients deficiencies. Trace elements additions affected the sulfide concentration and promoted the precipitation of insoluble metal sulfides and decreased the H_2S toxicity. At high doses, the trace elements could be more available for microorganisms and support the activity of fundamental enzymes (Demirel and Scherer, 2011; Jansen et al., 2002). Several authors (Moestedt et al., 2015; Nordell et al., 2015) have demonstrated the positive effect that the combined supplementation of Fe, Co and Ni when added into an anaerobic digester and have suggested different doses (Facchin et al., 2013).

To recover the biological process, feeding was stopped and the pH adjusted by the addition of lime to restore the optimal condition for anaerobic digestion. From day 170, a new start-up phase was implemented with the addition of a metal solution (Demirel and Scherer, 2011). Metals were provided to the feed to obtain concentrations of 4.3 mg Fe-$FeCl_3$/L, 0.46 mg Ni- $NiCl_2$ $6H_2O$/L and 0.51 mg Co- $CoCl_2$ $6H_2O$/L in the feed, as suggested by Takashima et al. (2011). A short start-up was conducted, and the process reached conditions similar to the period prior to failure event. The ammonium concentration stabilized approximately 630 mgN/L, the alkalinity decreased to 3360 mg$CaCO_3$/L, and pH ranged from 7.8 to 8.0 (Figure 4). For these operational conditions, the effluent solid concentration slightly decreased to 21.3 g TS/kg_{ww} and 57% volatile solids, whereas the particulate COD remained constant at 10.9 g/kg. The average concentration of soluble COD was 0.9 g COD/kg, which contained approximately 30% VFAs. This value was likely due to the high hydrolysis rate at 55° C and not from the accumulation of intermediate metabolites.

The concentration of nutrients in the solid fraction was stable and the COD:N:P ratio was 60:3:1, indicating elevated biological stability.

The average polyphenol content reduced from 160 to 66 mg GAE/L because the removal efficiency increased from 67% to 78%. Degradation efficiencies observed during the entire experimental process were higher than those reported by Cavinato et al. (2014) at 55 °C. Moreover, trace nutrient augmentation improved the microbial activity and allowed for better polyphenolic compound degradation.

The biogas production rate increased from 0.5 $m^3/(m^3_{reactor}d)$, at the beginning of second start-up, to 1.4 $m^3/(m^3$reactord) during steady state. The average specific gas production for these conditions was 0.450 m^3/kg COD (Figure 5) with 62% methane. Accordingly, with the increase in biogas production, solids and COD removal efficiencies increased to 34% and 88%, respectively. The high hydrolysis rate in the thermophilic range caused better solid reduction and organic matter stabilization.

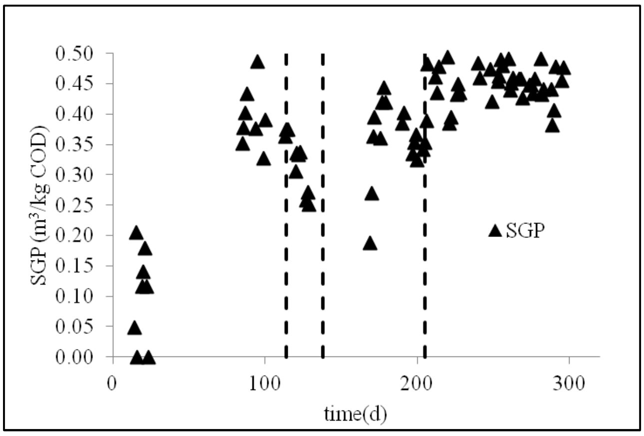


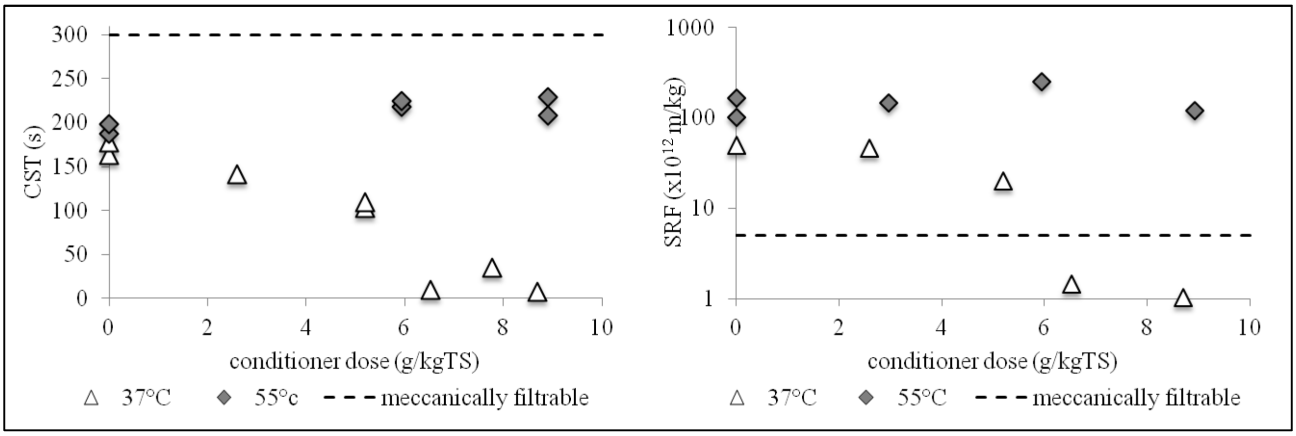
Figure 5. Trend of specific gas production under thermophilic condition.

3.4. Dewatering proprieties

Effluents from anaerobic digestion are usually dewatered to separate the liquid and solid fractions of the digestate for storage, transportation, post-treatment, and other purposes. Effective dewatering can significantly reduce the volume of digestate and the cost of further processing (Lü et al., 2015). The dewatering properties of digestate were determined by two testes: the capillary suction time (CST) and the specific resistance to filtration (SRF). The tests were conducted on mesophilic and thermophilic digestates at steady state and for different doses of chemical conditioners (Tillflock 6480 eTillmanns) to choose the optimal one for each digestate.

The mesophilic and thermophilic raw digestates had CST values of 171 and 193 s, whereas the SRF were $5.1 \cdot 10^{13}$ and $1.3 \cdot 10^{14}$, respectively. The CST values were significantly lower than those reported by Da Ros et al. (2014), likely due to the presence of bentonite in the winery wastes (lees). Generally, the CST was strongly affected by the free water content in the WAS and, in this case, the bentonite improved the release of water on the filter paper (Jin et al., 2004). In fact, bentonite is a mineral conditioner that, thanks to its surface charges, reacts with suspended organic matter and releases the bound water (Alvarenga et al., 2015). However, the SRF values were consistent with the literature data (Schafer, 2001), which described slightly worse dewaterability at 55 °C.

The application of a chemical conditioner to the mesophilic digestate improved the dewatering capability (Figure 6). The SRF reached a value of $1.5 \cdot 10^{12}$ with the addition of 6.5 g of chemical conditioner per kg of dry matter, indicating that the digestate can be dewatered mechanically. However, the thermophilic effluent required dosages higher than 10 g/kg_{dw}. In the same way, the CST of the mesophilic digestate decreased to less than 10 s, whereas the CST of the thermophilic digestate possessed values greater than 200 s. The presence of a porous conditioner such as bentonite in the sludge reduces the use of flocculants and the dosage used was similar to the typical dosage for waste activated sludge dewatering.



(a) (b)

Figure 6. Trends of CST (a) and SRF (b) increasing chemical conditioner dose

3.5. Comparison of the performances of the mesophilic and thermophilic processes

One of the main advantages of using thermophilic conditions was a higher waste stream reduction due to the improvement of the hydrolysis rate. In fact, the solid concentration in the reactor reduced from 24.3 to 21.3 g TS/kg$_{ww}$ when the temperature increased from 37 °C to 55 °C. The low values of TS reduction, 28% and 34% at 37 °C and 55 °C respectively, is due to nature of the largest organic structures presented in the WAS and that contributes to its low biodegradability (Battista et al., 2016). In agreement with the volatile solids and COD removal, the biogas production at 55 °C improved by 18% compared with the mesophilic process by applying the same operational conditions. The biological stabilization of the substrates appeared similar in the two reactors, with a VS/TS ratio of 57-58% and a particulate COD concentration of 613-640 mg/g$_{dw}$ (Table 3). The hydrolysis also affected the ammonification, resulting in a higher ammonium concentration at 55 °C (630 mg N/L) than at 37 °C (400 mg N/L). In both reactors, the free ammonia content was far lower than the inhibiting level (Gallert and Winter, 1997) because of the low nitrogen concentration in the inlet mixture. The solid fraction of the digestates appeared to be an interesting fertilizer for its nitrogen and phosphorus level, in particular, the COD:N:P was 70:4:1 and 60:3:1 at 37 °C and 55 °C, respectively.

The thermophilic process improved the effluent quality also in terms of pathogen removal (Da Ros et al., 2014; Sahlstrom, 2003). Polyphenols represented an important parameter for the reuse of the digestate because high concentrations of these compounds could reduce biological activity in the soils, affecting the fertility (Mosse et al., 2012). The process conducted at 37 °C was more efficient as polyphenols removal and resulted in a lower content in the downstream flow (Table 3).

Table 3 Characteristics of mesophilic and thermophilic digestates, average values and standard deviations. apCOD: particulate COD, bsCOD: soluble COD, c SGP: specific biogas production

Parameter (Unit)	Unit	37°C		55°C (with metals)	
		Average	**St.dev.**	**Average**	**St.dev.**
pH	-	7.46	0.19	7.91	0.09
Partial alkalinity	g $CaCO_3$/L	1.4	0.1	2.0	0.1
Total alkalinity	g $CaCO_3$/L	2.2	0.2	3.4	0.2
Ammonium	mg N/L	400	56	630	73
TS	g TS/kg_{ww}	24.3	2.9	21.3	1.7
VS	g VS/kg_{ww}	14.2	1.7	12.1	1.5
VS/TS	%	58	4	57	7
pCOD [a]	g/kg_{ww}	13.6	5.6	10.9	6.1
sCOD [b]	g/L	0.4	0.2	0.9	0.2
TKN	mg N/g_{dw}	36.3	4.5	32.8	5.4
Total phosphorus	mg P/g_{dw}	8.8	1.6	10.2	1.3
Polyphenols	mg GAE/L	26	7	66	28
SGP [c]	m^3/kgCOD	0.386	0.049	0.454	0.030
COD removal (%)	%	76		88	

To evaluate the best operational condition, the characteristics of digestates are not the only aspect to consider. The feasibility of the process should also be analyzed. The thermophilic process with metals augmentation had a higher buffer capacity, as defined by the total alkalinity (3560 mg$CaCO_3$/L) and, although the volatile fatty acid concentration in the bulk was higher, the pH remained between 7.8 and 8.0. The better efficiency, defined by higher biogas production and solids removal, can compensate the increase of some management costs. In particular the addition of metals under thermophilic condition roses the cost of 0.70 € per cubic meter of mixture fed into the digester. On the other hand the reduction of effluent mass allows for reduced the costs by the minor final solids mass, and consequently by the reduction of quantity of digestate to compost. Moreover, other inexpensive metals sources can be found in the surroundings: WAS from municipal wastewater or livestock effluents are widely available co-substrates that can be co-treated. Considering the several factors affecting the management costs, the best operational condition should be chosen on basis of availability of other co-substrates, possibility to operate the composting inside the company or spread the digestate on vineyards.

Both the processes could generate energy, which can be used inside the wastewater treatment plant, and the supernatant obtained by the dewatering of the digestates have high concentrations of ammonium and orthophosphate. Often, management of digestate liquid fraction is considered a cost because it needs a specific reactor for nitrogen removal/recovery or it leads to an increased nitrogen load in the wastewater treatment plant. For a winery, recirculation of the supernatant in the wastewater line may represent a way to reduce management costs, as it could limit the need for chemical nutrient additions in the aerobic bioreactor.

4. Conclusions

The cellar monitored in this work, which produced approximately 30,000 m^3 of wine per year, generated 1.96 m^3 of wastewater, 1 kg of WAS (dry matter) and 16 kg of lees per cubic meter of wine produced. Anaerobic co-digestion WAS and lees was feasible, both in mesophilic and thermophilic conditions, when operating with an OLR of 3.2 kg COD/(m^3d) and an HRT of 23 d. The mesophilic process was stable over a long period in terms of the stability parameters (pH 7.46, 400 mg N/L and 2248 mg $CaCO_3$/L) and biogas production (0.386 m^3/kgCOD). The thermophilic digestion process accumulated VFAs, and after one HRT, the process failed. Metal augmentation (Fe, Co and Ni) improved the stability and biogas yields (0.450 m^3/kgCOD) at 55 °C. Solid removal increased by 18% compared with the mesophilic process and ammonification resulted in a higher ammonium concentration in the thermophilic digestate (630 mg N/L). The dewaterability properties of the mesophilic process appeared to be better. A total of 6.5 g conditioner/kg_{dw} was sufficient for mechanical dewatering of the digestate, whereas the thermophilic digestate dewaterability did not change over the dosage range of 0-10 g polymer/kg_{dw}. Considering management costs, thermophilic conditions are still economically competitive thank to higher biogas production, even more if other co-substrates, derived from surrounding activities, are co-treated to supply the missing micro-nutrients.

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Appendix A. Supplementary data

Table 4. Winery wastewater production in the main production countries

Country	Wastewater production (L/L wine produced)	Reference
Sud Africa	1.5 - 6	Walsdorff et al., 2005
Chile	1.2 - 6	Aybar et al., 2007
Portugal	2 - 15	Oliveira et al., 2009
Portugal	1-3.7	Duarte et al., 2004
Italy	0.2 - 6	Berta et al., 2003
Italy	0.7 - 14	Andreottola et al., 2009
Greece	1 - 4	Vlyssides et al., 2005

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3.4. Treatment of winery waste inside on-farm biogas plant

Most of the on-farm biogas plants operating in Italy were fed with energy crops such as maize silage. Maize silage is a production that competes with food crops and thus is not ethically and environmental sustainable. Italian legislation encourages substitution of energy crops with agricultural by-production and catch crops. This study evaluated the use of wine lees as the main substitute of maize silage and the integration of catch crops in the feeding mixture. The results of this study were reported in master thesis of Antonella Tyrolt and the paper draft " Improve biogas production in on-farm digesters by winery waste and catch crops co-digestion" was written for a future publication.

Draft short communication

Improve biogas production in on-farm digesters by winery waste and catch crops co-digestion

Keywords: Anaerobic digestion, BMP test, Catch crops, Cattle slurry, Wine lees.

Abstract This study evaluates the substitution of maize silage with winery wastes and catch crops as co-substrates in on-farm biogas plant. Different biomasses (maize silage, sheat straw, *Lolium spp, Sorghum spp*. and wine lees) were compared analyzing their chemical-physical properties and potential methane production by batch tests. These preliminary tests showed that *Lolium spp.* had specific methane production (0.360 m^3 CH_4/kgCOD) comparable to maize silage while wine lees had lower methane yields (0.218 m^3 CH_4/kgCOD) but higher hydrolysis rate, in fact 90% of methane production was reached within 7 days. Co-digestion of cattle slurry and wine lees, and later with *Lolium spp.* addition, was evaluated in semi-continuous reactor. Mixture of cattle slurry and wine lees was balance in terms of nutrients and allow to reach the 90% of potential methane production. Biogas yield was 0.324 m^3/kgCOD with 73% of methane. Addition of *Lolium* increased organic loading rate from 2.6 to 3.2 kgCOD/(m^3d) without causing instability but the process performance was limited by mixing problem. Observed specific biogas production was 0.283 m^3/kgCOD with 70% of methane. Overall, both processes were feasible and were characterized by specific biogas production comparable with those obtained using maize silage.

1. Introduction

Number of on-farm anaerobic digestion plants recently increases in order to treat livestock effluents, reduce their environmental impact, obtain nutrients enriched amendment and produce renewable energy by biogas combustion.

Nonetheless, manure and slurry are often associated with poor methane production in continuous reactor because of two main factors. The first factor is the presence of lignin, a recalcitrant compound, and the second one is the low carbon to nitrogen ratio (C/ N) due to relatively high concentration of ammonia (Prochàzka et al., 2012). In order to improve the process performances and to make plants economically feasible, one or more co-substrates are used. Suitable co-substrates are carbon-rich biomasses and, when possible, with large amounts of easily biodegradable organic matter. The maize silage is the most used co-substrate because it has high methane yield (Jagadabhi et al., 2008). On the other hand it results in potential competition with food crops for available land (Murphy et al., 2011). In fact, maize for on-farm biogas plants needs water, energy and fertilizers for its growth (Hanegraaf et al., 1998) and decreases, at the same time, the amount of arable land surface available for food crop cultivation with a subsequent increase in the cost of food crops (Giuliano et al., 2013). Considering ethical problems and grant policy, residual biomasses and catch crops are being considered like potential co-substrates. Agro-industrial by-products are potential residual biomasses and are widely diffused because could derive from different processes (Schievano et al., 2009). On the other hand, the catch crops are vegetable species that could be planted in marginal zone not interested by agricultural purposes, then they don't compete with food and fodder crops. Moreover Italian policy encourage use of catch-crops (e.g. *Arundo donax L., Sorghum spp., Lolium spp., Mischantus spp.*) for energy plant fed with livestock manure and slurry. Several authors reported examples of anaerobic co-digestion of industrial by-products with livestock effluents (Giuliano et al., 2013) but few data are available about co-treatment with semi-solid winery wastes and catch crops (Molinuevo-Salces et al., 2014; Riaño et al., 2011).

The aim of this study is to evaluate the substitution of maize silage with winery wastes as co-substrates in livestock effluent anaerobic digestion; in fact some winery wastes, such as wine lees, content high concentration of highly biodegradable organic matter and low content of nitrogen and phosphorus (Da Ros et al., 2016). Moreover the catch crops could be integrated into inlet mixture when available. In order to choose the best catch crops, several species were evaluated in terms of chemical characteristic and potential methane production, and compared to cattle slurry and wine lees. Long-term anaerobic co-digestion of cattle slurry and wine lees was analyzed using semi-continuous CSTR reactor, and later the addition of the most promising biomass, *Lolium spp.*, was kept in consideration. These approaches can be applied in on-farm biogas plants equipped with anaerobic digestion and where cattle breeding and vine cultivation are carried out.

2. Materials and methods

2.1. Raw materials

All the substrates were collected in north-east of Italy. Cattle slurry (CS), maize silage (MS) and wheat straw (WS) were obtained from an on-farm biogas plant in Padua province where they were utilized as inlet co-substrates. Wine lees (WL) derived from a cellar in Treviso province and it is available throughout the year. Wheat straw and catch crops (*Lolium spp.* and *Sorghum spp.*) was cultivated in experimental farm of University of Padua. Wheat straw is the residue that remained in the field after cereal collection, while catch crops are plant, often perennial, that can be cultivated in marginal zones. These species prevent erosion and stabilize soil (Molinuevo-Salces et al., 2013); moreover Italian D.M.6 July 2012 encouraged their utilization by economical incentives.

Substrates were characterized in terms of total and volatile solids (TS and VS), COD on dry matter, total Kjeldahl and ammonium nitrogen, and total phosphorus as reported by American Public Health Association et al. (1999). Soluble COD fraction and ammonium concentration were determined in the wine lees.

2.2. Batch methanization tests

Preliminary methanization tests (BMP) were carried out in batch mode to evaluate their potential yields in term of biogas and methane as defined by Angelidaki et al. (2009). The sludge used as inoculum originated from a mesophilic full-scale digester co-treating cattle effluents and maize silage under mesophilic condition. The initial digestate was filtered on 2 mm sieve and diluted two times in order to reduce the residual biogas production of inoculum. The final digestate had an average solid concentration of 17 gTS/kg$_{ww}$, with 78% volatile solids. The particulate COD was 974 mg/g$_{dw}$, while nitrogen and phosphorus were 38 mgN/g$_{dw}$ and 9 mgP/g$_{dw}$, respectively. The stability parameters of seed sludge were optimal for anaerobic digestion, in fact it has sub-alkaline pH (7.97), high buffer capacity (9880 mg$CaCO_3$/L) and low volatile fatty acids content (234 mgCOD/L). Ammonium concentration was high (1,648 mgN/L) but it didn't reach inhibiting value for the anaerobic biomass.

The volume of produced biogas was determined by water displacement and the composition by a chromatography analysis using GC 6890N Agilent Technologies equipped with an HP-Molesieve column (30m x 0.3mm x 0.25 mm film thickness) and a thermal conductivity detector (TCD). Argon was used as carrier gas and the operating temperature of oven was 40 °C. The results were elaborated and expressed at operational temperature and pressure (35 °C and 1 atm). Blanks were also run in duplicate to determine the endogenous methane production. Average value of blanks was subtracted from the yield of tested samples to accurately assess the BMP yields. Biodegradability can be estimated comparing the measured methane production to theoretical one (0.350 Nm^3CH_4/kg COD). Finally hydrolysis constant was determined on first order model basis as described by Browne et al. (2014).

2.3. Semi-continuous test

Semi-continuous anaerobic co-digestion was carried out in a lab-scale reactor with working volume of 4.5 L and equipped with external jacket for maintain temperature at 35°C. The seed sludge used as inoculum was the same used in the BMP tests. The reactor was manually fed once a day, five time per week.

Different substrates mixtures were evaluated considering the substrate availability and the preliminary BMP tests results. Cattle manure is available throughout the year and it was the base of inlet micture. Wine lees are chosen because of their availability throughout the year and they are considered as feasible substitute of maize silage. CS and WL were thus the potential main co-substrates for a more sustainable on-farm biogas plant (RUN1). A further addition of *Lolium spp.* (RUN2) could improve process yield and economical balance of the farm, especially when there is a lack of availability of winery wastes. Contrary to winery waste, catch crops can be stored for long time as silage and used when their contribute is necessary. Hydraulic retention time was set at 40 d in both trials changing the water amount, and organic loading rates were 2.6 and 3.2 kgCOD/(m^3d) in RUN1 and RUN2, respectively (Table 1).

Table 1 Experimental design of semi-continuous test: organic loading rate (OLR), hydraulic retention time (HRT) and temperature

	OLR				HRT	Temperature
	CS	**WL**	**L**	**total**		
	kgCOD/(m^3d)				d	°C
RUN 1	0.7	1.9	0	2.6	40	35
RUN 2	0.7	1.9	0.6	3.2	40	35

Effluent was characterized in terms of stability parameters (pH, partial and total alkalinity at pH 6 and 4 respectively, ammonium concentration and volatile fatty acids content). Volatile fatty acids (VFA) content was monitored using a gas chromatograph (Carlo Erba Instruments) with hydrogen as the gas carrier, equipped with a Fused Silica Capillary Column (Supelco NUKOL™,15 m x 0.53 mm x 0.5 mm film thickness) and a flame ionization detector (200 °C). The analyzed samples were centrifuged and filtrated on a 0.45 mm membrane. Total and volatile solids, COD and nutrients content were determined weekly according with American Public Health Association et al. (1999). Biogas production was measured daily using a Ritter MilliGascounter® and its composition was evaluated by the same analytical method used for BMP tests.

