Water Resources and Industry 19 (2018) 25–34

Contents lists available at ScienceDirect

Water Resources and Industry

journal homepage: www.elsevier.com/locate/wri

The role of organic load and ammonia inhibition in anaerobic digestion of tannery fleshing

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ARTICLE INFO

Keywords:
Anaerobic digestion
Tannery waste
Ammonia inhibition
Organic load

ABSTRACT

In this study, batch tests on anaerobic digestion of tannery fleshing (skin-residue waste from hides' tanning process), as sole substrate, have been performed with the purpose of assessing the effects of high substrate concentration and consequent ammonia inhibition on the process. Co-digestion with tannery primary sludge was also evaluated. According to the results, no inhibition occurred at initial organic load up to 5 gVS/l; an inhibited steady state was observed at 10 gVS/l, and system failure and instability was showed at the highest load of 20 gVS/l. Co-digestion with tannery primary sludge proved feasible, probably due to dilution effect. The observed ammonia and VFA accumulation over the experimental time-lapse is also discussed. Results are intended to increase knowledge on the technological application of anaerobic digestion of sole tannery fleshing, in the perspective of its application as on-site treatment solution for decentralised tanneries.

1. Introduction

1.1. Tannery wastes

Tannery industry is among the most polluting activities due to the high production of wastewaters and solid wastes [1]. When tannery industries are organized as concentrated district, tannery wastewaters are treated in dedicated industrial wastewater treatment plants, where the production of tannery primary sludge and secondary sludge is massive. Leather tanning is a multi-step sequential process comprising pre-tanning, tanning and crusting, and refinishing operations [2]. Therefore, tannery solid wastes may vary widely in terms of quantity and quality depending on the process phase they have been generated from. Most pollutant load is generated in pre-tanning and tanning operations [2]. Pre-tanning solid wastes comprise mainly hairs, skin trimmings and fleshing. Tanned solid wastes such as wet-blue trimmings and shavings, carry the additional polluting load associated to tanning agents, i.e. chrome or tannins. Tannery fleshing (TF) is originated by the removal of the tissue adhered to the animal hide, usually after liming treatment and before tanning process and is characterized by high proteinaceous organic content, low C/N ratios, and high levels of chemical salts, since hides are usually preserved with sodium chloride and undergo liming and pickling treatments. TF and TPS have been traditionally handled trhough landfill disposal and incineration [3], due to the high content in chemical pollutants and the presence of recalcitrant compounds. Though, in