3. Results and discussion

3.1. Substrate characterization

Energy and catch crops had high solid concentration (> 150 gTS/kg_{ww}) thus they couldn't be treated by wet anaerobic digestion without dilution. Volatile fraction was greater than 85% indicating high organic matter content. Cattle slurry and wine lees showed a TS content of 91 and 51 gTS/kg_{ww}, respectively, then they are able to dilute other solid biomasses (Table 2). WL had the lowest VS/TS ratio because of bentonite presence, this type of clay was used to remove colloidal particles in wine. Instead of inert material content, 80% of COD was in soluble form (178 g/L) and readily biodegradable (Da Ros et al., 2016). The CS had the highest nutrients contents (36 mgN/g_{dw} and 11 mgP/g_{dw}) among the considered substrates, corresponding with

COD/N/P ratio of 71/3/1. Nitrogen in the other biomasses ranged from 15.4 to 29.3 mg N/g_{dw}, while total phosphorus varied between 2.1 and 6.9 mg P/g_{dw}. The main differences among the substrates were the COD content and COD/N; both these parameters could affect the stability of anaerobic process: in particular the low COD/N ratio observed in the CS could cause ammonium inhibition if the substrates is not diluted. MS had the highest COD concentration (355 g/kg_{ww}) and low lignin content: 2.9% of TS (Herrmann et al., 2016), that explain the high methane yields (0.40 m^3 CH_4/kgVS, Giuliano et al., 2013). Herrmann et al. (2016) reported that wheat straw had high amount of lignin (10% of TS) while *Lolium spp.* and *Sorghum spp.* had lignin amount comparable with maize (2-4% of dry weight).

Table 2 Characteristics of cattle slurry (CS), maize silage (MS), wheat straw (WS), *Lolium* (L), *Sorghum* (S) and wine lees (WL): total and volatile solids (TS and VS), particulate and soluble COD (pCOD and sCOD), total Kjeldahl nitrogen (TKN) and total phosphorus (Ptot).

	TS	**VS**	**VS/TS**	**pCOD**	**sCOD**	**TKN**	**$N\text{-}NH_4^+$**	**P_{tot}**
Substrate	g/kg_{ww}	g/kg_{ww}	%	mg/g_{dw}	mg/L	mg N/g_{dw}	mg N/L	mgP/g_{dw}
CS	91.0	67.6	74	789	-	35.8	-	11.1
MS	330,8	315,92	95	1074	-	15.4	-	2.5
WS	313.9	287.1	91	910.5	-	25.2	-	2.1
L	179.6	155.4	87	753.2	-	26.5	-	2.5
S	217.0	205.1	95	999.8	-	21.2	-	1.6
WL	50.7	33.0	65	608.0	178.4	29.3	34.6	6.9

3.2. Batch methanisation tests

BMP tests were carried out for least 30 days, until negligible biogas production was observed in all the tests. According with Giuliano et al. (2013), MS had the highest methane production (0.393 ± 0.062 m^3 CH_4/kgCOD) that indicated that it was completely biodegradable. On the other hand wheat straw had methane yields of 0.285 ± 0.070 m^3 CH_4/kg COD because higher lignin content that reduced biomass bioavailability (Kim et al., 2015).

Also catch crops (L and S) had elevated biodegradability, in fact methane production was comparable with theoretical one (0.360 ± 0.006 and 0.342 ± 0.078 m^3 CH_4/kgCOD, respectively). *Lolium spp.* had hydrolysis rate similar than energy crops (0.065 d^{-1}) while hydrolysis of Sorghum spp. was slow (0.004 d-1). Regarding these results, the Lolium spp. was the most promising catch crop for yields and hydrolysis constant as reported by Molinuevo-Salces et al. (2013). Wine lees generated 0.218 m^3 CH_4/kg COD and was characterized by an elevated hydrolysis constant (0.22 d^{-1}) due to presence of soluble organic compounds. Although the specific yield of WL was comparable to CS one, the high COD content and the elevated hydrolysis constant made this substrate particularly suitable as co-substrate.

Methane percentages in biogas and kinetic constants (Table 3) confirmed that substrates had different biodegradability characteristics. Organic matter in WL was readily converted into methane, in fact the 90%

of total biogas production was reached in only 7 days. Degradation of the other substrates (Table 3) was limited by hydrolysis step because of presence of particulate organic matter. In these cases hydrolysis step was relevant for biogas composition because in this phase only CO_2 was generated, then reduced the average methane percentage in the biogas. CS was characterized by the lowest kinetic parameter: the slow hydrolysis was due to high lignin content (14% of total solid, Normak et al., 2015) that is not biodegradable in anaerobic condition. Moreover it was subjected to a previous digestion in the animal digestive tract (Luste et al., 2012) then part of degradable substances were already consumed. For these reasons, CS had methane production of 0.237 ± 0.020 m^3 CH_4/kgCOD and the period needed to reach the 90% was about 28 days, that indicated slow degradation rate and difficulty to reach the same yield in continuous reactor.

Table 3 Results from BMP test carried out with cattle slurry (CS), maize silage (MS), wheat straw (WS), *Lolium spp*. (L), *Sorghum spp*. (S) and wine lees (WL). SGP: specific biogas production, SMP: specific methane production, CH_4: average percentage of methane in the biogas, k_H: hydrolysis constant.

	SGP	SMP	CH_4	k_H	Biodegradability
Substrate	m^3/kgCOD	m^3 CH_4/kgCOD	%	d^{-1}	%
CS	0.407 ± 0.041	0.237 ± 0.020	58	0.066	68
MS	0.830 ± 0.124	0.393 ± 0.062	47	0.061	100
WS	0.590 ± 0.123	0.285 ± 0.070	48	0.075	81
L	0.751 ± 0.082	0.360 ±0.006	48	0.065	100
S	0.670 ±0.126	0.342 ± 0.078	51	0.004	98
WL	0.345 ± 0.009	0.218 ± 0.003	63	0.220	62

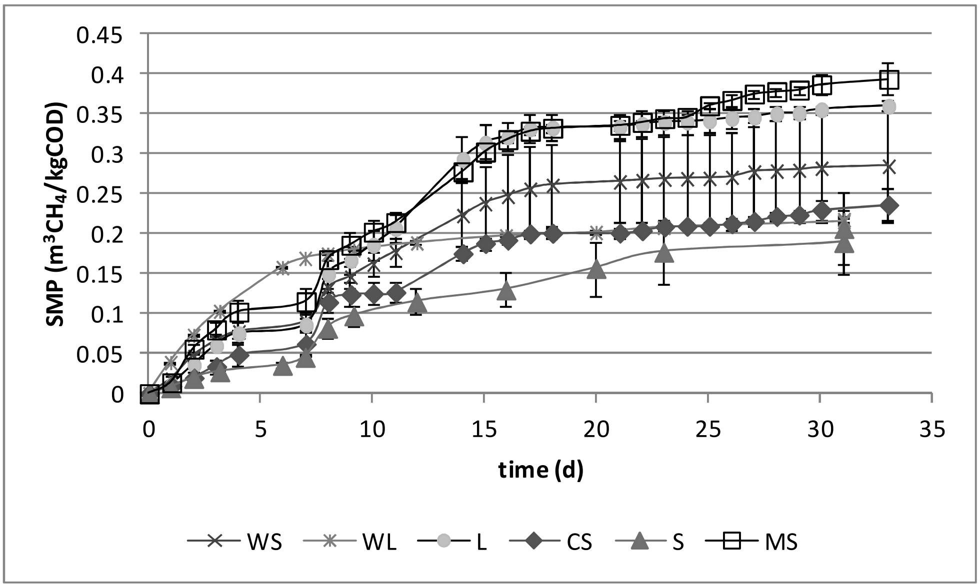

Figure 5. Specific biogas and methane production of wheat straw (WS), wine lees (WL), *Lolium spp.* (L), cattle slurry (CS), *Sorghum spp.* (S) and maize silage (MS).

3.3. Semi-continuous test

The potential biogas yields in many cases may not be realistic with respect to full-scale production plants (Schievano et al., 2009) because in continuous process the potential biogas production is not reached. Moreover the content of nutrients could affect the process performance: ammonium accumulation could inhibit methanogens and missing micro-nutrients could limit microbial growth. Hence semi-continuous process was evaluated by lab-scale CSTR reactor.

3.3.1 Anaerobic co-digestion of cattle slurry and wine lees (RUN 1)

Start-up of RUN1 (co-digestion of CS and WL) lasted about two weeks: wine lees was added to cattle slurry stepwise and OLR increased accordingly. The OLR reached the established value without causing stability problems and VFA/TA ratio reduced to value lower than 0.2 mgCOD/mgCaCO$_3$ (Figure 2). In fact during start-up pH slightly reduced to value 8.08, ammonium concentration decreased because of low nitrogen content in WL, and consequently also buffer capacity changed. At the end of transient period OLR of 2.6 and HRT of 40 d were set-up and pseudo-steady state was reached after 20 days operating in these conditions. The COD/N ratio of final mixture was 56, indicating high carbon content.

The total and volatile solid concentration was not constant because of mixing difficulties, TS concentration ranged from 29 to 36 gTS/kg$_{ww}$, while VS content was between 19 and 28 gVS/kg$_{ww}$. Average total solids concentration was 32.7 g/kg$_{ww}$ with 70% volatile, but mass balance on TS and VS basis indicates an error of 18% and 11%, respectively. Time profiles of pH, total and partial alkalinity (PA and TA), VFA/TA ratio and total ammonia (N-NH$_4^+$) showed substantial stability from the very test beginning (Figure 2). pH ranged from 7.87 and 8.20 while average total alkalinity was 5.5 gCaCO$_3$/L, value lower than those detected in anaerobic co-digestion of livestock effluents and energy crops (Giuliano et al., 2013) because of acidic pH in WL (Bustamante et al., 2007; Da Ros et al., 2016). Average VFA/TA value was 0.17 and it indicated that process could sustain higher organic load (Browne et al., 2014). Ammonium concentration ranged from 1.20 and 1.62 gN/L, lower than data reported by Giuliano et al., (2013) that considered co-digestion of livestock effluents and energy crops. This difference in ammonium concentration was due to WL, substrate characterized by negligible ammonium content and low organic nitrogen concentration. The COD content reduced comparing with inoculum: average value was 708 mg/g$_{dw}$, while average TKN and phosphorus concentrations were 29.6 mg N/g$_{dw}$ and 9.5 mgP/g$_{dw}$. Specific biogas production significantly increased feeding WL and reached average value of 0.324 m^3/kgCOD with 73% of methane. Considering the specific production calculated by BMP and the organic load substrates distribution, the biogas production represented the 90% of potential yields indicating that mixture of substrates equilibrated nutrient balance and warranted interesting process performance.

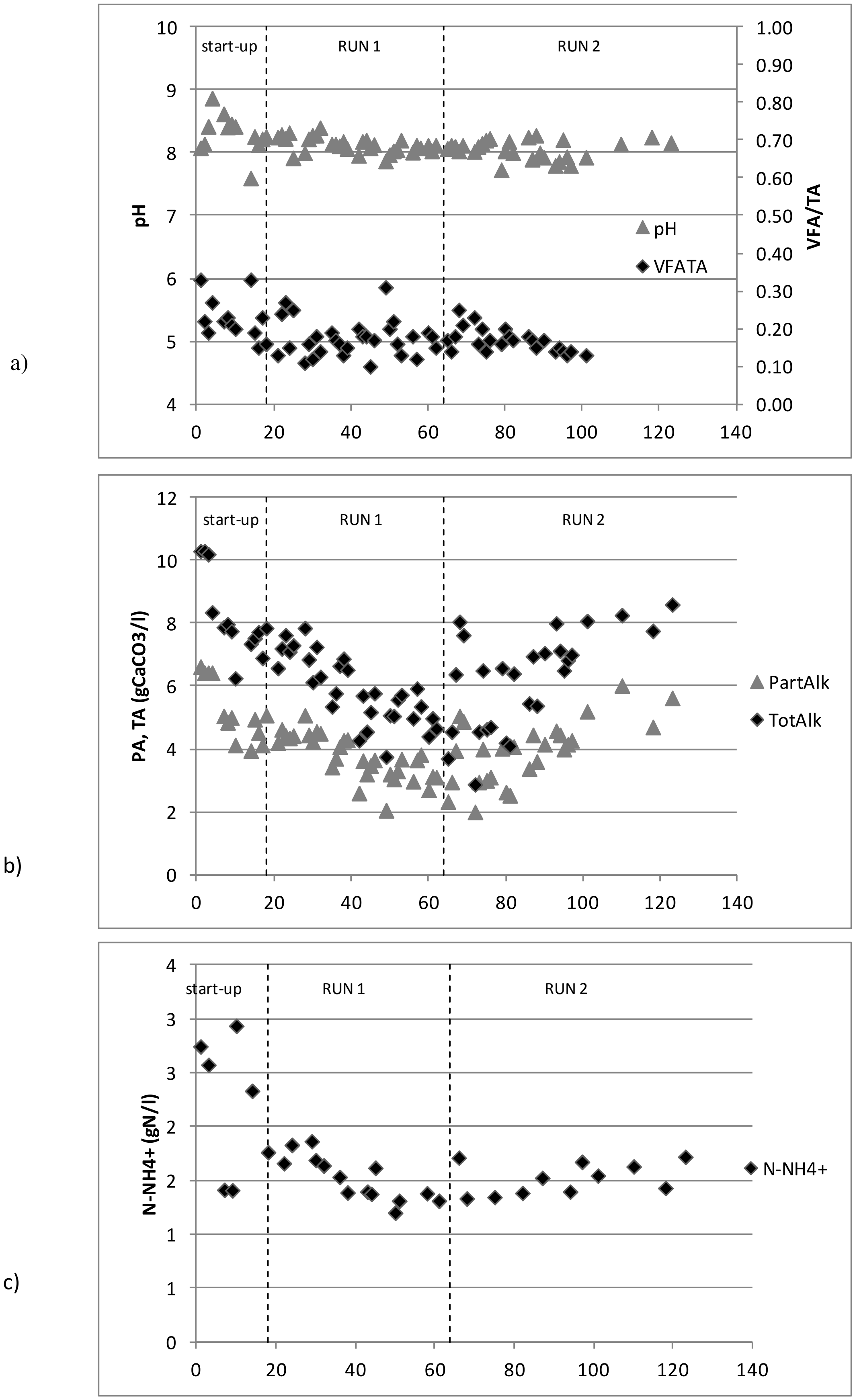

Figure 2. Time trend of pH and VFA/TA ratio (a), Partial and total alkalinity (b), and ammonium concentration (c).

3.3.2 Anaerobic co-digestion of cattle slurry, wine lees and *Lolium spp.* (RUN 2)

VFA/TA in RUN 1 showed that the process could support higher OLR then addition of *Lolium spp.* to feeding mixture was feasible. This supplement increased the OLR to 3.2 kgCOD/m^{3}d, the HRT remained at 40 d (Table 1) and the COD/N of feeding mixture became 35. The process reached a new steady state in about 20 days. The high solid content of L caused increase of the TS content inside the reactor and, despite the scattering of TS value, the effluent solid concentration reached 43.2 g/kg$_{ww}$ with 70% of VS. The long pieces of *Lolium spp.* increased the mixing problem observed during RUN 1 and the error of mass balance became 25%, which indicated that the mixing system should be adapted to these substrates in case of process up-scaling. pH was similar than previous period and ranged from 7.73 and 8.28, while VFA/TA decreased because of alkalinity increasing. Average PA and TA were 5.4 and 8.2 gCaCO$_3$/L probably because increasing buffer capacity and lower COD/N ratio. Feeding nitrogen amount was higher in RUN2 and caused an ammonium concentration increase of 9% (1.5 gN/L) without reaching inhibiting value. COD and TKN concentrations were 736 mg/g$_{dw}$ and 29 mgN/g$_{dw}$, respectively. They were equal to RUN1, while total phosphorus was lower (4.9 mgP/g$_{dw}$).

Specific biogas slightly reduced to 0.283 m^3/kgCOD with 70% of methane. The reason for lower biogas production with ryegrass should be that ryegrass tends to float upon the liquid surface inside the reactor as reported by Prochnow et al. (2009), leading to worse mixing effect. Probably improving mixing also process efficiency should increase. Probably improving mixing also process efficiency should increase.

4. Conclusions

Biological methanisation potential tests evidenced that wine lees should be a suitable co-substrate for substitute the maize silage although the methane production (0.218 m^{3}CH$_4$/kgCOD) was comparable to cattle slurry one. In fact the high hydrolysis constant and the elevated COD content of the winery waste could however improve the energy production in on-farm biogas. Inlet mixture with cattle slurry and wine lees was well balanced in terms of nutrients and allowed to reach the 90% of potential methane production in semi-continuous reactor. Biogas yield was 0.324 m^3/kgCOD with 73% of methane.

On the other hand *Lolium spp.* had a high biodegradability and average yield of 0.360 m^{3}CH$_4$/kgCOD. Considering the existing incentives that promote utilization of catch crops and the high potential methane production of this species, its integration appeared promising. Addition of *Lolium spp.* in CSTR reactor increased organic loading rate from 2.6 to 3.2 kgCOD/m^{3}d without causing any stability problem, but the process performance was limited by an inefficient mixing system. Observed specific biogas production was 0.283 m^3/kgCOD with 70% of methane. Overall, both processes were feasible with right mixing system and were characterized by specific biogas production comparable with those obtained with energy crops.

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3.5. Improve biogas production from winery wastes by automatic feeding mode

Mesophilic anaerobic digestion explained in section 3.3 was also analyzed in terms of kinetics using biogas production rate as main parameter. This investigation allowed for develop an automatic feeding system able to maximize organic loading rate and, consequently, the biogas production. The results were reported in the paper " Kinetic study of winery wastes anaerobic digestion and development of an automatic feeding control" but it should be also compared with a continuous process where flow rate has to be chosen with attention.

The same method could be applied to anaerobic processes fed by different types of substrate. Hence, this study could be considered as the starting point for create an automatic system based on kinetic study of biogas production rate in order to control anaerobic co-digestion processes and to optimize their management.

The work was setup and elaborated with the same co-authors of paper in section 3.3 with the exception of F.Micolucci that collaborated with the development of software. The software had to collect the data of biogas production and later, it elaborated the data and controlled the feeding pump.

Research paper

Kinetic study of winery wastes anaerobic digestion and development of an automatic feeding control

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Abstract This paper deals with the optimization of anaerobic digestion of winery wastes considering the behavior of biogas production rate between two consecutive feeds to the reactor. Processes operating with different hydraulic retention times (23 and 40 d) were monitored and the specific biogas productions were comparable (0.386 and 0.378 $m^3/kgCOD_{fed}$ for retention time of 23 and 40 d, respectively). The biogas production rate reduced after 11-13 hours in both the processes, this time corresponds to necessary period to consume readily biodegradable and easily hydrolysable COD. In order to maximize biogas production, a system able to increase feeding frequency was set-up, it activated feeding pump when a reduction of biogas production rate (below 0.417 $m^3_{biogas}/(m^3_{reactor}d)$) was detected. Consequently hydraulic retention time decreased to 21 d and organic loading rate reached 6.2 kg $COD/(m^3_{reactor}d)$. Moreover these conditions favored growth of microorganism involved into degradation soluble COD fraction and faster kinetics were observed in these conditions. Finally two kinetic models (first order and step-diffusional) were applied to understand how the retention time affects the degradation rate of the different types of compounds and to confirm the results obtained from the preliminary kinetic study. Step-diffusional model better predicted the trend of degradation rates (R^2 0.94-0.99%) because it considers three groups of compounds and the same number of constant.

Keywords: Anaerobic digestion, Kinetics, Step-diffusional model, First order model, Winery wastes, Process control.

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1. Introduction

Optimisation of anaerobic digestion (AD) process could be reached applying the operational conditions (organic loading rate, hydraulic retention time) able to maximise the biogas production without causing process instability. Hydraulic retention time (HRT) is one of most important operation parameters for AD systems control (Zhang and Noike, 1994). It is defined as the average time that bacteria or solids spend inside the reactor, and affects yields and microbiological community involved in the process (Rincón et al., 2008). Usually longer HRTs increase contact time between substrate and microbial biomass, so they are associated with higher destruction of volatile solids (Appels et al., 2008) and biogas production improving. The only way to increase the HRT without increasing the reactor volume and maintaining constant the organic loading rate (OLR), is to decrease the incoming flow rate and, at the same time, concentrate the total solids content in the feeding mixture.

Otherwise the characteristics of substrates fed to the reactor have great importance and could change the effects of long HRT application. Ruile et al. (2015) showed how the volatile solids removal in biogas plant treating cattle manure increased from less than 20% to about 75% when HRT was extended from 21 to 127 days. On the other hand Nges and Liu (2010) studied AD of dewatered sewage sludge and reported that volatile solid removed increased from 48% to 56% when HRT varied from 20 d to 35 d, while longer HRT did not appear economically advantageous. HRT has different effects on the process on basis of substrate biodegradability and microbial community developed in the reactor.

Considering the complexity of the process, usually it is difficult to find the right conditions that maximize the biogas production without stressing the microbial community. Combination of traditional process parameters (biogas yields and composition, removal efficiencies, stability parameters) and kinetic study can help to understand anaerobic process dynamics and evaluate the best operation conditions. The prediction of reactors behaviour, in the future or under other similar circumstances, is one of the objectives of AD modelling (Donoso-Bravo et al., 2011).

ADM1 and its modifications are promising models aimed to understand the system's behaviour and interaction of components; however identifying all the parameters and coefficients is not easy and their application appeared limited. Also simple models have been developed to predict the steady state and dynamic behaviour of digester (Husain, 1998); which parameters have a physical interpretation and are adjustable, for instance by a parameter estimation procedure (Lauwers et al., 2013). They are called grey-box or mechanistically inspired models and the most diffused are Monod, first order, Contois, Singh. All these models consider a sole limiting-rate step (hydrolysis or methanogenic) and consequently they are defined by one kinetic constant. Otherwise degradation of complex substrates, characterized by simultaneous presence of materials with different degradability, depends on the interactions existing among hydrolytic, acidogenic, acetogenic and methanogenic populations (Converti et al., 1999), then higher number of constants should be considered. For this reason the most common grey-box models proposed for anaerobic digestion are inadequate to represent the actual situation inside the digester fed by complex substrates (Cecchi et al., 1990a; Converti et al., 1999). Step-diffusional model represents the evolution of linear model because takes into account the nature and chemical characteristics of compounds present in complex substrates, including the extent to which they are removed. In fact it considers four specific groups of compounds on basis of different utilisation rate, clearly identified observing the plotting of biogas production rates (GPR) versus time during the semi-continuous degradation of complex wastes. The step-diffusional model was mainly applied to AD of organic fraction of municipal solid waste (Cecchi et al., 2013, 1997; 1991), but recently this model was applied also to AD of pre-hydrolysed woody wastes (Converti et al., 1999) and to winery wastes (Da Ros et al., 2014).

The aim of the present work was to evaluate the effect of different HRTs on anaerobic co-digestion of winery wastes in terms of process performances and kinetics. By now the information obtained by kinetic models were used to describe the process but they could be useful to develop control system able to maximize the removal efficiency and the biogas production. This manuscript describes the development and application of this type of control system based on kinetic study of AD treating waste activated sludge and wine lees. Processes working with one feed per day and different HRTs were compared with automatic controlled one in terms of digestate characteristics, process performances and kinetics using first order and step-diffusional models.

2. Material and Methods

2.1.Substrates characteristics

Dewatered sludge used during the experimentation was collected in a winery wastewater treatment plant located in the North-East of Italy: total solid concentration generally ranged from 129.0 to 193.7 g TS/kg$_{ww}$ but some outliers were detected because of some technical reasons (conditioner doses, filter press setting). Volatile Solids (VS) to Total Solid (TS) ratio in winery sludge (88%) was higher than typical value of sludge from municipal wastewater, probably due to high biodegradability of raw wastewater. Moreover COD concentration (868 mg/g$_{dw}$) was indicative of low biological stability of the sludge. Sludge nutrients ratio is well balanced for Anaerobic Digestion biological stabilisation (Table 1), with COD:N:P ratio of 124:7:1. Chemical analysis of sludge showed limited contamination of metals (Cd <0.5 mg/kg$_{dw}$, Cr^{6+} <0.5 mg/kg$_{dw}$, Cr 46 mg/kg$_{dw}$, Hg <0.1 mg/kg$_{dw}$, Ni 18 mg/kg$_{dw}$, Pb 7 mg/kg $_{dw}$, Cu 280 mg/kg$_{dw}$, Zn 97 mg/kg$_{dw}$), thus it is suitable for land application as amendment (D.Lgs. 99/1992; European Commission 2010).

Wine lees were produced during wine decanting step, adding bentonite. Both wine lees from red and white wine processing were used during the experimentation to evaluate substrate variability and how it affects the process. About the 90% of wine lees samples had solid concentration between 37.9 and 77.2 g TS/kg$_{ww}$, but extreme values were also detected. Generally these winery residues were characterized by low content of volatile solids (57% of total solids) due to bentonite presence. COD was concentrated in the soluble form (sCOD was the 83% of total COD) while particulate fraction was typically between 417 and 627 mg COD/g$_{dw}$. Nitrogen and phosphorus levels were limiting for bacterial growth if compared with COD concentration, in fact the COD:N:P ratio was 502:5:1.

Waste activated sludge is often associated with poor methane yields because of low biodegradability of microorganisms cells. On the other hand wine lees had unbalanced COD:N:P ratio; anaerobic co-digestion of these two wastes together should balance the nutrient content improving biogas conversion efficiency and consequently economical sustainability. Hence this process configuration was tested at pilot-scale, working in semi-continuous mode.

Table 1 Waste activated sludge and wine lees characteristics (nd: not detected)

Parameter	**Unit**	**Waste Activated Sludge**	**Wine Lees**
TS	gTS/kg$_{ww}$	158.9 ± 49.3	62.0 ±27.9
VS	gVS/kg$_{ww}$	143.5 ±41.6	33.6 ± 15.1
VS/TS	%	88% ± 3%	57% ±13%
COD	mg/g$_{dw}$	868 ± 69.4	559 ± 151
sCOD	g/L	nd	167 ± 45
TKN	mg N g$_{dw}$	52.7±16.3	30.3 ±12.7
NH_4^+	mg N/L	nd	33.9±22.7
P_{tot}	mg P/g$_{dw}$	7.3 ±2.0	6.2 ±2.9

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2.2. Experimental setup

The AD experimentations were carried out at pilot scale. A completed stirred tank reactor of 0.23 m^3 working volume was applied. The operational temperature was 37 ± 2 °C and it was controlled by external jackets hot water recirculation systems. PT100 probe monitored process temperatures and managed water recirculation pumps. At the beginning of the trials the reactor was filled up with digestate derived from anaerobic process treating winery wastes. Initial digestate has pH of 7.53 and low ammonium concentration

(193.4 mg $N\text{-}NH_4^+/L$). The reactor was fed once a day with a mixture of winery wastes (waste activated sludge -WAS- from winery wastewater treatment and wine lees -WL-) diluted with tap water to reach the desiderated flow rate and sludge solid concentration.

During the start-up the organic loading rate was stepwise increased to 3.2 kg COD/($m^3_{reactor}$d) while HRT of 23 d was maintained (Run1). Semi-steady state lasted 9 HRTs. After that, HRT was set at 40 d in order to evaluate the retention time effect (Run 2). This reactor operated until a steady-state condition.

Biogas production was measured by drum-type gas flow gas meter (Ritter, Bochum, Germany) connected with real-time data recording system, cRIO^{TM} interface (National Instruments^{TM}). GPRs were automatically calculates as slope of biogas production curve over time. This approach was performed using cRIOTM hardware and a specific graphic interface (Virtual Instrument from LabVIEW^{TM} software). Considering the results obtained with these conditions, the automatic feeding system, interfaced with on-line biogas production monitoring, was set-up. The software automatically controlled a pump that fed the reactor as soon as the GPR decreases to value lower than a defined set-point. This approach minimized the time between two consecutive feeds and increases the feeding loading frequency. In order to avoid microbial biomass withdraw, the mixture of substrates was prepared reducing water dilution, corresponding with higher solid concentration in the waste activated sludge.

The authors did not choose the operational conditions (not HRT neither OLR) but it was linked to the pump functioning and process reaction rate. The average operational parameters observed in this third Run were HRT of 21 d and OLR of 6.2 kg COD/($m^3_{reactor}$d).

2.3. Analytical methods

The substrates and the digesters effluents were collected and monitored once a week in terms of total and volatile solids content (TS and VS), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and total phosphorus (P_{tot}) (American Public Health Association et al., 1999). The process stability parameters (pH, volatile fatty acids -VFAs- content and composition, total and partial alkalinity, and ammonia concentration) were checked two or three times per week. VFAs content was monitored using a gas chromatograph as reported by Cavinato et al. (2014). At steady state conditions, total phenols were spectrophotometrically analyzed by the Folin–Ciocalteu assay and concentration was reported in terms of Gallic acid equivalent per litre (mg GAE/L, Lafka et al., 2007). Biogas composition (CO_2, CH_4, H_2 and O_2) was determined by a gas chromatograph (GC Agilent Technology 6890 N) equipped with the column HP-PLOT MOLESIEVE, 30 × 0.53 mm ID ×25 μm film, using a thermal conductivity detector and argon as gas carrier. The gascromatograph had a thermal conductivity detector (TCD) with a temperature of 250 °C, while the injector temperature was at 120°C and pressure in the injection port was 70 kPa. Samples were taken using a syringe gas type with an amount of 200 μL of biogas. Once vaporized the whole sample, the separation of the peaks takes place within the column with a constant temperature of 40 ° C (8 min).

2.4. Kinetic study

For the kinetic study, the behaviour of biogas production of several days was analyzed for each tested operational condition. In particular at least eight days were considered and average behaviour was considered. The result of this elaboration was the average biogas production profile against the time between two subsequent feeds of each steady-state. The biogas production was converted into COD removed and the trends of COD concentrations inside the reactor were determined. GPR, COD concentration and consumption rate profiles were used in order to describe the kinetics of the processes by two linear kinetic models (first-order and step-diffusional).

First order model considers the microorganisms as 'catalysts' and represents an overall mass transfer kinetic model for a 'catalyzed' reaction (Cecchi et al., 1990a). Although this is not a sophisticated model, it can provide a single and useful kinetic constant called hydrolysis constant (*k*). The equation 1 describes the model; *S* is the substrates and *t* the elapsed time.

$$\frac{dS}{dt} = -k\,S \qquad (1)$$

This equation, however, is clearly simplifying the system: in fact if several substrates, with different characteristics, are fed to the system, their removal follows different constants. To overcome this restriction the step diffusional model was introduced. Here, the anaerobic digester is described like a semi-continuous system, and the different substrate nature is taken into account. The degradation rate of each group of compounds can be therefore described by a differential kinetic equation (Cecchi et al., 1990a; Cecchi et al., 1991). In general, given the assumptions reported above, the degradation equation for the use of a substrate, *S*, with time, *t*, is reported in Equation 2, where v_i is the maximum degradation rate and *x* is a generic kinetic constant.

$$\frac{dS}{dt} = v_i \; - \frac{4x(t-t_i)}{2} \qquad (2)$$

This equation assumes a different form depending on the kind of substrate used in the process: in general at least three main groups of substrates can be detected and they are associated with the related equations.

The first group was mainly constituted by acetic acid, methanol and compounds directly convertible into biogas by methanogenic microorganisms (Cecchi et al., 1991). Its consumption can be described by the linear equation 3, where S_0 represents the overall organic matter content of the substrate, v_0 is the maximum degradation rate and $4a$ the kinetic constant representing the proportionality constant between degradation rate and time for the methanogenic step.

$$dS_0/dt = v_0 - 4at/2 \qquad (3)$$

The second group, containing mainly volatile fatty acids with more than three carbon atoms and ethanol, is degraded more slowly of the easily digestible fraction, according with Eq. 4. S_1 represents the residual organic matter content of the substrate, once the easily degradable compounds (acetate, methanol, etc.) have been removed. At this point the degradation rate is v_1 which is indicated by an inflexion in the plot of rate against time at $t = t_1$.

$$dS_1/dt = v_1 - 4b(t - t_1)/2 \qquad (4)$$

The degradation of the last group, constituted of recalcitrant complex biopolymers, is controlled by hydrolysis rate. The slowest degradation step is described by equation 5:

$$\frac{dS_2}{dt} = v_2 \ - \frac{4c(t-t_2)}{2} \qquad (5)$$

The equations 5 is applied for $t_2 < t < t_3$ where S_2 represents the residual organic matter content of the substrate, once the soluble degradable compounds have been removed. At this point the degradation rate is v_2 producing an inflexion in the rate curve at $t = t_2$. At the end of this period, the degradation rate (denoted as v_3) corresponds to the hydrolysis of particulate organic matter (equation 6):

$$\frac{dS}{dt} = v_3 \qquad (6)$$

The linear regressions, minimizing sum of square differences between the predicted and experimental values in each point, were carried out on these data to obtain the model constants. The regression coefficients, together with the lacks of fit, expressed as the sum of square differences between the real and the model predicted values, have been used to test the best fitting model.

3. Results and discussion

3.1 Anaerobic digestion processes performances

During the start-up the organic load was gradually increased from 0.6 to 3.2 kg COD/($m^3_{reactor}$d) as reported by Da Ros et al. (2016). In particular, a constant quantity of sludge was used (0.6 kg COD/($m^3_{reactor}$d)), and the wine lees feeding amount increased gradually from 0 to 2.6 kg COD/($m^3_{reactor}$d). The anaerobic process started using a HRT of 23 d (Run 1), corresponding to a hydraulic flow rate of 10 L/d.

At steady state the average pH was 7.46 and total alkalinity stabilized to 2,248 mg$CaCO_3$/L. Low level of sCOD (360 mg/L) indicated the complete removal of readily biodegradable fraction present in WL. About 40-50% of residual sCOD was due to volatile fatty acids: acetic acid was the dominant volatile fatty acid (52% of total VFAs) while propionic acid was the second most abundant with 12% of VFAs. The process, in this condition, was able to remove 76% of total COD. Total solids and COD concentrations reduced with time down to average values of 24 gTS/kg_{ww}, with 58% volatile solids, and 640 mg COD/g_{dw} (Table 2). Most of biogas production derived from degradation of soluble compounds and the solid fraction was partially degraded. For this reason total and volatile solids removal were 28% and 40%, respectively. Nutrients concentrations generally reduced until steady values were reached (37 mgN/g_{dw} and 9 mg P-PO_4^{3-}/g_{dw}). Ammonium concentration stabilized around 400 mgN-NH_4^+/L: considering negligible ammonium nitrogen concentration in inlet substrates (less than 1% of total nitrogen in the feed is due to ammonium ion), it is possible to observe that about 30% of organic nitrogen was converted in soluble form. As reported by Owamah et al. (2014), digestate contains more readily available nutrients than the undigested products which make it better for crops fertilisation. Biogas production scattered a lot, depending on the type of wine lees in the inlet mixture: average biogas production was 0.386 m^3/kg COD_{fed} with 64-73% of methane.

Table 2 Characteristics of digestates from process working with 23 and 40 days of HRT

Parameter	Unit	Run 1	Run 2
HRT	d	23	40
TS	gTS/kg_{ww}	24.3 ± 2.9	33.4 ± 2,43
VS	gVS/kg_{ww}	14.2 ± 1.7	21.7 ± 1.1
COD	mg/g_{dw}	640 ± 46	752 ± 73
sCOD	mg COD/L	360 ± 152	349 ± 74
TKN	$mg\ N/g_{dw}$	36.3 ± 4.5	52.8 ± 3.3
$N\text{-}NH_4^+$	mg N/L	400 ± 56	638 ± 49
Ptot	$mg\ P/g_{dw}$	8.8 ± 1.6	8.4 ± 0.8
pH	-	7.46 ± 0.19	7.51 ± 0.07
Total Alkalinity	$mg\ CaCO_3/L$	2248 ± 200	3,332 ± 124
Polyphenols	mg GAE/L	26 ± 7	56 ± 26
SGP	$m^3/kgCOD$	0.386± 0.049	0.378 ± 0.036
TS removal	%	28%	22%
VS removal	%	40%	36%
COD removal	%	76%	78%

In the second Run (HRT 40 d) the flow rate has been reduced to 5.75 L/d and HRT increased to 40 days. Flow rate was reduced without changing the organic loading rate, in fact only the amount of water was reduced in the feeding mixture. The total solid concentration of substrates mixture increased from 3% to 5%. Change in operational conditions caused a transient period of about one HRT, defined by an increased ammonium concentration and alkalinity. As reported in Table 2 the stability parameters were in optimal range for AD: pH was 7.51, similar than previous period, while alkalinity increased to 3,332 $mgCaCO_3/L$ because of higher concentration of ammonium (638 $mgN\text{-}NH_4^+/L$). Concentration of sCOD was around 349 mg/L and VFA contributed for less than 50 mg/L. Acetic acid was the only volatile fatty acid detected during this period. The different VFA concentration and distribution could be due to effect of HRT. In fact long HRT allows for higher content of acetoclastic methanogens bacteria, characterized by lower growth rate than bacteria degrading other organic compounds (Amani et al., 2012; Metcalf and Eddy, 2003). Moreover longer HRT increased the methanogenic population and promoted the efficient propionate and butyrate oxidation (Schmidt and Ahring, 1995), consequently reduced the total VFA concentration.

Total solid concentration increased from 24.3 to 33.4 g/kg_{ww} due to higher solid concentration in the inlet mixture. Percentage of volatile to total solids was about 61% and particulate COD was 752 mg/g_{dw}. These values were slightly higher than those observed during period with 23 d of HRT, but the difference was not significant and it was probably due to substrates variation. In fact also the mass balance on TS, VS and COD basis showed similar removal performances (22%, 36% and 78% respectively). Nitrogen fate didn't change increasing HRT, in fact the same amount of organic nitrogen was converted into ammonium form (28% of total nitrogen) and the higher concentration in the second condition was due to minor dilution. Considering average and standard deviation of phosphorus concentration, no significant difference for this parameter can be detected in the two periods. Biogas production yield (0.378 m^3/kg COD) was like previous trial, with methane content of 65%.

3.2 Kinetic study

Comparing mass balances and SGP between Run 1 and Run 2(Table 2), it is evident that hydraulic retention time did not affect the degradation efficiency but reduced the effluent sCOD concentration. The higher concentration of solids and minor flow rate could reduce the downstream volume to be disposed and consequently the management costs.

On the other hand, some interesting differences were observed in cumulative biogas production curves (Figure 1). In both the conditions the curves could be separated in three parts, corresponding with different slopes and GPR values (Figure 2). As suggested by Cecchi et al. (1997), the higher slope values correspond to conversion of readily biodegradable carbon source to biogas. The readily biodegradable substrate (rbCOD) consisted of acetate and compounds readily transformed into acetate. These compounds were transformed into biogas in few hours and the complete consumption was highlighted by a sharp change in the slope (Pavan et al., 2000). During the next 6-10 hours, degradation of ethanol and VFAs became important (Cecchi et al., 1997). This group of compounds could be defined as easily hydrolyzed COD fraction (rhCOD), term borrowed by wastewater COD fractionation (Cokgor et al., 2009; Fall et al., 2012). It is composed by biodegradable sCOD that is quickly converted in volatile fatty acids: organic acids such as tartaric and malic ones. The amount of rhCOD fraction was the same in the two processes and contributed for similar percentage to daily biogas production (37% and 42% in Run 1 and Run 2 respectively). Otherwise the higher GPR observed in Run 2 caused faster consumption of rhCOD fraction.

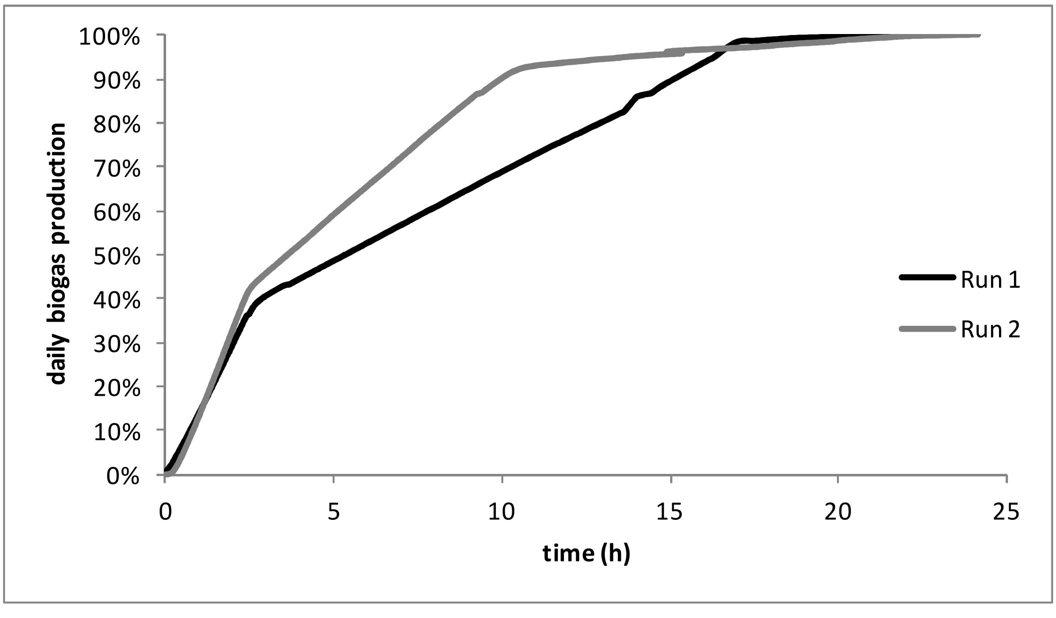

Figure 6. Cumulative biogas production plotted against the time between two consecutive feeds in Run 1 and Run 2

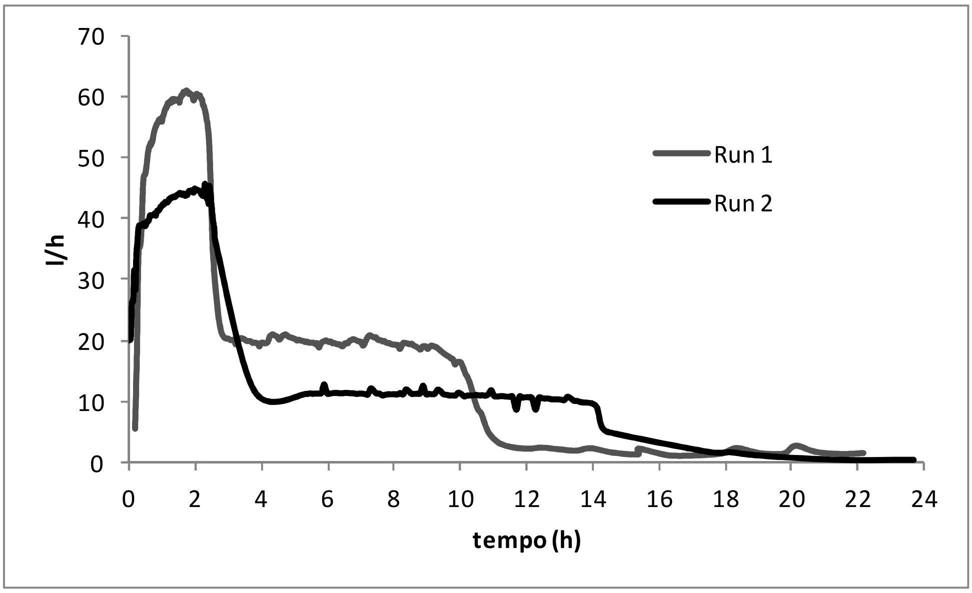

Figure 7. Biogas production rates detected in Run 1 and Run 2 between two consecutive feeds

Another sharp change in gas production rates indicated consumption of rhCOD and start of slowly biodegradable substrate (sbCOD) degradation (Figure 2). In Run 1 GPR suddenly reduced from 1.0 to less than 0.5 $m^3/(m^3_{reactor}d)$ after 14 hours from feeding, while in Run 2 biogas production rate decreased from 2.0 to 0.3 $m^3/(m^3_{reactor}d)$ around the 11th hour from feeding. SbCOD was linked to particulate matter, then hydrolysis and solubilization steps controlled its degradation. Consumption of particulate organic matter was carried out during the whole day because the substrate for these biological reactions was never limiting but it became the bottleneck step of the whole process when the other groups of compounds were totally consumed.

The behaviours of the reactors during the two tested conditions were qualitatively similar and the different slopes were probably due to biomass concentration. The biomass production (P_x) was proportional to flow rate (Q) and COD removal (S_0 - S), where S_0 and S are influent and effluent COD concentrations (Eq. 7). In this equation parameters *Y, b* and *SRT* indicate respectively the specific biomass yield, the decay coefficient and the solid retention time, that in a system without recirculation corresponds to HRT.

$$P_x = \frac{Q\,Y\,(S_0 - S)}{1 + b\,(SRT)} \tag{7}$$

The organic loading rate was the same in both processes hence also the biomass growth can be considered equal. However the higher HRT determined minor withdrawn of microorganisms and higher biomass concentration in the reactor. The biomass concentration (X) can be calculated with equation 8, where V_r stands for reactor volume. The mass is expressed as grams of suspended volatile solids (VSS).

$$X = \frac{P_x\,SRT}{\mathrm{V_r}} \tag{8}$$

The calculated biomass concentrations were 2.6 and 3.8 g VSS/L and allowed for determine the maximum specific GPRs (m^3/(kg VSS d)) for the three group of compounds. As resumed in Table 3, the specific biogas production rate (SGPR) were similar in the different applied HRTs: 1.632 - 1.800 m^3/(kg VSS d) for rbCOD, 0.552 - 0.432 m^3/(kg VSS d) for rhCOD and 0.048 - 0.072 m^3/(kg VSS d) for sbCOD, with HRT of 40 and 23 d, respectively. The presence of easily biodegradable and hydrolysable COD didn't promote the growth of biomass able to hydrolyse particulate substrates. Neither application of long HRT in processes treating substrates with easily biodegradable organic matter doesn't improve the process yields.

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Table 3 Absolute and specific biogas production rate for rbCOD, rhCOD and sbCOD fractions, determined on basis of active biomass concentration (VSS).

Parameter		**Unit**	**Run 1**	**Run 2**
HRT		d	23	40
GPR	**rbCOD**	$m^3/m^3_{reactor}d$	4.7	6.2
	rhCOD	$m^3/m^3_{reactor}d$	1.2	2.0
	sbCOD	$m^3/m^3_{reactor}d$	0.1	0.1
VSS		g/L	2.60	3.82
SGPR	**rbCOD**	m^3/(kgMLVSS d)	1.800	1.632
	rhCOD	m^3/(kgMLVSS d)	0.432	0.552
	sbCOD	m^3/(kgMLVSS d)	0.072	0.048

In both the processes appeared clear that biogas production was not continuous during the day and became negligible when rhCOD was totally consumed. Theoretically a higher organic load could be treated by the AD process without accumulation of intermediate metabolites. The organic load can be increased feeding more substrate once a day or feeding the reactor more frequently, when the easily hydrolysable COD was completely removed. In the first case the concentration of COD and volatile fatty acids in the reactor, just after the feeding, increased and probably methanogenic-inhibiting conditions should establish. Overloading due to higher OLR application was also reported by Da Ros et al. (2014) that worked with OLR of 4.5 kg COD/($m^3_{reactor}$d). The second option should split the feeding flow in more times in order to avoid inhibiting conditions. On the other hand, the higher organic load, couple with greater flow rate, should reduce the HRT then it has effects on microorganism concentration in the reactor and degradative rates, as observed in Table 3. Kinetic study can help to decide the best moment for the feeding. In fact the sharp GPR reduction, corresponding with easily degradable/hydrolysable COD consumption, could be used as signal to feed the reactor. Considering this approach biogas flow rate has been used as the control variable for the development of an automatic control system.

Several researchers have used methane or biogas production as inner-loop variable for AD process control because it shows faster response to perturbation compared with liquid phase variables. Otherwise the existing studies considered the GPR as a stability parameter and combined this information with a secondary variable (Chynoweth et al., 1994; García-Diéguez et al., 2011; Liu et al., 2004). They never considered the instantaneous biogas production rate as a consequence of process kinetics and as the sole control variable.

3.3 Automatic feeding system

Feeding system was controlled using GPR as the sole variable of process control: the feeding pump was automatically activated when biogas production rate was lower than a fixed set point. Set point was chosen on basis of previous kinetic results (RUN 1 and RUN 2). In particular it was observed that GPR decreased from 1.1 -2 m^3/($m^3_{reactor}$d) to 0.11-0.14 m^3/($m^3_{reactor}$d) when rhCOD fraction was completely consumed. For this reason biogas production rate of 0.4 m^3/($m^3_{reactor}$d), corresponding to production of 1 L every 15 min, was chosen as set point. Considering that the data-logger received a digital signal every litre of biogas produced, if after 15 minutes no signal was detected, the pump has been activated. As consequence the reactor produced minimum 0.4 m^3/($m^3_{reactor}$d) and there was almost no biogas production for maximum a quarter of hour.

In order to avoid excessive reduction of HRT and to make the comparison easier, in RUN 3 the characteristics of substrates mixture and volume for each feed (5.75 L) were similar to RUN 2. The reactor operating with this feeding mode worked for more than 90 days. The automatic control fed 5.75 L of substrate every 14.4 h in average, with average OLR of 6.2 kg COD/($m^3_{reactor}$ d) and HRT of 21 d. Some fluctuations of stability parameters were observed, but they were more affected by variability of substrates than by different feeding mode. pH values were quite constant as reported in Table 4, while alkalinity increased according with ammonium concentration and biogas production (higher solubility of CO_2 in the liquid phase). Although the higher nitrogen load, the ammonium concentration did not reach inhibiting values (Table 4).

Table 4 Characteristics of digestates from automatic fed process

Parameter	Unit	Run 3
HRT	d	21
TS	gTS/kg_{ww}	50.0 ± 5.1
VS	gVS/kg_{ww}	30.0 ± 1.8
COD	mg/g_{dw}	693 ± 73
sCOD	mg COD/L	1803 ± 931
TKN	mg N/g $_{dw}$	62.0 ± 7.3
$N\text{-}NH_4^+$	mg N/L	818 ± 83
P_{tot}	mg P^-/g $_{dw}$	8.8
pH	-	7.38± 0.14
Total Alkalinity	mg $CaCO_3$/L	3775 ± 202
Polyphenols	mgGAE/L	nd
SGP	m^3/kgCOD	0.457 ± 0.041
TS removal	%	24%
VS removal	%	35%
COD removal	%	69%
Polyphenols removal	%	nd

Lower influent dilution caused effluent solid concentration higher than previous trials (50 g TS/kg_{ww}). Percentage of volatile over total solids (60%) and particulate COD concentration (693 mg/g_{dw}) indicated that stabilisation of organic matter was at the same degree of those reached under other conditions. On the other hand the soluble COD was around 1,803 mg/L but the high value is not due to VFAs accumulation.

The biogas production reached mean value of 0.457 $m^3/kgCOD_{fed}$ with 60% of methane. The total and volatile solids removal were respectively 24% and 35%, while the COD was removed for the 69%. COD conversion was lower than Run1 and Run2 because of low methane content in the biogas.

Also in this trial, the three fractions of COD were well identified by different biogas production rates. At steady state the degradation rates were constant: 7.6, 3.2 and 0.5 $m^3/(m^3_{reactor}d)$ for rbCOD, rhCOD and sbCOD. The absolute degradation rates were higher than previous trials because greater biomass concentration (4.40 kgMLVSS/m^3). In fact the biomass was proportional to consumed substrates (Eq. 7) that in the third Run was almost the double of other Runs.

Considering active biomass concentration, the specific biogas production rate associated with rbCOD was 1.718 m^3/(kgMLVSS d), value comparable with the others Runs. While the SGPR of rhCOD and sbCOD improved to 0.736 and 0.106 m^3/(kgMLVSS d), respectively (Figure 3). SGPR for rhCOD and sbCOD increased of 67% and 75% compared with the trial operating with HRT of 23d, of 35% and 93% compared with results of trials with 40 d of HRT. Higher OLR improved the degradation rate of slow biodegradable fraction because of population shift or stimulating effect of high concentration of organic matter, quite constant in the time.

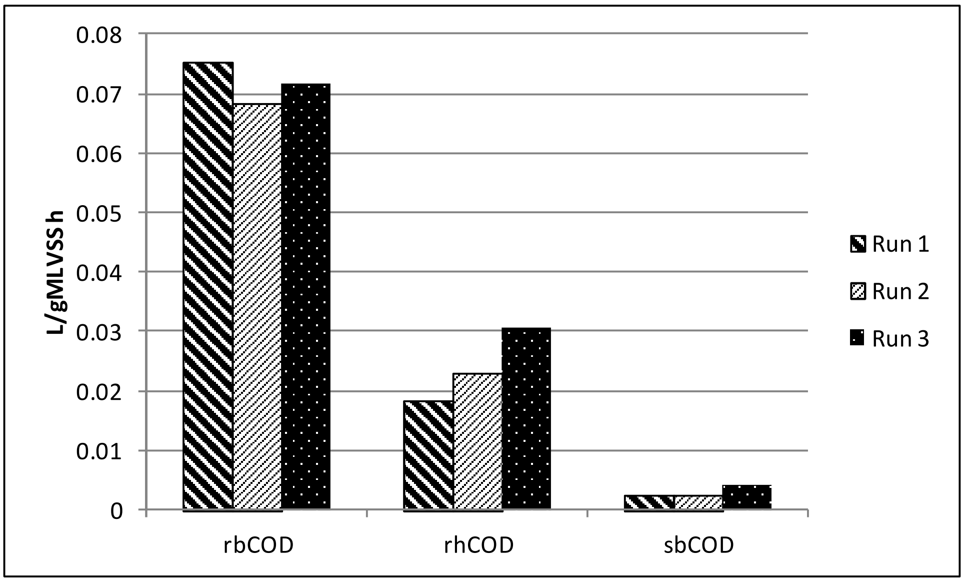

Figure 8 Specific degradation rates of rbCOD, rhCOD and sbCOD detected in Run 1, Run 2 and Run 3

3.4 Kinetic models application

In order to describe and compare the AD processes working with different operational conditions, two different linear models were applied to the biogas production data: first-order and step-diffusional models. Values of COD concentrations inside the reactor and degradation rates between one fed and the next one were considered for application of the models.

As can be seen from Table 5 the fit of the first-order model was not satisfactory (R^2 between 0.84 and 0.90), in fact this model cannot describe the sharp changes in biogas production rates because it had one sole constant. Anyway the values of constant model emphasized the increasing degradative capacity at longer HRT and higher OLR: kinetic constant increased from 1.40 to 2.56 d^{-1} with HRT of 23 and 40 d (OLR 3.2 kgCOD/($m^3_{reactor}$d)), respectively, and reached 3.88 d^{-1} with HRT of 21 d and OLR of 6.2 kgCOD/($m^3_{reactor}$d). As a final remark, it can be pointed out that biomass concentration became the limiting factor when the substrate concentration in the reactor was very high and substrate to microorganism ratio was elevated. In fact few hours after feed the curve reached a plateau corresponding to maximum degradation rate and this part be described by zero-order reaction (Cecchi et al., 1997).

Table 5 Linear regression parameter applying first order model.

		Run 1	Run 2	Run 3
		First-order model		
k	d^{-1}	1.40	2.56	3.88
R^2		0.90	0.84	0.89
Lack of fit		679	772	643
Average lack of fit		3.31	2.55	2.16

The step-diffusional model introduced different kinetics parameters for rbCOD, rhCOD and sbCOD, in particular for each COD fraction the maximum degradation velocities (v_0, v_1 and v_2) and diffusional rate (*4a, 4b* and *4c*) were identified (Cecchi et al., 1997, 1990b). The velocities increased according with longer HRT and higher OLR applied (Table 6), and were positively affected by concentrations of active biomass involved into COD fraction degradation. Comparison of velocity values determined in this study and those reported by Da Ros et al. (2014) showed similar results operating with comparable operational conditions. Da Ros et al. (2014) reported that using HRT of 21 d the velocities v_0, v_1 and v_2 were 4.11, 1.17 and 0.45 mgCOD/L min, and in this study they are 4.92, 1.45 and 0.77 mgCOD/L min.

Table 6 Regression parameter applying step-diffusional model.

		Run 1	Run 2	Run 3
		Step diffusional model		
v_0	mgCOD/L min	4.92	6.20	8.89
v_1	mgCOD/L min	1.45	2.85	4.12
v_2	mgCOD/L min	0.77	0.97	1.01
4a	mgCOD/L min^2	-0.007	-0.191	0.006
4b	mgCOD/L min^2	0.000	0.003	0.005
4c	mgCOD/L min^2	0.007	0.002	0.006
R^2		0.99	0.94	0.96
Lack of fit		20.7	216	152
Average lack of fit		0.10	0.72	0.55

Considering the biomass concentration the maximal specific degradation rates of each COD fraction were calculated, as v_i/X, and compared with those estimated by preliminary kinetic study (Figure 3). Increasing OLR from 3.2 to 6.2 kgCOD/($m^3_{reactor}$d) and maintaining similar HRT (23 and 21 d) the velocity associated with soluble fractions (v_0 and v_1) became higher, while the *v2* values were comparable (Figure 4).

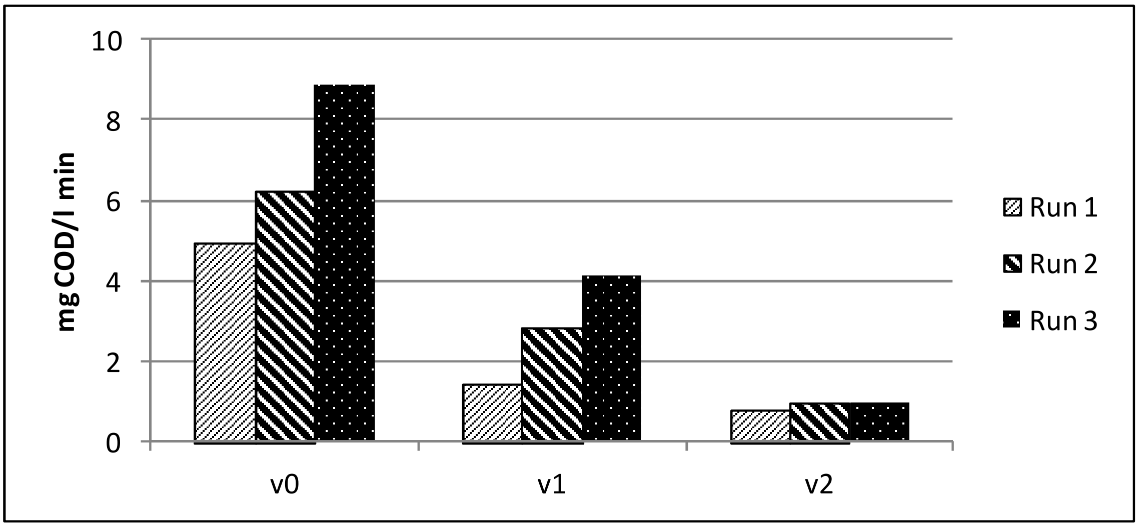

Figure 4 Degradation rate obtained applying step-diffusional model in the process operating with HRT of 23 and 40 d (Run 1 and Run 2) and HRT of 21 d (Run 3).

The direct application of the model to the data caused the determination of wrong value of diffusional rate of acetate throughout the cell (4*a*); in fact considering the physical meaning of this parameter it should have positive values. Its calculation was affected by slow increase of velocity just after the feed and the constants in this condition assumed negative values (Table 6). On the other hand, if this initial period was not considered, the available points where velocity decreased due to consumption of rbCOD, were not enough for constant estimation. In Run 3 the maximum velocity was reached in minor time, probably due to high concentration of biomass and to higher feeding frequency, and all the parameters *4a, 4b* and *4c* had positive values. These values had the same magnitude than those reported in the literature (Cecchi et al., 1991; Da Ros et al., 2014). The values are slightly higher than those derived from OFMSW anaerobic digestion indicating that diffusion of soluble compounds and of extracellular enzymes were less limiting than with other substrates. Although the model limits described above, the correlation coefficients were higher than those of first-order model, they ranged from 0.94 and 0.99. Also the average lack of fit reduced of one magnitude order (0.1-0.72).

4. Conclusions

Application of long HRT to AD of winery waste didn't affect the specific biogas production but increased microbial biomass concentration, and consequently improved the observed degradation rates. The behaviour of biogas production rate between a reactor feed and the next one was considered and the negligible production after 11-13 hours was observed due to complete consumption of soluble biodegradable COD. Consequently the feeding frequency was increased through a control system able to feed the reactor when GPR reduced below 0.4 $m^3/(m^3_{reactor}d)$. This system managed the feeding pump by a simple on/off control. The proposed approach allowed for increase the OLR from 3.2 to 6.2 $kgCOD/(m^3_{reactor}d)$, without any stability problems. The average GPR increased from to 1.1 to 2.8 $m^3/(m^3_{reactor}d)$, mainly due to faster degradation of rhCOD and sbCOD. Two kinetic models were used to describe the process: first order and step-diffusional models. First order model could not describe the degradation of different COD fraction (R^2 0.84-0.90) because of its simplicity but it was able to detect total degradation rate variation. Step-diffusional model previewed better the trend of degradation rates (R^2 0.94-0.99) although for diffusion rate of acetate, due to slow increase of degradation rate just after feeding. Step diffusional model showed how the diffusion of substrates and enzymes through the bacteria membrane has minor effect in AD of winery waste than of OFMSW, because of high biodegradability of wine lees and to microbial community selection.

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3.6. Role of trace elements augmentation on thermophilic anaerobic digestion of winery wastes

In section 3.3 stability problems, related to thermophilic anaerobic digestion, were introduced. The lack of micro-nutrients is not easy to demonstrate because several factors should be kept in consideration. In fact the availability of metal depends on total concentration and chemical form, moreover also microbial species in the reactor affect the nutrients requirement. The hypothesis of missing micro-nutrients derived from literature review and the relationship between metal addition and COD removal in this study confirmed the proposed hypothesis. In this section the benefit of metal augmentation is analyzed and discussed. The paper " Optimization of thermophilic anaerobic digestion of winery bio-waste by micro-nutrients augmentation" deals with effect of addition of iron, cobalt and zinc into feeding mixture. Several doses were tested and biogas production, COD removal and effluent characteristics were kept in consideration.

As other papers, C. Cavinato and P. Pavan contributed into setup the experimental design and elaboration of data. As well as D. Bolzonella collaborated into data elaboration and paper writing. I carried out all the experimental work, elaborated the results and wrote the manuscript.

Research paper

Optimization of thermophilic anaerobic digestion of winery bio-waste by micro-nutrients augmentation

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Keywords*:* mesophilic anaerobic digestion, micro-nutrients, minimum requirement, thermophilic anaerobic digestion, winery waste

Abstract Thermophilic anaerobic digestion is a suitable technology to treat agricultural waste because of its higher biogas production, hygienisation effect and solids removal efficiency. Although these benefits, poor effluent quality and instability are encountered in some cases. The anaerobic co-digestion of winery waste and waste activated sludge at 55°C, operating at 23 days HRT and organic loading rate of 3.2 kg COD/m^{3}d, was charaterized by accumulation of volatile fatty acids, pH fall and reduction of biogas production, while mesophilic process was steady at long term. The study evaluated the effect of trace elements (iron, cobalt and nickel) augmentation in the thermophilic reactor at different concentration of micro-nutrients. The addition improved the process stability: pH became constant and average volatile fatty acids concentration was below 1000 mg COD/L. The biogas production increased from 0.38 to 0. 45 m^3/kg COD, corresponding to 90% of COD removal, while mesophilic reactor removed the 78% of total COD. Digestate had interesting characteristics as fertilizer in fact the higher solids removal (28%) allowed to concentrate the phosphorus in the particulate fraction and nitrogen was transformed into more available form for plants growth.

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1. Introduction

Agro-industrial activities represent important sources of environmental impact because fruit and vegetable processing generates high amount of organic wastes. Among these bio-waste, the winery residues have great relevance in terms of quantity and of potential pollution. In fact their uncontrolled field spreading could cause relevant environmental problems due their high biodegradability, expressed by high level of chemical oxygen demand (COD). The raw wastes disposal on soils can consume large quantity of oxygen consequently they create anoxic conditions, reducing soil fertility. Moreover winery residues content antibacterial compounds produced by plants such as polyphenolic compounds, and toxic compounds like pesticides and heavy metals that can reduce crops growth (Moldes et al., 2008). For these reasons winery wastes need a treatment before the disposal, the most diffused process is composting but it is economically and energetically expensive. Considering the costs of the composting and of disposal, the production companies are seeking alternative biological treatments with a low environmental impact and economically sustainable for winery residues valorization, consisting in the recovery or transformation of the present components into high value-added resources.

Anaerobic digestion represents a suitable technology to reduce pollutant load of winery residues and to produce biogas, a renewable energy source. This technology is commercially proven and is widely used for treating high moisture content organic wastes (Ciubota-Rosie et al., 2008). Mesophilic processes, with a working temperature of 35-37°C, are the most applied conditions but the interest for thermophilic condition (55°C) is increasing for its several advantages. Kafle et al. (2014) demonstrated, in terms of digestate characteristics and kinetic constant, that VS removal was significantly higher under thermophilic temperature conditions than under mesophilic temperature conditions. Since reaction rates increase with temperature, significantly higher organic loads and considerably shorter hydrolysis retention times are expected at 55°C (Van Lier et al., 1997). Therefore, a smaller reactor volume will be sufficient at thermophilic temperatures compared to mesophilic conditions (Lens and Verstraete, 2001). Usually higher temperature improves solid and organic matter removals due to faster hydrolysis rates, and consequently biogas productions increased and effluent stream quantity reduced. Pathogens removal is guaranteed by maintaining 55°C for few days (Sahlström et al., 2004) and also the phytotoxicity of thermophilic digestate seems lower than mesophilic one (Vallini et al., 1993).

Although these benefits few full scale plants operate in thermophilic range, this is due to the higher heating costs and less stable operation (De Baere, 2000). The causes of instability are several: with regard to manure digestion, high ammonia concentrations may limit the thermophilic anaerobic treatability, owing to toxicity problems (Angelidaki and Ahring, 1993; Zeeman et al., 1985). As the free ammonia fraction increases with temperature and pH, the ammonia concentration tolerated at high pH and at thermophilic temperatures would be expected to be low (Ahring, 1994). Another drawback is the often-found high effluent volatile fatty acids concentration but the etiology is not clear. Variation of temperature determined a change in methanogenic population (Van Lier et al., 1992) explained by a rapid die-off of mesophilic organisms at temperature exceeding the maximum growth temperature of these bacteria. Thermophilic anaerobic population is composed by a lower numbers of species and probably has different nutrients requirements.

The treatment of industrial substrates emphasizes these aspects because usually these wastes have low concentration of micro-nutrients and an inadequate amounts of bio-available trace elements, metals in particular (Rittmann and McCarty, 2001; Speece, 1983; Zandvoort et al., 2006). In fact some metals are involved into biochemistry of anaerobic microorganisms and play important roles as co-factors of various enzymes involved in anaerobic reactions and transformations as reported by Fermoso et al. (2009).

Depending on the pathway, metal requirements may differ, but the general trends remain the same: Fe is the most abundant metal, followed by Ni and Co, and smaller amounts of Mo (and/or W) and Zn. The function of these metals on the growth and metabolism of anaerobic bacteria is well documented in the literature (Agler et al., 2008; Fermoso et al., 2009; Oleszkiewicz et al., 1990) but the effect of missing nutrients occasionally could be not evident. In fact the anaerobic microorganisms respond quite slowly and have long lag phase time after the supplementation of micronutrients, therefore batch tests results usually are not sufficient to understand the real nutrients necessity.

The experiments have to be carried out in continuous mode for quite long period as made by Takashima et al. (2011). That work reported a complete study on continuous reactor working at 55-57°C with metals supply and demonstrated that trace elements requirements in thermophilic digestion is greater than in mesophilic one, implying more requirements for biomass growth and activity and/or less bioavailability of those trace metals at higher temperature. The experiment was carried out using a solution of sole glucose as feed, then the effect of the substrate was not considered in that study. In general the variations in optimal concentration of trace elements at mesophilic and thermophilic temperature ranges are explained by the variety of methanogens, each having a unique trace metal requirement, which also depends on the type of substrate utilized (Paulo et al., 2004). For example Qiang et al. (2013) determined the metals requirements in thermophilic digestion of solid food waste and the values were lower than ones reported by Takashima et al. (2011).

In present study the anaerobic digestion of winery wastes, originated from wine making process, was carried out in thermophilic condition with different supplementation of trace elements. In order to evaluate the process, the stability parameters and the specific biogas production were compared with the same parameters of stable mesophilic reactor working with the same operational conditions but without necessity of metals supply. Two parallel experiments, with different temperature conditions, were carried out in order to verify that the operational conditions were not extreme and that inhibition of thermophilic reactor was not due to overloading of the reactor.

2. Materials and methods

2.1. Analytical methods

Substrates and effluents were monitored once a week in terms of total and volatile solids content (TS and VS), COD on particulate and soluble fraction (pCOD and sCOD respectively), total Kjeldahl nitrogen (TKN) and total phosphorus (Ptot). The process stability parameters, namely pH, volatile fatty acids (VFAs) content and speciation, total and partial alkalinity (TA and PA) and ammonium ion, were checked twice a week. All the analyses, except for VFAs, were carried out in accordance with the Standard Methods (APHA–AWWA–WEF, 2011). Volatile fatty acids content was monitored using a gas chromatograph (Carlo Erba instruments) hydrogen as gas carrier, equipped with a Fused Silica Capillary Column (Supelco NUKOLTM, 15m×0.53mm×0.5 µm film thickness) and with a flame ionization detector (200°C). The temperature during the analysis started from 80°C and reaches 200°C trough two other steps at 140 and 160°C, with a rate of 10°C/min. The analyzed samples were centrifuged and filtrated on a 0.45 µm membrane. The concentration of total polyphenols was measured using a modified version of the Folin–Ciocalteu reaction as reported in Laftka et al. (2007) and converted into Gallic acid equivalent (mg GAE/L). Gas productions were monitored continuously by two gas flow meters (Ritter Company, drum-type wet-test volumetric gas meters), and their composition (CH_4, CO_2 and O_2) were monitored by portable biogas analyzer (Geotechnical Instruments, GA 2000). This instrument was calibrated using air with content of oxygen of 21%, and a certificated mixture of CH_4 and CO_2 (60% and 40% respectively).

2.2. Experimental setup

The experimental trial was carried out by two identical continuous stirred pilot-scale bioreactors each with working volume of 230 L. The reactors included a water jacket connected to a heating recirculation system to maintain a constant temperature of 37°C and 55°C. The digesters were fed once a day with a mixture of waste activated sludge and wine lee to reach HRT of 23 d and organic loading rate (OLR) of 3.2 kg COD/(m^3d). The organic loading rate was chosen considering the results obtained by Da Ros et al. (2014), that demonstrate this OLR could inhibit the thermophilic process. The composition of feeding mixture derived from the real availability of winery wastes in a cellar able to receive about 300 thousand liters of wine per year: the mixture was composed by 0.6 kg COD/(m^3d) of waste activated sludge (WAS), and the remaining fraction (2.6 kg COD/(m^3d)) of wine lees (WL). Both the reactors started without metal elements addition, but after the failure of thermophilic process, a solution of Iron, Cobalt and Nickel was added to the reactor working at 55°C to reach the concentration suggested by Takashima et al. (2011) of 4.3 mg Fe-$FeCl_3$/L, 0.46 mg Ni-$NiCl_2$ $6H_2O$/L and 0.51 mg Co- $CoCl_2$ $6H_2O$/L. In order to evaluate the best concentration of metals, the doses were lowered step-by-step and maintained for at least a HRT for each dosage. In Table 1 tested doses in thermophilic reactor were reported, while the stable mesophilic system worked without metals addition and was used as control.

Table 1. Tested metals doses in thermophilic reactor

	RUN 0	RUN 1	RUN 2	RUN 3	RUN 4
Fe (mg/L)	0	4.3	3.01	2.15	0.86
Ni (mg/L)	0	0.46	0.32	0.23	0.09
Co (mg/L)	0	0.51	0.36	0.25	0.10

2.3. Inoculum and substrates characteristics

The reactors were initially filled-up with mesophilic and thermophilic digestates deriving from previous experimentation. The inocula were well stabilized, solids content was lower than 10 gTS/kg_{ww} and stability parameters were in the optimum ranges of anaerobic digestion (Table 2). Bio-wastes from wine-making process were used as feeding in this experimentation because of their low concentration of trace elements. Waste activated sludge (WAS) derived from a wastewater treatment plant working mainly with winery wastewater; the plant treated about 170 m^3/d of wastewater with average concentration of 3,747 mg COD/L. The treatment process was characterized by food to microorganisms ratio of 0.26 g COD/g MLVSS and long sludge retention time (35 d).

Table 2. Mesophilic and thermophilic seed digestates characteristics (TS: total solids, VS: volatile solids on wet and dry weight, pCOD: COD on particulate fraction, sCOD: COD on soluble fraction, pH, TKN: total Kjeldahl nitrogen on particulate fraction, NH_4^+: ammonium concentration in soluble fraction; P_{tot}: total phosphorus, Polyphenols).

Parameter	Unit	37°C	55°C
TS	gTS/kg_{ww}	8.84	9.37
VS	gVS/kg_{ww}	5.92	4.69
VS	% TS	67%	50%
pCOD	mg/g_{dw}	552	751
sCOD	g/L	910.7	1072.5
pH	-	7.53	8.33
TKN	$mg N/g_{dw}$	41.63	33.09
NH_4^+	mg N/L	193.4	539.4
P_{tot}	$mg P/g_{dw}$	47.0	26.8
Polyphenols	mg GAE/L	83.75	58.35

The WAS had high VS/TS ratio (88%) probably due to characteristics of raw wastewater, and well balanced nutrients ratio for biological treatment (Table 3). The wine lees were collected in same cellar that produced the WAS and it was formed by wine decanting after addition of bentonite. The presence of this inert material determined low content of volatile solids (57% of total solids) and the COD was concentrated in the soluble form (sCOD was the 83% of total COD). The levels of nitrogen and phosphorus were limiting for bacterial growth if compared with pCOD concentration. Considering WL (Table 3), variability ranges of total and volatile solids were larger than ones of WAS, because of the variability of produced wine. Both substrates were poor in micro-nutrients because of their origin.

3. Results and discussion

The initial start-up period, in both reactors, consisted with stepwise increases of organic load maintaining constant the contribution of WAS (0.6 kg COD/m^{3}d) and increasing the amount of WL. In the same time the HRT was lowered from 46 d to 23d. During this period the specific gas production increased agree with OLR (Figure 1), in fact supplied sCOD from WL, which was easily biodegradable COD, was completely converted to biogas. This transient period lasted 114 days.

Table 3. Waste activated sludge and wine lees characteristics (TS: total solids, VS: volatile solids on wet and dry weight, pCOD: COD on particulate fraction, sCOD: COD on soluble fraction, pH, TKN: total Kjeldahl nitrogen on particulate fraction, NH_4^+: ammonium concentration in soluble fraction; P_{tot}: total phosphorus)

Parameter	Unit	Waste Activated Sludge				Wine Lees			
		average	CV %	min	max	average	CV %	min	max
TS	gTS/kg_{ww}	158.9	31%	22.7	267.8	62.0	45%	12.3	120.0
VS	gVS/kg_{ww}	143.5	29%	20.7	237.3	33.6	45%	10.3	73.0
VS/TS	%	88%	3%	79%	93%	57%	23%	29%	86%
pCOD	mg/g_{dw}	868	8%	749	1008	559	27%	312	919
sCOD	g/L	-				167	27%	111	204
TKN	mg N/g_{dw}	52.7	31%	14.5	80.3	30.3	42%	9.7	68.7
NH_4^+	mg N/L	-				33.9	67%	6.7	95.3
P_{tot}	mg P/g_{dw}	7.3	27%	2.5	10.7	6.2	46%	2.6	14.3

3.1. Comparison of mesophilic and thermophilic processes without trace-elements

Mesophilic process reached steady state after two HRTs at constant conditions and was characterized by good stability parameters for the monitored period (9 HRTs). In particular pH ranged from 7.2 to 8.1, the soluble COD concentration was around 360 mg/L with less than half due to VFAs, and the ammonium content was about 400 mg/L. The process guaranteed the complete soluble COD removal but only a part of the particulate COD was converted into biogas. The 81% of COD was converted into biogas while solids were reduced of 19%. The average biogas production was 0.386 m^3/kgCOD$_{fed}$ with 78% of methane. On the other hand thermophilic process did not show instability problems during the start-up period but started to accumulate VFAs after reaching the fixed conditions. In particular the VFAs concentration ranged from 476 mgCOD/L, at the end of start-up, to 6,825 mgCOD/L after 23 days of regular feeding.

The dominant volatile fatty acids were acetic and propionic acids, corresponding to the 66 and 15% of total COD, respectively. Consequently partial alkalinity was consumed, pH dropped down to 5 and methanogenesis was totally inhibited. The biogas production was reduced in this period from 0.39 to 0.25 $m^3/kgCOD_{fed}$, and later stopped. Figure 1 highlights the process instability in RUN0, detected by VFAs increase, and consequently specific biogas production (SGP) reduction. After feed suspension the hydrolysis of organic matter continued, in fact the VFAs concentration increased and degradation of proteins enhanced ammonium content.

The possible inhibitors were examined (free ammonia, polyphenols, sulfide). The free ammonia (FA) at 55 °C was about 177 mg N/L and concentrations below 200 mg N /L are generally believed beneficial to anaerobic process since nitrogen is an essential nutrient for anaerobic microorganisms (Liu and Sung, 2002). Polyphenols were present in the winery waste at concentration of 1,496 mg GAE/L and, although their degradation was more difficult at thermophilic temperature than at mesophilic one (Levén and Schnürer, 2005), the measured concentration in the thermophilic effluent was 152.8 mg GAE/L, far lower than inhibiting level (Melamane et al., 2007). Utilization of $CuSO_4$ and SO_2 during wine-making process can cause high concentration of sulphates in WL. During the anaerobic digestion the sulphates were reduced to sulfides and H_2S was formed. The H_2S is the most toxic sulfide form for the microorganisms involved into methanisation, and the inhibiting concentration range was 50–400 mg H_2S/L (Parkin et al., 1990). The content of H_2 was monitored in the biogas of both the reactors and resulted similar at different temperature (800 ppm). Considering the Henry's law the concentration in liquid phase of H_2S was lower at 55°C than at 37°C, and mesophilic reactor did not show inhibition effects. Moreover the pH > 7 determined the dominance of HS^- specie, less toxic than unionized sulfide.

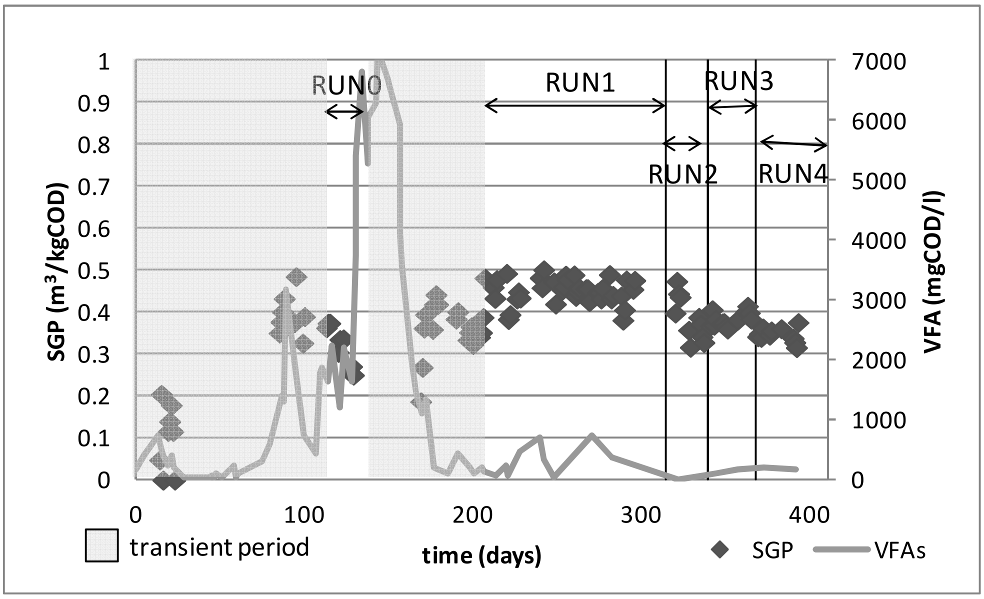

Figure 1. Trend of biogas production in thermophilic reactor, comparison with mesophilic process (continuous line)

3.2. Thermophilic anaerobic digestion with trace-elements augmentation

High concentrations of VFAs and mainly of propionate were indicative of less bioavailability of trace elements at thermophilic operating temperature as suggested by Takashima et al. (2011). In order to verify the effect of trace elements supply, the thermophilic reactor was recovered and the second start-up was designed with trace elements addition to obtain concentration of 4.3 mg Fe/L, 0.46 mg Ni/L and 0.51 mg Co/L. The same metals augmentation was tested in the feed during RUN1 from day 205. The thermophilic process with these dosages was carried out for 4 HRT and it appeared steady in terms of stability parameters, effluents characteristics and biogas production. The pH ranged from 7.7 to 8.0, average ammonium concentration was 664 mg N/L and the corresponding free ammonia value was 158 mg N/L. Comparing VFAs in RUN0 and RUN1 (Figure 1), it is clear the VFA concentration reduction and the corresponding increase of biogas production to 0.45 $m^3/kgCOD_{fed}$ with 77% of methane. On the other hand, comparison of mesophilic and thermophilic process (RUN1) showed an improved biogas production of 18% and increase of solid removal from 17% to 28%. In fact the average solid concentration was 20.6 g TS/kg_{ww} because of greater hydrolysis of particulate matter at this temperature. The higher hydrolysis rate explained also the value of sCOD (995 mg COD/L) that was higher than in mesophilic effluent. Just the 30% of soluble COD was due to VFA, but they did not accumulate and the buffer capacity (total alkalinity 3,390 mg $CaCO_3$/L) was enough to maintain optimum pH value for anaerobic digestion. The addition of trace elements also improved polyphenols degradation, in fact the concentration has been reduced to 66 mg GAE/L, slightly higher than in mesophilic effluents. The difference in degradation efficiencies between the operational temperatures were due to presence of the different microbial populations in the two environments and to partial inactivation of enzyme involved into phthalate-degrading pathways at 55°C (Levén and Schnürer, 2005). In order to evaluate the best dosage, the amount of added metals were reduced to 70% of initial quantities (3.01 mgFe/L, 0.32 mgNi/L and 0.36 mgCo/L) in the RUN2. The stability parameters remained in the suggested range for anaerobic digestion during whole HRT. The monitoring results showed a slightly reduction of ammonium concentration (590 mg/L), alkalinity and pH, also the solid concentration was lower. These changes were not due to process but to wine lees variability. In fact the WL at the beginning of this period had low solid concentration (20.6 mg/g_{ww}) and consequently the inlet nitrogen into reactor has been reduced. The results showed that the process could support nitrogen load variation and macro-nutrients were not limiting.

During RUN2 the biogas production reduced of 14% and reached values equal of mesophilic process (0.39 m^3/kgCOD) with 71% of methane. Also the COD removal (73%) was quite similar to process at 37°C. Beside the reduction of biogas production the process remained stable and metabolites remains at low concentration, in fact average soluble COD was 740 mg/L. Instead, in terms of energetic and economical balance, thermophilic process became not advantageous compared with mesophilic one, even considering the major cost of metals supplementation.

In RUN3 metals addition had been reduced again to 50% of initial dose (2.15 mgFe/L, 0.23 mgNi/L and 0.25 mgCo/L) and the process did not change significantly its performances if compared with RUN2. The soluble fraction in the effluent remained below than 900 mgCOD/L, pH values ranged from 7.6 to 8 and ammonium stabilized around 650 mgN/L, corresponding to about 150 mgN/L of free ammonia. Total alkalinity was strongly affected by ammonium and increased from 2,440 mg$CaCO_3$/L, of previous condition, to about 3,160 mg$CaCO_3$/L in RUN3. The biogas production slightly reduced to 0.381 m^3/kgCOD with 69% of methane. Considering these results, significant differences between RUN2 and RUN3 were not detected, but anaerobic digestion is really complex process and is affected by many factors such as variability of substrates.

Finally trials with 20% of initial dose were carried out (RUN4) and the main variation was in terms of biogas production that reduced to 0.347 m^3/kgCOD, but the methane percentage remained good (70%). Although the decrease in biogas production was a sign of instability, the other parameters were consistent with anaerobic digestion range. pH was 7.9, total alkalinity stayed around 3,023 mg$CaCO_3$/L and ammonium concentration was 644 mg/L. Soluble COD was similar to the value obtained in RUN3 and indicated that methanogenic bacteria were not inhibited but probably the biological activity slowed with low metals addition. Considering performances of this condition the failure of process was expected with further reduction in metals dose.

3.3. Comparison of anaerobic digestion operational conditions

Monitored parameters of all tested conditions were reported in Table 4. Thermophilic process with metals augmentation had suitable stability parameters for anaerobic digestion without any significant differences among the tested dosages. Comparing with mesophilic process, ammonium concentration was higher, because of greater hydrolysis rate, and the free ammonia was one magnitude order different. Ammonium and volatile fatty acid concentration determined values of alkalinity greater than 3,000 mg $CaCO_3$/L and pH around of 7.9. The degradation of solid particles also affected the solid content in the digestates, which reduced at least of 10% respect tomesophilic effluent, and the nitrogen distribution. In fact the nitrogen is for 39-49 % in soluble form at 55 °C, while at 37 °C less than 30% was ammonium nitrogen.

Greater solids removal efficiency also determined concentration of phosphorus into digestate, in fact the content in thermophilic effluent was always higher than in mesophilic one. The nutrients concentration in thermophilic digestate became it more interesting in terms of fertilization capacity.

Comparing metals requirement obtained by linear correlation between removed COD and metals addition, reported in Figure 2 (0.352 $mgFe_{added}/gCOD_{rem}$, 0.042 $mgCo_{added}/gCOD_{rem}$ and 0.038 $mgNi_{added}/gCOD_{rem}$), with those reported by Takashima et al. (2011), they were in the same magnitude order but slightly lower probably because the metals content of the substrates increased the available metals concentration in the reactor feed.

The addition of metals, also in low concentration, allowed better degradation of polyphenols probably because trace elements were involved into polyphenolic degradation pathway. The process yields were the most interesting results of this study, the specific gas production and COD removed at 55°C went over mesophilic yields only with maximum tested dose, while in the other cases the productions were comparable or minor. Relationships between metals addition and COD removal were showed in Figure 2. Considering the trials with lower additions of metals (RUN2, RUN3 and RUN4), micro-nutrients augmentation was well correlated with COD removal (R^2 99%), while in RUN1 the COD removal was higher than expected value from linear correlation.

Table 4. Comparison of stability parameters, digestate characteristics and yield at different operational conditions (pH, PA: partial alkalinity, TA: total alkalinity, NH_4^+: ammonium concentration in soluble fraction, FA: free ammonia, TS: total solids, VS: volatile solids on wet and dry weight, VS/TS percentage, pCOD: COD on particulate fraction, sCOD: COD on soluble fraction, TKN: total Kjeldahl nitrogen on particulate fraction; P_{tot}: total phosphorus, Polyphenols, SGP: specific gas production, percentage of CH_4, COD removal)

Parameter	Unit	37°C	55°C				
			RUN0	RUN1	RUN2	RUN3	RUN4
Stability parameters							
pH	-	7.38	6.7	7.91	7.78	7.82	7.9
PA	mg $CaCO_3$/L	1370	1688	2043	1678	1941	1944
TA	mg $CaCO_3$/L	2287	3673	3390	2439	3062	3023
N-NH_4^+	mg N/L	373	820	630	455	665	644
FA	mg N/L	11.8	110	154	90	145	155
Digestate characteristics							
TS	gTS/kg_{ww}	24.7	31.9	20.6	19.8	22.1	20.3
VS	gVS/kg_{ww}	14.3	19.5	12.1	13.3	11.6	11.5
VS/TS	%	58	61	59	67	52	61
pCOD	mg COD/g_{dw}	614	671	615	680	602	556
sCOD	mg COD/L	391	5394	995	740	870	882
TKN	mg N/g_{dw}	37.9	40.4	33.1	35.9	37.6	33.1
P_{tot}	mg P/g_{dw}	8	11.1	10.6	11.3	9.8	8.5
Polyphenols	mg GAE/L	26	153	66	61	57	-
Yields							
SGP	m^3/kgCOD	0.386	0.390	0.450	0.386	0.381	0.347
CH_4	%	78%	72%	77%	71%	69%	70%
COD removal	%	79%	-	92%	73%	70%	65%

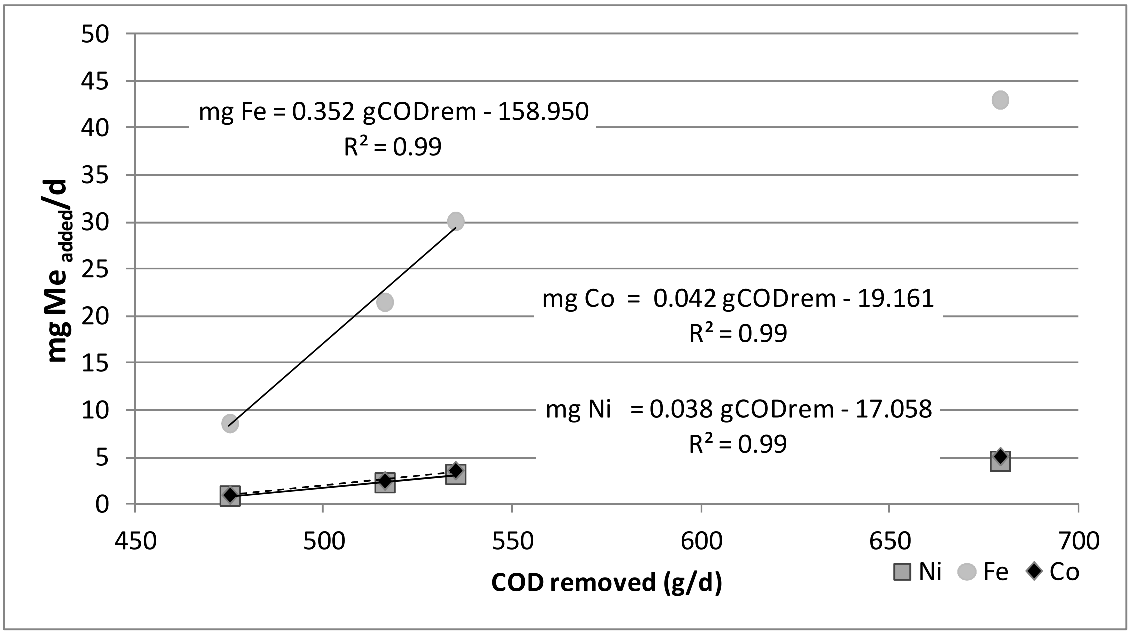

Figure 2. Metals requirements in different tested conditions

It is clear that micro-nutrients have positive effect on anaerobic degradation of organic matter but they were also involved in complex chemical reaction in the reactor. Probably the metals at high concentration react with potential inhibiting agents as reported by Gustavsson et al. (2013) and have synergistic effect on anaerobic process.

Naturally the metals dosages and the consumption of energy to maintain a higher operational temperature should increase management cost, then the economical aspect has to be evaluated deeply in order to apply thermophilic process at full-scale. Metal salts costs depend on location of the treatment plant and transportation cost, quality of salts and quantity purchase. Quoted prizes range from 0.29 to 7.10 \$/kg $FeCl_3$ (Schafer, 2001) and are 147 \$/kg $NiCl_2$ $6H_2O$ and 1440 \$/kg $CoCl_2$ $6H_2O$ (Pfluger, 2010). More attractive nutrient sources could be some wastes with good content of metals such as livestock effluents or waste activated sludge from civil or industrial wastewater treatment. In fact sludge, deriving from wastewater treatment of fruit and vegetable processing, has low concentrations of metals. It is important to note that both sludge and manure have characteristics other than nutrients which may aid digestion: they increase bacteria population by continuous system inoculum, add alkalinity to the system and are a source of degradable organic matter. It is more effective to mix two or three organic wastes to prepare a nutrient sufficient feed-stock for a high-solids anaerobic digestion process (Kayhanian et al., 1995). Hinken et al. (2008) reported that anaerobic digestion of silage failed after the removal of manure in the feeding of reactor and demonstrated that trace elements concentration in biomasses depends on amount of manure in the substrate for digestion plants.

On the other hand thermophilic process could reduce effluent disposal costs because of better hygienisation effect. Several studies reported the greater pathogens depletion were reached at 55°C because the *E.coli* and *Salmonellae spp.* were significantly removed (Da Ros et al., 2014; Sahlstrom et al., 2004).

4. Conclusions

Mesophilic anaerobic digestion fed with winery wastes was steady and SGP reached 0.386 m^3/kgCOD, while thermophilic one failed because VFAs accumulated. The cause of instability was the different requirement of thermophilic bacteria. The augmentation of iron, cobalt and nickel in thermophilic process at different concentrations was carried out. Higher trace-elements augmentation (4.3 mg Fe /L, 0.46 mg Ni/L and 0.51 mg Co/L) increased biogas production to 0.450 m^3/kgCODfed and COD removal reached 92%. While reducing metals addition, stability process remained in the optimum ranges for anaerobic digestion but yield reduced to value equal or lower than mesophilic one.

Relationship between metals addition and COD removal was linear only for the lowest three doses: 0.352 $mgFe_{added}/gCOD_{rem}$, 0.042 $mgCo_{added}/gCOD_{rem}$ and 0.038 $mgNi_{added}/gCOD_{rem}$. In the case of the highest addition maybe other chemical equilibria, not considered in this study, interact with trace-elements availability.

Thermophilic anaerobic digestion had several benefits, but metals and heat costs should be kept into account.

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3.7. Assessment of applicability of digestate as fertilizer on agricultural soil

Several studies reported that digestate is a suitable fertilizer because of its content of nutrients and properties of organic matter. Application of digestate on agricultural soil improves the economic balance of anaerobic digestion plant and of the whole cellar when the process is applied as described in section 3.3. In order to assess the feasibility of this activity, the digestate has to meet some characteristics defined by legislation. Among these characteristics, Italian legislation inserted germination index. This is a parameter that evaluate the toxicity on macrophytes and keep in consideration the complexity of the digestate.

Considering the few data about phytotoxicity of digestate, in paper " Assessing the potential phytotoxicity of digestate from winery wastes" has the aim to analyze the digestate deriving from process described in section 3.3 for this issue.

The experimentation was carried out with the analytical support of G. Libralato and M. Radaelli. All the authors collaborated into elaboration of the results and paper writing.

Research paper

Assessing the potential phytotoxicity of digestate from winery wastes

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Keywords*:* anaerobic digestion, digestate, germination index, phytotoxicity, winery wastes

Abstract Anaerobic digestion has widely diffused to treat organic wastes such as winery residues and recovery of its effluent increases the economical and environmental process sustainability. Spreading of digestate on soil could represent a suitable approach to recycle nutrients and organic matter, creating an on site circular economy. In this study, evaluation of digestate quality was carried out considering both chemical-physical characteristics and biological toxicity applying germination test. The effluent did not meet all the amendment quality standard defined by D.Lgs 75/2010 (germination index >60% with solution of 30% v/v of digestate), but bio-stimulation was observed at low doses (3.15-6.25 % v/v) for *S. alba* and *S. saccharatum*. The beneficial concentration agreed with Nitrate Directive dose and suggested that limited addition of digestate could have several positive effects on soil characteristics and on crop growth. Specific test using ammonium and copper solutions showed that these pollutants were not directly correlated to observed phytotoxicity. In fact the EC50 values with ammonium solutions were about 500 mgN/L exception for *S. saccharatum* (EC50 37 mgN/L); Cu concentration measured in the digestates (< 10 mg Cu/L) had no effect on germination.

1. Introduction

Anaerobic digestion (AD) has been widely diffused in the last decades to treat several type of organic waste such as organic fraction of municipal waste (Jain et al., 2015), waste activated sludge (Appels et al., 2008), livestock effluents (Ward et al., 2008) and winery wastes (Da Ros et al., 2016). The effluent of AD process is called digestate and its recovery can increase the economical and environmental process sustainability. The direct application of digestate to soil is currently considered an inexpensive option for its disposal and for recovery of their mineral and organic constituents for agricultural systems (Alburquerque et al., 2012). In fact, during the anaerobic process, part of organic nitrogen is transformed into ammonium, while phosphorus is partially converted in orthophosphate; both chemicals are easily available for plants growth. Digestate application can consequently substitute or reduce the use of chemical fertilizer. On the other hand, nutrients could be readily released polluting underground and surface water. For this purpose, the European Community defined a maximum nitrogen fertilization rate according to the Nitrate Directive (Directive 91/676/EEC). Considering the organic constituents, the labile fraction was mostly degraded during the AD process and lignin-like material, complex lipids and steroids became concentrated (Lorenz et al., 2007) reported that these compounds are humos precursors, consequently supply organic carbon in the soil. Moreover application of digestate leads to enhanced microbial processes such as nitrogen mineralization and ammonia oxidation (Abubaker et al., 2012; Odlare et al., 2008), and enzymatic activity (Galvez et al., 2012), which further increases the long-term nutrient release in soils (Abubaker et al., 2012; Odlare et al., 2008). Digestate improves soil physical properties (Różyło et al., 2015) increasing water balance and soil structure (Abubaker et al., 2012). In spite of digestate beneficial properties, it has to meet also quality standards in terms of heavy metals, polychlorinated byphenyls (PCBs), pathogens and phytotoxicity. Phytotoxicity is an interesting parameter evaluating the real digestate spreading impact on crops and it represents an index of its overall ecotoxicological impact. In fact the combined effect of the different contaminants mixed together, as well as their bioavailability, is difficult to estimate by chemical analysis while biological assays could supply the missing information (Alvarenga et al., 2007). Additionally, efforts should be made to identify the doses that will produce the desired fertilization effects ensuring the safety of agro-ecosystems (Różyło et al., 2015). To date, many countries introduced germination index (GI) to assess the quality of amendment as the result of the combination of macrophytes germination and root elongation. Generally it is an indicative limit value is provided in existing guidelines but only in Italy is a parameter enforced by law. The threshold for digestate acceptability as amendment according to the Italian legislation (D.Lgs 75/2010) was set at GI ≥ 60% in a digestate samples diluted at 30%.

GI was chosen for its simplicity, short time requirement (up to 72 h) and sensitivity, being the germination phase strongly affected by environmental conditions (Wang, 1991). It was applied mainly to compost (Komilis and Tziouvaras, 2009; Teglia et al., 2011a; Young et al., 2016) and recently to digestate (Di Maria et al., 2014; Pivato et al., 2016). Phytotoxicity test uses a matrix-based approach that considers the overall source of pollutants in the matrix and toxicants interaction. In most studies, it is applied as an indirect test, using an extract of the solid sample to identify its impact (Alvarenga et al., 2007) and the results depend strongly on the solid-to-liquid ratio assumed. Instead direct test deals with the raw sample (Kapanen and Itävaara, 2001) and gives more realistic results, because all kind of interactions between contaminants, soil matrix and test organisms are included and all site specific effects are integrated.

The presence of so many complex chemicals in the digestate (e.g. including metal ions, macro and micro-nutrients, organic pollutants) caused ecotoxicological interactions varying from synergism to antagonism (Gupta and Kelly, 1990), making toxicity etiology difficult to identify (Tam and Tiquia, 1994). Generally, phytotoxicity test carried out on digestate from livestock effluents showed stimulation at high dilution rate (Alburquerque et al., 2012; Pivato et al., 2016), while high concentrations showed germination inhibition. In contrast Gell et al. (Gell et al., 2011) didn't observe any differences from the control using digestate deriving from cow manure, pig slurry and human excreta, and three plant species (*Lactuca sativa L.*, *Raphanus sativus L.* and *Triticum aestivum, L.*). Germination index is usually inversely correlated with conductivity and ammonium concentration (Alburquerque et al., 2012; McLachlan et al., 2004; Tam and Tiquia, 1994). High ammonium concentration can reflect potential phytotoxicity (Teglia et al., 2011b; Tigini et al., 2016; Wong et al., 1983), but a threshold limit is not well defined. Di Maria et al. (2014) reported that concentration of 16-25 mg $N\text{-}NH_4^+/kg_{dw}$ inhibited seed germination in *Lepidium sativum*, while Tigini et al. (Tigini et al., 2016) indicated that the inhibiting concentration was higher than 2000 mg/L of $N\text{-}NH_4^+$ for *Lepidium sativum* and *Cucumis sativum*.

Salinity limits the germination of many plant species through osmotic effects or through ion toxicity (Brenchley and Probert, 1998). It is reported by Boluda et al. (2011) that salinity levels higher than 2.0-2.6 mS/cm can inhibit the number of *Lactuca sativa* germinated seeds and delay the germination process. Germination inhibition correlated by high conductivity level in the digestate was detected by several authors (Alburquerque et al., 2012; Pivato et al., 2016; Tigini et al., 2016). It can be associated with high concentration of sodium, chlorine, ammonium, and also metals. About metals in digestate, copper (Cu) and zinc (Zn) are the most recurrent (Alburquerque et al., 2012; Teglia et al., 2011a).

Phytotoxicity is not only correlated to chemical characteristics, but it depends on i) type of feedstock, ii) AD operational conditions (Abubaker et al., 2012; Tambone et al., 2010) and iii) macrophyte species used during the experimental phase. Di Maria et al. (Di Maria et al., 2014) demonstrated that operational conditions could affect toxicity, in particular high organic loading rate (OLR) and short hydraulic retention time determined higher concentration of volatile fatty acids (VFAs), reducing the biological stability and, hence, the digestate germination index.

Considering the several parameters affecting digestate phytotoxicity, prediction of residual toxicity is difficult and experimental tests have to be carried out taking in consideration chemical characteristics and operational AD conditions.

Winery wastes are interesting substrates for AD in wine producing countries because of their high biodegradability and pilot-scale experimentation showed that mesophilic process is the easiest to manage using hydraulic retention time higher than 20 days and organic loading rate of about 3 kg COD/(m^3d) (Da Ros et al., 2014). Digestate spreading on vineyards could represent a suitable approach to recycle nutrients and organic matter creating an on site circular economy, but the phytotoxicity evaluation has never been made.

In this study, digestate from winery wastes was investigated focusing on phytotoxicity with macrophytes looking for the potential contribution of ammonium and copper.

2. Material and methods

2.1. Digestate production and sampling

Two winery wastes, called D1 and D2, were considered: D1 was waste activated sludge (AS) from winery wastewater treatment and D2 was wine lees. They were collected in a cellar in Conegliano (Italy) producing about 30,000,000 L of wine per year. The75% of sold wine is white one and most of it is producing by Charmat method along the whole year. Throughout the year it generates 1.6 kg of wine lees and 2.0 L of wastewater per L of wine. The wastewater has high COD concentration (3,747 mg/L in average) and was treated inside the cellar borders by conventional activated sludge (AS) process. As reported by Da Ros et al. (Da Ros et al., 2016), the AS process operated with average hydraulic and sludge retention times (HRT and SRT) of 6.7 d and 35 d, respectively. The oversized biological reactor volume allowed to operate with long HRT and SRT values, in order to withstand the load picks due to. The MLVSS was 3,010 mg/L and the corresponding food to microorganisms' ratio was 0.26 kg COD/kg MLVSS per day. The COD was completely removed (95%) during the treatment and, in turn, 613 kg of dewatered waste AS was produced weekly. The substrate characteristics were reported in Table 1 and described in detail by Da Ros et al. (2016b).

Table 1 Wine lees and waste activated sludge characteristics; AS = Activated Sludge; nd = not detected. TS = total solid; VS = volatile solids; EC = electrical conductivity; pCOD = particulate COD; sCOD = soluble COD; TKN = total Kiendhal nitrogen; P_{tot} = total phosphorus.

Parameter	Units	Wine lees	Waste AS
TS	gTS/kg_{ww}	62.0 ± 27.9	158.9 ± 49.3
VS	gVS/kg_{ww}	33.6 ± 15.1	143.5 ± 41.6
VS/TS	%	57% ± 13%	88% ± 3%
pH	-	3.43 ± 0.14	nd
EC	mS/cm	1.94 ± 0.19	nd
$N\text{-}NH_4^+$	mg N/L	33.9 ± 22.7	nd
pCOD	g/kg_{ww}	30.7 ± 18.0	130.5± 38.2
sCOD	g/kg_{ww}	167 ± 45	nd
TKN	$mg\ N/g_{dw}$	30.3 ± 12.7	52.7 ± 16.3
P_{tot}	mgP/g_{dw}	6.2 ± 2.9	7.3 ± 2.0
Polyphenols	mg GAE/L	1537 ± 1189	nd

A continuous stirred tank reactor (CSTR) with a working volume of 0.23 m^3 was employed for anaerobic co-digestion of waste AS and wine lees. The temperature was maintained at 37 °C using an external jacket. PT100 probes (OMEGA Engineering Inc., Norwalk, CT, USA) monitored the temperature trend during process and managed the water recirculation pumps. The reactor operated with an organic loading rate of 3.2 kg/(m^3 d) of chemical oxygen demand (COD) and HRT of 23 d. The organic load distribution between the two co-substrates considered the real waste flow characteristics: 80% of wine lees and 20% of waste AS.

The operational conditions were reached by a long start-up period (140 d) that consisted in slowing the increase of organic loading rates. The steady state was maintained for more than one year. Stability process parameters and biogas composition were analyzed twice per week. Nutrients content and COD concentration was measured once per week, while the phytotoxicity was evaluated twice in the whole period, eleven months far from each other.

2.2. Analytical methods for digestate characterization

2.2.1 Physico-chemical analyses

The substrates and the digester effluents were collected and monitored once a week to determine the total and volatile solid content (TS and VS), COD, total Kjeldahl nitrogen (TKN), and total phosphorus (P_{tot}) (American Public Health Association et al., 1999). The process stability parameters, pH, VFAs content and composition, total and partial alkalinity, and ammonia concentration were checked two or three times per week. VFAs content was monitored using a gas chromatograph as reported by Cavinato et al. (2014). At steady state conditions, the total polyphenols were analyzed spectrophotometrically using the Folin Ciocalteu assay (Lafka et al., 2007). The concentration was reported in terms of gallic acid equivalent per liter (mg GAE/L). Biogas was collected by a Tedlar® gas sampling bag and the biogas composition (CO_2, CH_4, H_2, and O_2) was determined by a gas chromatograph (GC Agilent Technology 6890 N) equipped with a column HP-PLOT MOLESIEVE, 30 x 0.53 mm ID x 25 mm using a thermal conductivity detector and argon as gas carrier.

Dry milled digestate samples were analyzed to determine Cu and Zn content. Sample digestion was carried out using a microwave oven (Ethos l-Milestone S.r.l Advance Microwave Digesting Labstation, Italy) in acid conditions (ultrapure hydrofluoric and nitric acids). Concentration of metals was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) equipped with a collision/reaction cell (ICP-ORS-MS) (Agilent 7500 ORS).

2.2.2. Experimental design and phytotoxicity test

Phytoxicity tests were carried out according to Beltrami (Baudo et al., 1999) and OECD (OECD, 2006). A battery of three macrophytes was selected including two dicotyledonous (*Lepidium sativum* and *Sinapis alba*) and one monocotyledon (*Sorghum saccharatum*) species (Baudo, 2012). Certified seeds were purchased from Ecotox Ltd. (*L. sativum*-lot LES290311; *S. alba*-lot SIA051011; *S. saccharatum*-lot SOS140611). Germination (G, %), seedling elongation (SE, mm), germination index (GI) expressed as percentage ($GI = [100x(G \times SE)_{treatment}/[(G \times SE)_{control}]$). (Beltrami et al., 1999) were considered as endpoints. All endpoints were assessed in triplicate, otherwise explicitly indicated, including negative controls (ultrapure water). The common accepted threshold level when ten seeds are exposed per replicate in negative controls is 10% (Beltrami et al., 1999; OECD, 2006). The GI can assume values greater or lower than 100%, where a value equal to 100% means that the seedling average length and germination rate between a specific treatment and the negative control are exactly the same (Baudo, 2012). If values are between 80% and 120%, effects are likely the negative controls, otherwise values > 120% indicate biostimulation and < 80% inhibition effects (Cesaro et al., 2015). Polystyrene Petri dishes equipped with a Whatman no. 1 filter were used as testing chambers containing 5 ml of digestate, or a dilution of it with distilled water. Ten seeds were incubated per Petri dish for 72 h at 25 °C in the dark. Results were acquired using a digital camera corrected for objective distortion. The number of germinated seeds was registered and the whole length of seedling measured.

Experimental design considered phytotoxicity characterization of two digestate samples (D1 and D2), and ammonium and copper synthetic solutions (Figure 1).

Both digestates were analyzed using different dilutions obtained by distilled water (3.125, 6.25, 12.5, 25, 50 and 100% v/v for D1, 5, 10, 25 and 50% v/v for D2) and evaluating the overall toxicity of digestate via dilution-response relationship.

Several authors reported that ammonium is one of the most toxic compounds in the digestate, but they did not define its toxicity. In order to confirm literature data and estimate ammonium effect, phytotoxicity tests were carried out on $(NH_4)_2SO_4$ (10, 100, 500, 1000 and 10000 mg N/L) using the same battery of macrophytes.

The results of germination assays on digestate samples were thus elaborated considering ammonium content and, finally, the biological assay was repeated with D1 after partial ammonium stripping by air bubbling for 24 h. The long bubbling simulated a post-treatment able to reduce ammonium concentration, remove volatile organic compounds and consequently increase the pH; on the other hand this process did not modify the persistent compounds content such as heavy metals and salts. Neutral pH was corrected by diluted HCl addition and this dilution was considered to calculate real dilution (2.9, 5.8, 11.5, 23.1, 46.1, 92.2% v/v) and ammonium concentration.

In order to evaluate the role of copper in seed germination, the results obtained with D1 exposure was analyzed considering Cu content and compared with response using solution of copper sulfate ($CuSO_4$) with concentration ranging from 1 mg Cu/L to 1000 mg Cu/L.

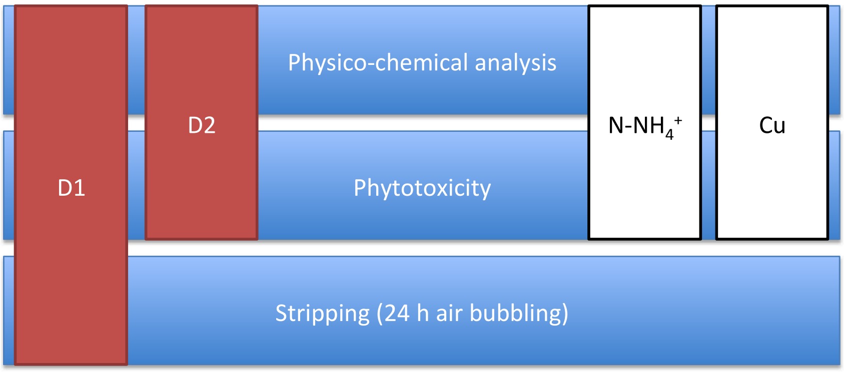


Figure 1 Experimental design of this study. D1 e D2 = digestate 1 and 2

2.3 Data analysis

Root elongation was carried out with ImageJ (Schneider et al., 2012). Whenever possible, toxicity was expressed as effective median concentration generating a 50% in the treated population (EC50). Otherwise, toxicity was expressed as percentage of effect at its relative exposure concentration. The significance of differences between average effect values of different experimental treatments and controls was assessed by the analysis of variance (ANOVA) considering a significance threshold level always set at 5%. When ANOVA revealed significant differences among treatments, post-hoc tests were carried out with Dunnett's method and Tukey's test. Statistical analyses were performed using Microsofts Excel 2013/XLSTAT©-Pro (Version 7.2, 2003, Addinsoft, Inc., Brooklyn, NY, USA).

Two parametric models were used to calculate EC50 and presence of stimulation effects. As suggested by Vanewijk and Hoekstra (1993), logistic model was used when concentration-response toxicity data followed a sigmoidal curve, while linear logistic model (Brain and Cousens, 1989) was applied when a stimulation for low concentrations (hormesis) of otherwise toxic compounds was detected. The logistic (Eq. 1) and linear-logistic (Eq. 2) models were used to describe experimental data.

$$y = \frac{k}{1+(x/x_0)^b} \qquad \text{Eq. 1}$$

$$y = \frac{k\,(1+fx)}{1+(2fx_0+1)*(x/x_0)^b} \qquad \text{Eq. 2}$$

Where *y* is the effect expressed as GI, *x* the digestate concentration in terms of percentage over total solution volume and *k* stands for the y value at x = 0. The parameter *b* relates to the slope of the tangential line in the point of inflection on response-dose curve or stands for the slope of the line on logit-log-scale. x_0 is the EC50 value and *f* stands for hormesis, when it has positive value the curve shows an increase of response value at low concentrations.

A nonlinear least-square regression analysis was performed using Excel™ to determine the two models equations parameters (*k*, x_0, *b* and *f*) and the EC50 defined by x_0 value. The correlation coefficient (R^2) was calculated to assess the goodness-of-fit of each model, like as the significance of stimulation. When the equation model is known, the effect for each dilution could be calculated.

3. Results and discussion

3.1 Chemical-Physical characteristics of digestate

Two digestate samples (D1 and D2) were collected from pilot-scale reactor eleven months far between each other. No dewatering was carried out consequently the samples had low dry matter content (22.4 and 22.7 gTS/kg_{ww}). They can be classified as liquid substrate because dry matter was lower than 15% and can be evaluated without operating an extraction. As reported in Table 2, they were characterized by pH values > 7; D2 had a more alkaline value because of the greater ammonium concentration (Figure 2). Also buffer capacity could affect pH, but in this case partial and total alkalinity can be considered comparable. The highest conductivity was observed in the second sample (5.74 mS/cm), probably due to higher ions concentration such as potassium ion and ammonium concentration (Table 2). Both digestates had EC values considered able to inhibit seed germination (Boluda et al., 2011).

The organic matter content, expressed as COD, was comparable in D1 and D2 (696 and 639 mg COD/g_{dw}, in that order) and similar to other digestates from different origin (Tigini et al., 2016). Regarding the plant nutrient content and hence the fertilizer value, total nitrogen content (sum of ammonium and TKN content on dry matter) was 1.4 and 1.7 gN/L in D1 and D2, respectively. The difference was mainly due to the ammonium content that was 23% and 37% of the total nitrogen. Hence, this nutrient is mainly in the organic form (76 and 63% of total nitrogen), less available for the plant and slowly released to the environment. Total and volatile solids content and particulate COD, TKN and P_{tot} were comparable, because they are correlated with operational conditions applied (i.e. organic loading rate, HRT and temperature) and affected by waste AS.

Table 2. Characteristics of digestates used for phytotoxicity tests; D1 e D2 = digestate 1 and 2, nd = not detected , TS = total solids, VS = volatile solids, VS/TS = volatile to total solids ratio, pH, EC = electrical conductivity, partial and total alkalinity determined at pH 6 and 4 respectively, $N\text{-}NH_4^+$ = ammonium nitrogen, sCOD = soluble COD, pCOD = particulate COD, TKN = total Kjeldahl nitrogen, P_{tot} = total phosphorus.

Parameter	Units	D1	D2
TS	mg/g_{ww}	22.4 ± 0.7	22.7 ± 1.4
VS	mg/g_{ww}	14.6 ± 1.1	13.3 ±1.7
VS/TS	%	62 ± 11	58 ± 5
pH	-	7.35 ±0.12	7.70 ± 0.11
EC	mS/cm	4.20 ± 0.42	5.74 ± 0.21
Partial Alkalinity (pH 6)	$mgCaCO_3/L$	1,326 ± 20	1,475 ± 102
Total Alkalinity (pH 4)	$mgCaCO_3/L$	2,121 ± 10	2,331± 99
$N\text{-}NH_4^+$	mg N/L	321 ± 37	639 ± 51
sCOD	mgO_2/L	217 ±72	350 ± 24
pCOD	mg/g_{dw}	696 ± 34	687 ± 65
TKN	mg/g_{dw}	46.8 ± 1.5	48.0 ± 10.2
Ptot	mg/g_{dw}	11.1 ± 0.2	7.5 ±1.8
Polyphenols	mg GAE/L	43.3 ± 29.3	30.7 ± 4.4
Na^+	mg/L	90.79 ± 6.72	64.63 ± 5.60
K^+	mg/L	298.81 ± 28.73	591.37 ± 2.59
Mg^{2+}	mg/L	nd	2.29 ± 0.90
Ca^{2+}	mg/L	nd	54.88 ± 20.77
Cu	mg/kg_{dw}	430.7 ± 24.6	nd
Zn	mg/kg_{dw}	141.1 ± 5.73	nd

The characteristics associated with liquid fraction (i.e. pH, alkalinity, conductivity, soluble COD and ammonium nitrogen) were different. In D2, the ammonium nitrogen concentration was higher than D1, probably affecting the greater values of pH, conductivity and alkalinity (Table 2, Figure 1). The differences were due to wine lees that had a great variability range (Table 1). The sCOD was slightly higher in the second sample (Figure 3), but both D1 and D2 had VFAs <1,500 mg/L, which is the proposed threshold limit for digestate fertilizer use within the end-of-waste criteria (Saveyn and Eder, 2014)

Presence of polyphenols < 50 mgGAE/L was characteristic of digestate from winery waste (C Da Ros et al., 2016). The polyphenolic compounds could inhibit or delay the germination, anyway they are degraded in aerobic conditions and could serve as precursor for the formation of humic acids in soil (Mekki et al., 2007).

Copper is used in the vineyard for plant health and during the winemaking process. In the digestate Cu concentration was around 431 mg/kg_{dw} and derived from wine lees (Da Ros et al., 2014). The digestate did not meet the threshold limit for fertilizer in Italy (230 $mgCu/kg_{dw}$, D.Lgs 75/2010) and proposed end-of-waste criteria from 3rd Working Document (100 mg Cu/kg_{dw}, Saveyn and Eder, 2014).

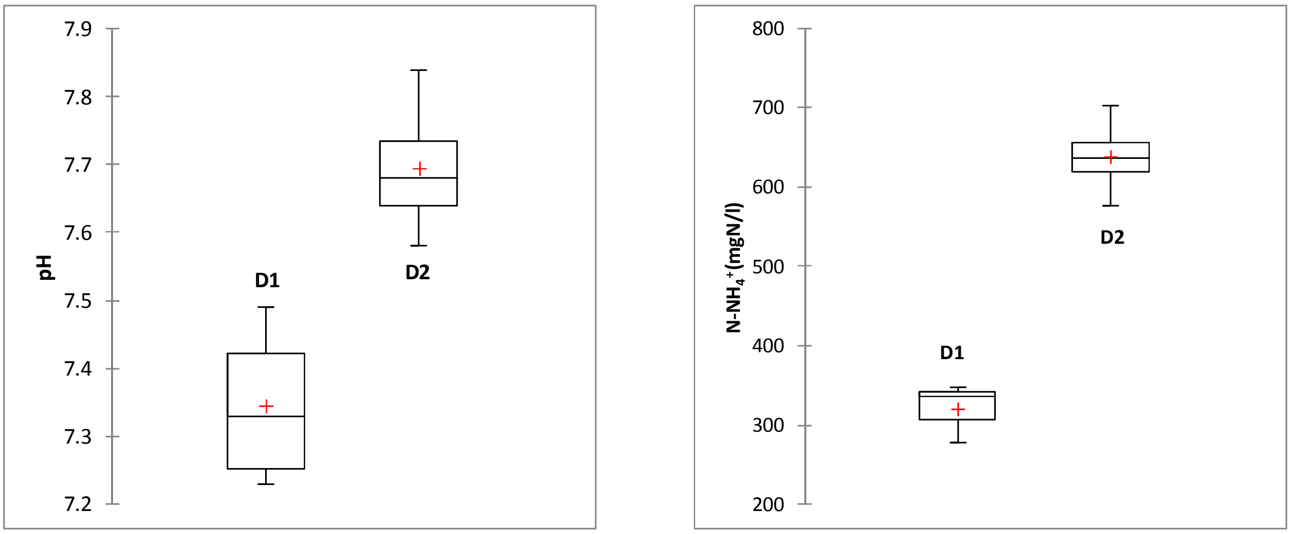


Figure 2. Comparison of pH values and ammonium concentration in the digestate samples (D1 and D2)

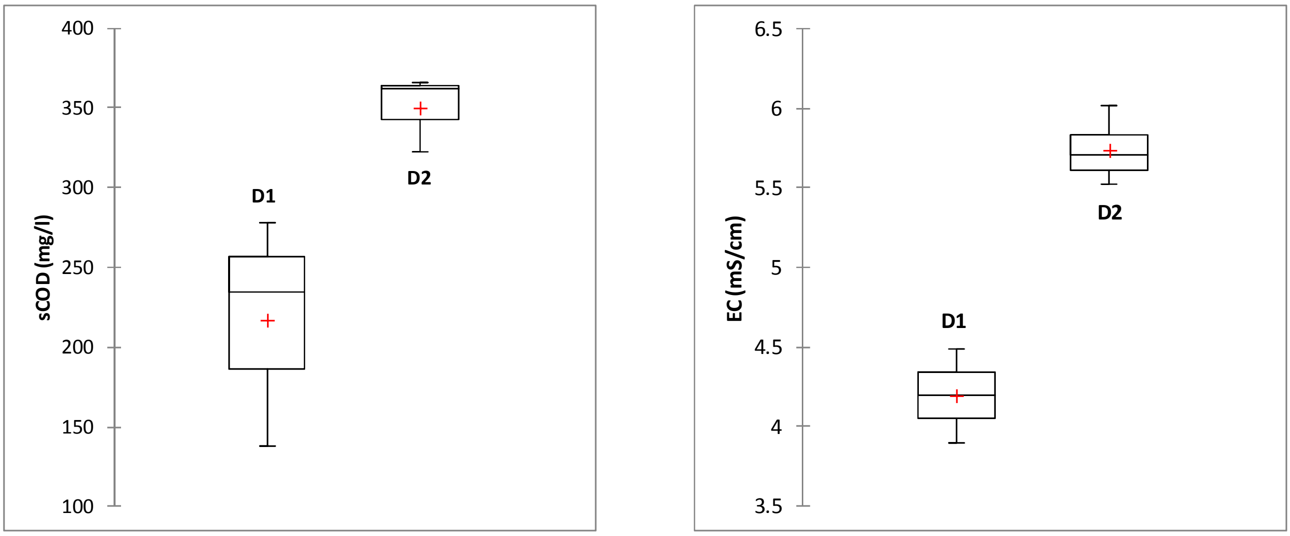


Figure 3. Comparison of soluble COD (sCOD) concentration and conductivity (EC) values in digestate samples (D1and D2).

3.2 Digestate phytotoxicity

3.2.1 Phytotoxicity of D1

The number of germinated seeds of *L. sativum* reduced from 93% in the control test to about 80% when digestate solutions at 3.125, 6.25 and 12.5% were used. Negative controls (< 10%) were acceptable for all testing species according to Libralato et al. (Libralato et al., 2015). Less diluted samples significantly decreased the number of germinated seeds (Table 3). Seedling elongation increased when 3.125% of D1 was applied (+35%) and dilutions of 6.25 and 12.5% had no effect on elongation after normalization to the negative control. Higher D1 concentrations inhibited root development and seedling development (Table 3). GI showed a slight stimulation at the lowest D1 concentration (3.125% v/v). ANOVA evidenced no significant differences after the exposure from 0% to 12.5% ($p < 0.05$), while inhibition was detected for higher concentrations (25, 50 and 100% v/v).

S. alba was less sensitive than *L. sativum* in terms of germinated seeds, in fact the germination rate was about 90% up to 25% of the digestate. The most interesting effect of digestate was observed on seed elongation: root length increased from 29.3 mm up to 50 mm with D1 dilutions of 3.125, 6.25 and 12.5%. The difference between the control and treatments was not relevant up to 25% of D1. Higher concentrations (25, 50 and 100% v/v) inhibited both seed germination and elongation. GI agreed with these observations: important stimulation (74-78%) was observed at lower digestate concentration (3.125 and 6.25 % v/v), the effect was not significant at 25% of D1, while germination was completely inhibited at 50 and 100% of digestate.

Table 3. Germinated seeds (GS), root elongation (RE) and germination index (GI) measure with different exposition to D1.

L. sativum

D1 (% v	**0**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
GS (%)	93 ± 6	80 ± 10	83 ±6	83 ± 15	77 ± 6	13 ± 12	13 ± 6
RE (mm	48 ± 3	65 ± 3	43 ± 15	47 ± 8	17. ± 2	2 ± 1	1 ± 0
GI (%)	100 ± 0	117 ± 20	81 ± 28	89 ± 30	29 ± 4	0 ± 1	0 ± 0

S. alba

D1 (% v	**0**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
GS (%)	90 ± 0	93 ± 12	90 ± 10	87 ± 15	87 ± 6	3 ± 6	0 ± 0
RE (mm	29 ± 5	49 ± 7	51 ± 5.	54 ± 14	27 ± 3	1 ± 2	0 ± 0
GI (%)	100 ± 0	179 ± 52	174 ± 15	174 ± 15	89 ± 22	0 ± 1	0 ± 0

S. saccharatum

D1 (% v	**0**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
GS (%)	97 ± 6	100 ± 0	83 ± 6	100 ±0	97 ± 6	77 ± 21	40 ± 20
RE (mm	50 ± 12	71 ± 4	42 ± 7	29 ± 11	34 ± 8	16 ± 10	5 ± 2
GI (%)	100 ± 0	151 ± 31	78 ± 35	71 ± 28	61 ± 29	25 ± 17	5 ± 2

The number of *S. saccharatum* germinated seed was not significantly different considering 3.125%-25% D1 treatments ($p < 0.05$), while greater dilutions inhibited germination. Germination was observed also with raw D1 while the other species didn't germinated at the same conditions (RE<1 mm), then *S. saccharatum* appeared more tolerant to raw digestate. Elongation stimulation was detected with 3.125% of D1, while gradual inhibition was observed for increasing D1 concentrations. GI showed stimulation (up to 51% at 3.125% v/v of D1), while lower dilution rates (> 6.25%v/v) had inhibiting effect.

Dilution-response relationships were analyzed using two models (logistic and linear-logistic) in order to evaluate which model fitted better the experimental data according to the absence or presence of biostimulation event (Figure 4). The linear-logistic fitted best except for *L. sativum*. The fitting of logistic model with the *L. sativum* data (R^2 0.98) confirmed the absence of biostimulation with an EC50 value of

20% of D1. Linear-logistic model fitted with *S. alba* (R^2 0.98) and *S. saccharatum* (R^2 0.95). The EC50 values calculated on this model basis were 30% and 19% for *S. alba* and *S. saccharatum*, respectively.

3.2.2 Phytotoxicity of D2

D2 inhibited the germination also at lowest concentration; in fact at dilution of 5% v/v germinated seeds are the 67% of total seeds. The difference between dilutions of 5% and 10% v/v is not significant, while at higher digestate concentration (25 and 50% v/v) only 10-13% of seeds germinated (Table 4). RE was similar in the control and in the test carried out with digestate most diluted (5%), latter it gradually reduced increasing digestate dose. GI gradually reduced increasing the digestate content in the tested solution. The analysis of variance indicated that results with digestate at 5% and 10% were statistically similar ($p < 0.05$). Hence, the toxicity was significant for D2 dilution > 10% and appeared comparable at 25% and 50% of D2.
The percentage of *S. alba* germinated seed was comparable with the control test up to 25% of D2, while a significant inhibition on root elongation was observed at lower concentration (up to -78%). This indicated that the substrate affect more the root development than germination. Higher concentrations (> 25%) significantly reduced both seed germination and root elongation. Statistical analysis clustered GI results in two groups: i) < 10% of D2: treatments had no effect on plant development; ii) > 10% of D2: significant phytotoxicity including both germination inhibition and/or root elongation inhibition. Total inhibition was observed when digestate was diluted two times (Table 4).

Table 4. Germinated seeds (GS), root elongation (RE) and germination index (GI) measure with different exposition to D2.

L. sativum					
D2	**0**	**5.0**	**10**	**25**	**50**
GS (%)	93 ± 6	67 ± 15	83 ± 12	10 ± 10	13 ± 6
RE (mm)	26 ± 8	28 ± 7	14 ± 3	4 ± 4	2 ± 1
GI (%)	100 ± 0	80 ± 23	52 ± 20	2 ± 1	1 ± 1
S. alba					
D2	**0**	**5.0**	**10**	**25**	**50**
GS (%)	93 ± 12	93 ± 6	100 ± 0	97 ± 6	3 ± 6
RE (mm)	55 ± 13	46 ± 7	45 ± 3	12 ± 3.2	1 ± 1
GI (%)	100 ± 0	88 ± 24	94 ± 33	25 ± 14	0 ± 0
S. saccharatum					
D2	**0**	**5.0**	**10**	**25**	**50**
GS (%)	83 ± 12	87 ± 15	80 ± 10	77 ± 12	67 ± 12
RE (mm)	38 ± 6	37 ± 12	29 ± 16	16 ± 10	14 ± 4
GI (%)	100 ± 0	105 ± 33	75 ± 40	43 ± 38	30 ± 8

The lower sensitivity of *S. saccharatum* was confirmed also in the case of D2. The percentage of germinated seed was reduced from approximately 80% (5-10-25% of D2) to 67% at 50% of D2. The root elongation reduced by 23% considering a 10% of D2, with inhibition increasing at higher D2 concentrations. The effect at 25% and 50% of D2 were not significantly different. The average GI values indicated that toxicity was inversely correlated to digestate content. Standard deviations observed on results using concentration form 10% and 25% v/v were higher than 30% and indicated a wide response variability of this macrophyte to digestate. Moreover no significant differences ($p<0.05$) between the highest evaluated doses (25% and 50% v/v) were evidenced.

D2 data fitted better with the logistic model (R^2 0.996 for *L. sativum* and *S. alba*, R^2 0.95 for *S. saccharatum*), because no hormesis was detected. EC50 values determined were 10%, 23% and 18% of D2 for *L. sativum, S. alba* and *S. saccharatum*, respectively (Figure 5).

3.2.3 Comparison of D1 and D2

In all the tests the toxicity is related to digestate concentration. Low doses (3.125% v/v of D1 and 5% v/v of D2) caused GI comparable to controls, germination reduced increasing digestate content until to totally inhibit the germination at 50% v/v of digestate. *S. saccharatum* is the less sensitive species because germination was observed also with 50% v/v of digestate concentration (GI of 25% and 30% using D1 and D2, respectively).

D1 and D2 were collected from the anaerobic reactor working at the same operational conditions (e.g. temperature, HRT, OLR, substrate types) at a time-distance of eleven months. Inconstancy on wine lees characteristics (Table 1) affected the final digestate parameters, despite that long HRT (23 d) moderated the effluent variability. The differences observed in terms of pH, conductivity, ammonium concentration and soluble COD, were due to wine lees fed to the reactor and had consequence on the digestate quality and its phytotoxicity. As consequence of different digestates characteristics, also phyto-toxicity changed using D1 and D2. Significant stimulation at low doses (3.125-5% v/v) was observed on *S. alba* and *S. saccharatum* when D1 was applied, while hormesis was not detected in D2. The EC50 values (Table 5) confirmed the higher toxicity of D2 exception for *S. saccharatum.* Germination inhibition of 50% of *L. sativum* was detected with 20% v/v of D1 and 10% v/v of D2, while EC50 values are less different for *S. alba* (30% v/v for D1 and 23% v/v of D2). *S. saccharatum* appeared less sensitive to digestate variability and more tolerant to high concentrations, in fact the complete inhibitions was observed only using the raw digestate (D1) while solution with 50% v/v of digestate inhibited germination for 70% and 75% for D1 and D2, respectively. On the other hand it appear the most variable macrophyte in fact the standard deviation values were often around the 30%.

Table 5.6 EC50 values along with 95% confidence for D1 and D2 using *L. sativum, S. alba and S.saccharatum*. The values were estimated using the model (logistic or linear-logistic) that better fits experimental behaviour.

	D1	**D2**
L. sativum	20% ± 7%	10% ± 3%
S. alba	30% ± 4%	23% ± 6%
S. saccharatum	19% ± 13%	18%± 16%

Germination tests results agreed with inhibiting effect of increasing concentration of ammonium and salinity level reported by studies on AD effluents (Di Maria et al., 2014; Pivato et al., 2016; Tigini et al., 2016). Despite the relationship found by Di Maria et al. (2014), the inhibitions of germination were not related to presence of readily biodegradable COD: in fact sCOD values were not relevant in the digestates (< 400 mg/L). While the presence of metals, mainly Cu, should be taken into consideration because its concentration was higher than law limits (230 mgCu/kg_{dw}) even if it is difficult to estimate their bioavailability and bioaccessibility in digestate.

The toxicity effect of solution containing 30% of both D1 and D2, as requested by D.Lgs. 75/2010, had a GI < 60% on *L. sativum*, meaning that an excess toxicity could be present for crops (Di Maria et al., 2014). In order to reach the GI of 60% the applied dilution should be 18% v/v of D1 and 8% v/v of D2.

Nitrate Directive should be taken in consideration in addition to D.Lgs. 75/2010, because it defined the nitrogen fertilization in order to protect groundwater from nutrients' pollution and avoid eutrophication. The maximum rate of nitrogen allowed by Directive on Nitrate Vulnerable Zones, such as Po Valley, is 170/kg N/hectare year. Considering this limit and that the soil depth interested by fertilization is equal to 30 cm, the amount of D1 and D2 used per hectare would be respectively 124 and 98 m^3, corresponding to 4.1-3.3% of dilution. In this concentration range no significant inhibition was detected, moreover stimulation could be sometimes observed. Comparing the dilution obtained on Nitrate Directive basis (3.3-4.1 % v/v) with that defined by D.Lgs.75/2010 for germination test (30% v/v), the GI limit appeared strongly preventive for digestate case and does not consider nitrogen amount. Considering the end-of-waste approach recently suggested at European level (Saveyn and Eder, 2014), a revision of threshold limit for digestate should be taken into account.

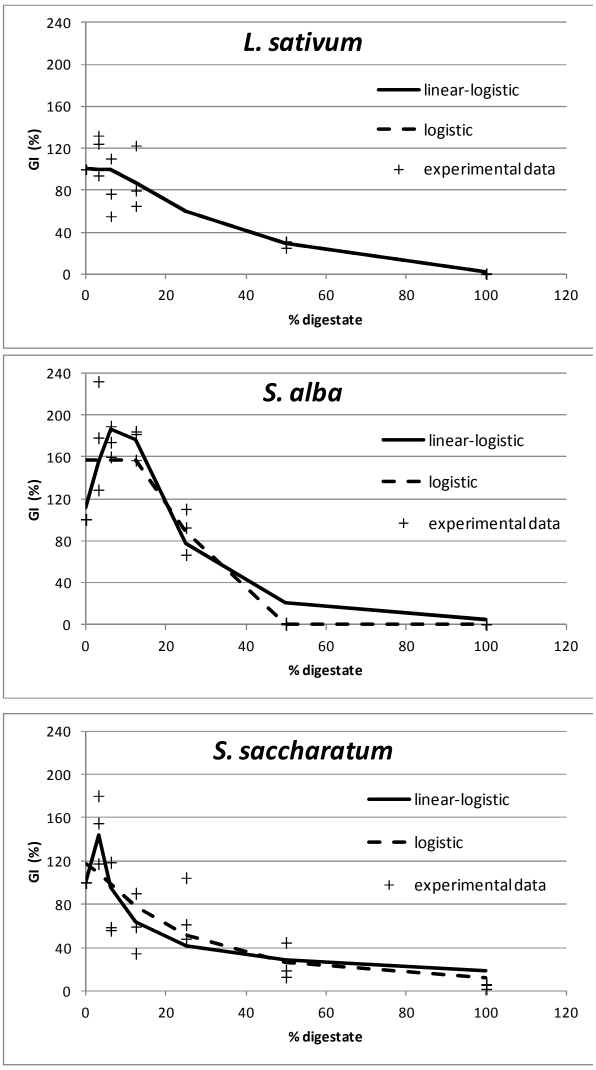


Figure 4. Germination index values determined using D1, trend predicted by logistic and linear-logistic models

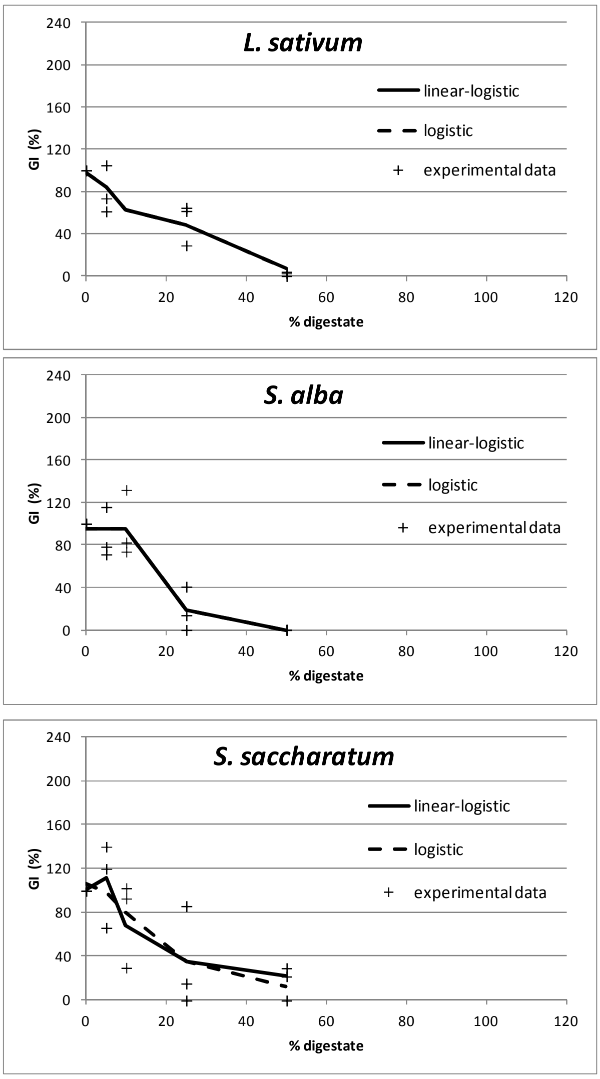


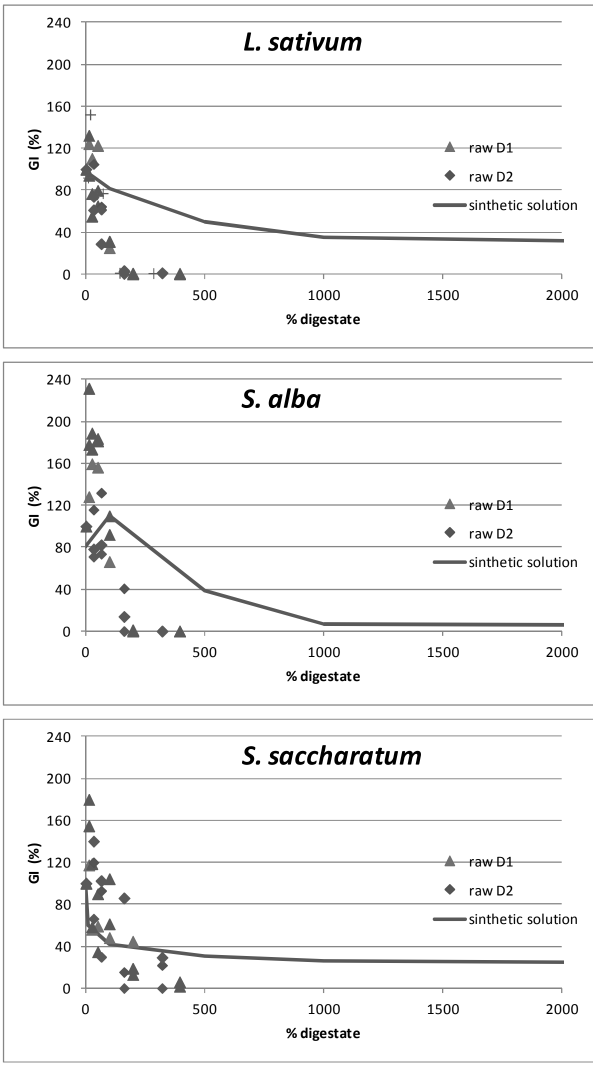
Figure 5. Germination index values determined using D2, trend predicted by logistic and linear-logistic models

3.3 Ammonium phytotoxicity

Ammonium solutions (10, 100, 500, 1000 and 10000 mg N/L) were analyzed by germination tests in order to evaluate the effect of this sole compound. *L. sativum* germinated seeds percentage was higher than 90% in all conditions, RE and GI followed the logistic model trend (Figure 6). EC50 for this species is 514 mg N/L, that is a concentration higher than in D1. Concentration of 10,000 mg N/L completely inhibited *S. alba* germination, while percentage of germinated seeds was higher than 80% at lower concentrations. In terms of RE, 100 mgN/L slightly stimulated root development (11%). Although the stimulation at 100 mg N/L is not significant compared to negative controls, the overall trend was better described by linear-logistic model and the corresponding EC50 was 490 mg N/L. *S. saccharatum* seeds germinated up to 1,000 mg N/L, while were completely inhibited at highest concentrations. RE and GI decreased according to ammonium concentration and evidenced higher sensitivity to ammonium than other seeds. In fact, also the lowest concentration (1 mg N/L) inhibited seed elongation up to 48%, while inhibition was 6% and 38% for *L. sativum* and *S. alba*.

Logistic model indicated that the EC50 was 37 mg N/L: the concentration was one order of magnitude lower than value estimated using the other species.

Toxicity data showed that ammonium could not be considered as the main toxicant inhibiting seed germination because result obtained with digestate and synthetic solution did not agree. *L. sativum* and *S. alba* appeared the two species most sensitive to ammonium, with an EC50 of approximately 500 mg N/L. This value alone did not explain the whole inhibition using digestates diluted two times and corresponding to 197 and 320 mg N/L, using D1 and D2, respectively. Figure 6 confirmed that concentration-response curves had different trend using the digestate and the synthetic solutions. In particular using ammonium solution the hormesis was not detected at low concentration and inhibition to *L. sativum* and *S. alba* was higher than that observed with D1 and D2, except for *S. saccharatum*. EC50 values of *S. saccharatum* were quite similar (37 mgN/L for synthetic solution, 59 mgN/L for D1 and 121 mgN/L for D2) but total inhibition using the digestate was observed at concentration lower than 500 mg/L while with synthetic solution limited germination was also observed at highest concentration (10,000 mg N/L). Other toxicants in digestate inhibited germination or a synergistic effect could increase ammonium toxicity.



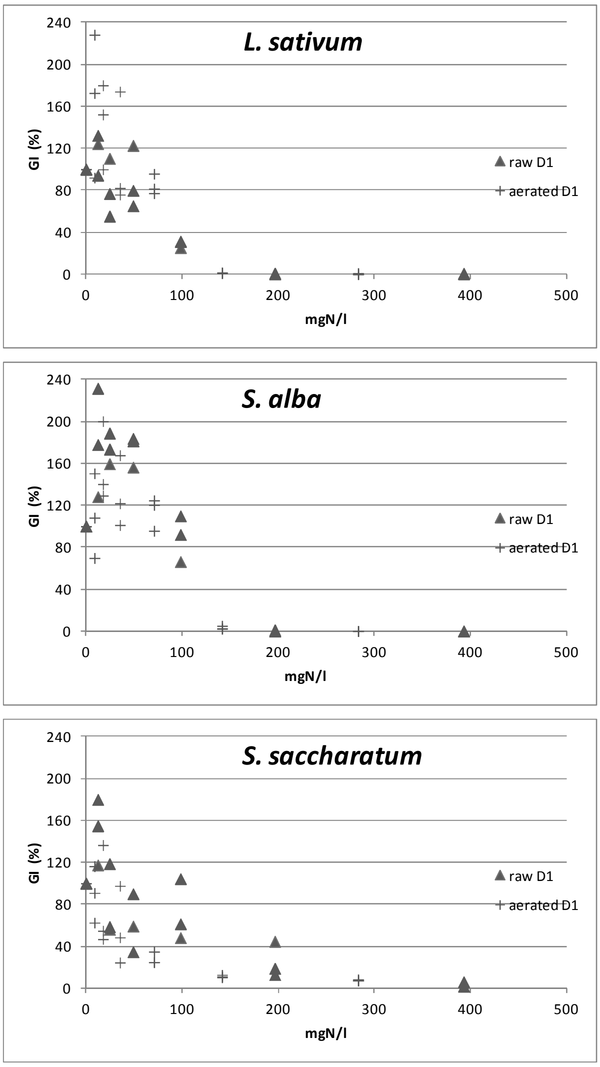


Figure 6. Effect of D1, D2 and synthetic solution of ammonium sulfate

Figure 7. Effect of raw and aerated D1

Since the analysis of synthetic solution is interesting but reductive compared to the complexity of digestate, the authors tried to strip ammonium out of the digestate D1 via an overnight aeration. Ammonium concentration reduced from 393 to 307 mgN/L using this treatment while salinity and heavy metals content could be considered constant. On the other hand, during aeration more chemical-physical reaction occurred, like the oxidation of reduced compounds (e.g. hydrogen sulfide, organic compounds) and their sub-sequent volatilization. The concentration-response trend using aerated sample changed on basis of macrophyte species.

L. sativum inhibition reduced according with ammonium concentration in fact the EC50 was constant in the tests carried out with raw and aerated D1 (79 mg N/L), while treated digestate concentration-response curve showed stimulation at low concentrations. Probably, the removal/oxidation of other toxicants reduced phytotoxicity for this species. The toxicity of *S. alba* was not related to ammonium concentration (Fig 7) but to pollutants that were not lost during the stripping. In fact the EC50 values were comparable in term of digestate dilution (30% v/v for both D1 samples) but different on ammonium concentration basis (118 and 92 mg N/L for raw and aerated D1, respectively). The concentration-response curve of *S. saccharatum* changed: D1 showed bio-stimulation at low concentration and hormesis was detected, while aerated D1 is better described by logistic model. As consequence the EC50 in test with aerated D1 was lower 13% v/v and 40 mg N/L (versus 19% v/v and 75 mgN/L using raw D1). Phytotoxicity to *S. saccharatum* slightly increased by short period of aeration as reported by Vallini et al. (Vallini et al., 1993) probably because the macrophyte was more sensitive to oxidized compounds, generated during the aerobic process

3.4. Copper phytotoxicity

Metals are considered toxic for microorganisms, plants and animals, but it is difficult to estimate the amount of bioavailable metals, because some of them are borderline between micro-nutrients and toxicity. By date, legislation defined threshold limits expressed as total metal content on dry matter basis but the toxicity should consider chemical forms and behavior in environment. The most hazardous form is soluble one such as copper ion (Cu^{2+}), then phytotoxicity of Cu^{2+} was analyzed by the synthetic solution and the results were compared with those from digestates exposure.

The dose-response curves with synthetic solution followed a logistic model on all the seed types and did not follow the trend of test with digestate (Figure 8). The EC50 values were 5.9, 9.9 and 2.7 mg Cu/L for *L. sativum, S. alba* and *S. saccharatum*, respectively. No bio-stimularion was detected with low concentration of Cu and it totally inhibited the germination at highest dose (1,000 mg Cu/L for *L. sativum* and *S. saccharatum,* 100 mg/L for *S. alba*). *S. alba* was the most sensitive specie to Cu, in fact GI was near 0% at 100 mg Cu/L while the index was > 10% for other species (Figure 8). Content of Cu comparable with digestate (<10 mg Cu/L) did not affect the germination in a significant way; hence the metal was not the direct cause of digestate phytotoxicity.

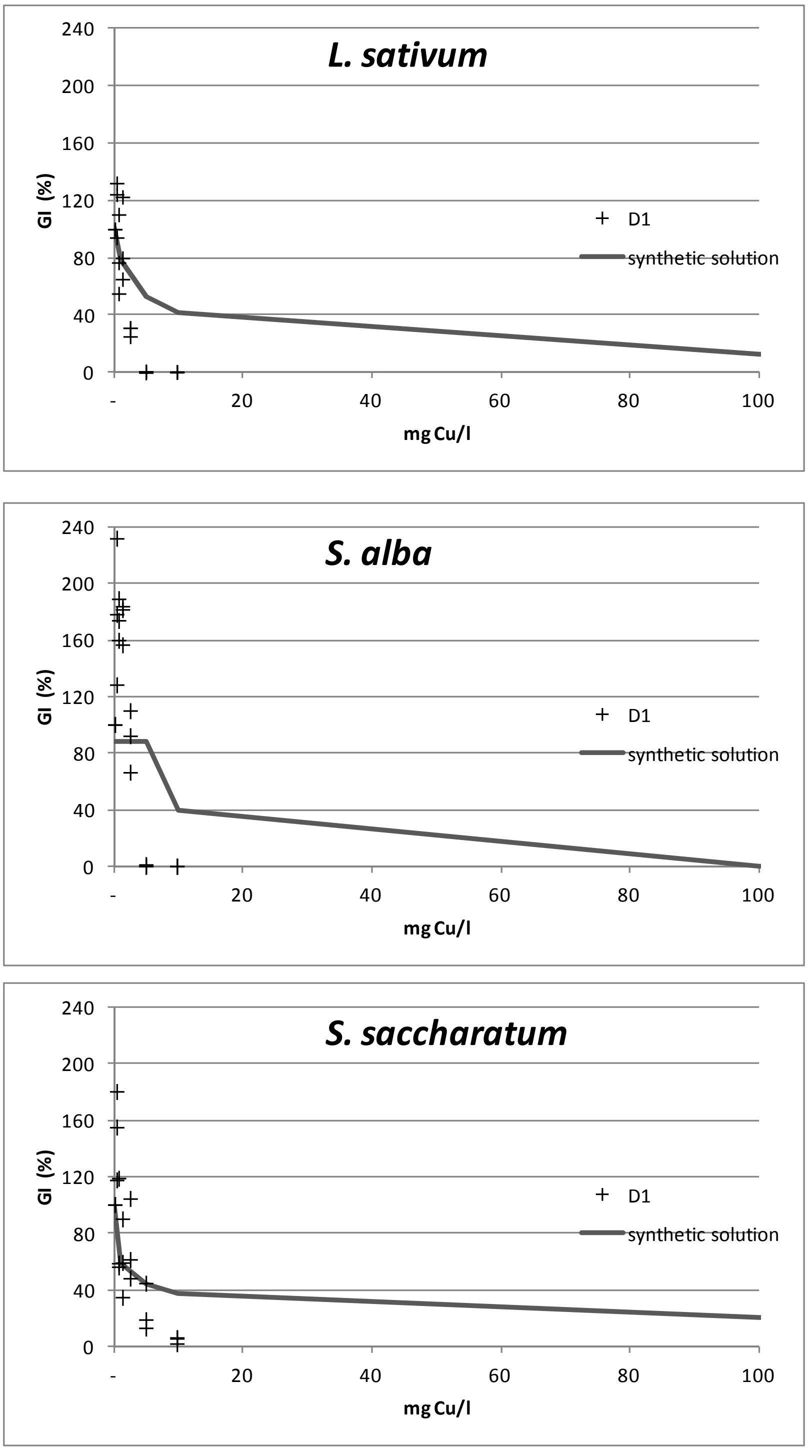


Figure 8. Effect of digestate D1 and synthetic solution of copper sulfate.

4. Conclusions

The phytotoxicity of digestate from winery wastes was analyzed considering the germinated seeds percentage, root elongation and germination index in three macrophyte species. Results showed that effect on seed germination was not constant over time because of variability on substrates fed to the reactor. Low doses of digestate (3-5 %) stimulated the germination of *S. alba* and *S. saccharatum* and had no significant effect (difference to control lower than 20%) on *L. sativum*. Higher doses reduced germination index until total inhibition with 50% of digestate. *S. saccharatum* appeared the less sensitive to this substrates, in fact the 40% of seed germinated also with raw digestate. Overall, the digestate didn't meet the phytotoxicity criteria of Italian legislation (IG>60% using solution of 30% v/v of digestate) that is a protective limit. In fact considering the limit of Nitrate Directive the maximum applicable digestate dose on soil should be of 4.1-3.3%, corresponding to concentration range without significant inhibition.

Effect of ammonium and copper content were deeper investigated because they characterized this type of digestate. The macrophytes had EC50 of about 500 mgN/L exception for *S. saccharatum* (EC50 37 mgN/L), hence the concentration in the digestates (393-639 mg N/L) can't justify the observed inhibition. Neither Cu appeared as the main cause of inhibition, because test carried out with solution of ion Cu^{2+} totally inhibited germination at concentration higher than 100 mgCu/L while the digestate has content lower than 10 mg Cu/L. Direct correlation between ammonium/copper and phytotoxicity was not observed, probably there was a synergic effect of different compounds and metals in the digestate that is difficult to evaluate.

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4. Conclusions

The objectives of the thesis were reached during the projected and the main conclusions are summarized using the same order proposed in the section 2.

- The lack of knowledge about thermophilic anaerobic digestion of winery wastes was filled by BMP tests. Grape marcs are the main by-products from wine-making process and thermophilic digestion (55 °C) was able to increase their potential methane potential of 84% (0.360 $Nm^3CH_4/kgVS_{fed}$) comparing with mesophilic process. Grape stalks had low specific yields of 0.133 $Nm^3/\ kgVS_{fed}$ because of lignin content, while wine lees has specific production of 0.370 $Nm^3CH_4/kgVS_{fed}$ for WL, values comparable to yields obtained at 37°C. All methane production cumulative curves exhibited inhibition at the beginning of the batch tests except for grape stalks.
- Anaerobic digestion semi-continuous trial of sole grape marcs was characterized by low biogas production (0.114 to 0.145 $Nm^3CH_4/kgVS_{fed}$). The addition of waste activated sludge improved process stability but did not increase volatile solids removal and methane production. Higher performances were reached using fermented grape marcs as a unique substrate and a longer HRT (0.290 $Nm^3CH_4/kgVS_{fed}$). Considering the best biogas yields, potential electricity and heat were calculated using a typical CHP unit efficiency. The combustion of biogas generated by GM could generate, globally, 1520 GWh/y of heat and 1245 GWh/y of electricity that could be used by wine cellar facilities.
- The three approaches to co-treat wine lees in anaerobic co-digestion were examined at pilot scale.

1. The anaerobic co-digestion of municipal waste activated sludge and winery waste (lees) can be considered stable for an OLR of 2.8 $kgCOD/m^3d$ where winery lees represented 22% of the feeding volume (but 70% of the inlet COD). The observed biogas yields were 0.38 $Nm^3/kgCOD_{fed}$ in the mesophilic reactor and 0.40 $Nm^3/kgCOD_{fed}$ in the thermophilic one. Thermophilic process was inhibited at higher organic load: VFAs accumulated, polyphenols and ammonia concentrations is higher than mesophilic ones, because of higher degradation rates. Effluents were characterized against criteria defined in the EU Directive 86/278, the 3rd draft of the new Directive on sludge (EEC, 2000) and the criteria defined in the document on End of Waste of digestate and composts proposed by the Joint Research Center of Sevilia. It was observed a good level of stabilization, with a reduction of about 60% of total and volatile solids. With reference to pollutants it was found a removal of 50% of TCDD, while content of other PCDDs/Fs and PCB remained quite constant or increased slightly because of the total solids reduction. Concentrations of Cu and Zn in the digestates were about 930 and 1200 mg/kg_{dw} respectively, because of high content of these metals in raw substrates, especially wine lees: these two elements, with their concentrations, determine the real limit for the co-digestion process. The amount of wine lees to be co-digested is determined on a

mass balance basis according to Cu and Zn presence. As for pathogens, *Salmonellae spp.* indicator was not present in the effluent and anaerobic digestion reached 1.3Log and 2.7Log reduction in mesophilic and thermophilic conditions, clearly showing the importance of the use of 55 °C instead of 37 °C in this specific aspect.

2. Anaerobic co-digestion of WAS from winery wastewater treatment and wine lees was feasible in mesophilic condition, when operating with an OLR of 3.2 kg COD/(m^3d) and a HRT of 23 d. The thermophilic digestion process accumulated VFAs, and after one HRT, the process failed. The process was recovered by metal additions. The dewaterability properties of the mesophilic process appeared to be better than thermophilic one. A total of 6.5 g conditioner/kg_{dw} was sufficient for mechanical dewatering of the digestate, whereas the thermophilic digestate dewaterability did not change over the dosage range of 0-10 g polymer/kg $_{dw}$. Considering management costs, thermophilic conditions are still economically competitive thank to higher biogas production, even more if other co-substrates, derived from surrounding activities, are co-treated to supply the missing micronutrients.
3. Substitution of maize silage with wine lees and catch crops in on-farm biogas plant appeared feasible and able to solve some ethical problems related with competition with cultivation of food biomasses. Biological methanisation potential tests evidenced that *Lolium spp.* had methane potential production of 0.360 m^3CH_4/kgCOD, comparable to maize silage yield. Wine lees had lower methane production (0.218 m^3CH_4/kgCOD) but higher hydrolysis rate able to transform 90% of biodegradable fraction within 7 days. Mixture of cattle slurry and wine lees was balance in terms of nutrients and allows for reach the 90% of potential methane production in semi-continuous reactor. Winery waste was able to dilute ammonium load due to slurry and determinate biogas yield was 0.324 m^3/kgCOD with 73% of methane. Addition of *Lolium spp.* increased organic loading rate without causing instability but the process performance was limited by mixing problem. Observed specific biogas production was 0.283 m^3/kgCOD with 70% of methane.

- Mesophilic anaerobic digestion of wine lees and winery WAS was optimized on basis of results of kinetic study. In fact the application of different hydraulic retention times (23 and 40 d) showed that long HRT didn't affect the specific biogas production but increased microbial biomass concentration, and consequently improved the observed degradation rates. Biogas production rate, between a feeding and the next one, significantly reduced after 11-13 hours, when soluble biodegradable COD was completely consumed. Consequently the feeding frequency could be increased through a control system able to feed the reactor when GPR reduced below a defined set-point (0.4 m^3/($m^3_{reactor}$d)). Application of this approach constituted by a simple on/off control, allowed for increase the OLR from 3.2 to 6.2 kgCOD/(m^3d), without any stability problems. The average GPR increased from to 1.1 to 2.8 $m^3/m^3_{reactor}$d, mainly due to faster degradation of rhCOD and sbCOD.
- The failure of thermophilic process treating wine lees and winery WAS was deeper investigated and the cause appeared the scarce trace-elements. Metal augmentation at doses of 4.3 mg Fe /L, 0.46 mg

Ni/L and 0.51 mg Co/L improved the stability and biogas yields (0.450 m^3/kgCOD). Solid removal increased by 18% compared with the mesophilic process, COD removal reached the 92% and ammonification resulted in a higher ammonium concentration in the thermophilic digestate (630 mg N/L). Reducing metals addition, stability process remained in the optimum ranges for anaerobic digestion but yield reduced to value equal or lower than mesophilic one (0.380 m^3/kgCOD$_{fed}$). Relationship between metals addition and COD removal was linear only for the lowest three tested doses. In the case of highest addition maybe other chemical equilibria, not considered in the study, interact with trace-elements availability.

- The quality of digestate was analyzed to evaluate the applicability as amendment. Germination index was used as parameter able to considered the whole properties of the digestate, included synergic effects of pollutants. Results showed that digestate had a residual phytotoxicity but the effect on seed germination was not constant over time because of substrate variability. Low doses of digestate (3-5 %) can stimulate the germination of *S. alba* and *S. saccaratum* or had no significant effect (difference to control lower than 20%). Higher doses reduced germination index until total inhibition with 50% of digestate. Comparison of results with legislation indicated that D.Lgs. 75/2010 has protective limits, in fact evaluates dilution of 30% that is not applicable on soil because of limits defined by Nitrate Directive. Considering the maximum nitrogen soil application the dilution on soil should be 3-4% v/v, value that should have benefic effect on germination. Ammonium and cooper are pollutants that characterized digestate from winery wastes and, consequently, their phyto-toxicological effect was analyzed. They did not appear the main causes of digestate phytotoxicity, probably there was a synergic effect of different compounds and metals in the digestate that is difficult to measure.

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Dottorato: SCIENZE AMBIENTALI ____________________

Ciclo: XXIX

Titolo della tesi[1] **:** ***Assessment of energy and material recovery from winery wastes***

Estratto:

La vinificazione genera un ingente quantitativo di rifiuti organici. Lo scopo del progetto è valutare la fattibilità della digestione anaerobica come trattamento di questi scarti usando reattori discontinui e continui in condizioni mesofilica e termofilica. Lo studio ha dimostrato che sono possibili differenti approcci. La feccia può essere conferita agli impianti già esistenti come nei impianti di trattamento acque municipali dotati di digestori per i fanghi o negli impianti a biogas alimentati con reflui zootecnici. Infine anche la crezione di nuovi impianti all'interno del comparto vinicolo risulta possibile. Analisi cinetiche hanno evidenziato margini di incremento del carico organico e quindi della produzione di biogas nel processo mesofilo grazie a un sistema di controllo automatico dell'alimentazione. Invece il trattamento termofilo ha mostrato problemi di instabilità che possono essere controllati dall'aggiunta di metalli utili per la crescita batterica. Infine l'uso del digestato come ammendante agricolo è stato analizzato anche dal punto di vista eco-tossicologico mediante l'applicazione del test di germinazione

Abstract:

Wine-making process generates a great amount of organic wastes (grape stalks, grape marcs, wine lees and waste activated sludge). The aim of this study is to evaluate winery wastes anaerobic digestion feasibility using batch and semi-continuous tests at mesophilic and thermophilic temperature. The study demonstrate that three different approaches were feasible: integration of wine lees treatment in digesters already built such as reactors located inside wastewater treatment plant and those treating livestock effluents (on-farm plants), and application of anaerobic digestion to support wine sector utilities and activities inside the cellar borders. Kinetic investigation allowed for setup an automatic feeding control able to increase organic loading rate and improve mesophilic process performances using an automatic feeding mode. On the other hand thermophilic process had some stability problems that were controlled by metal augmentations. Finally phytotoxicity of digestate and its applicability on soil was evaluated by germination tests.

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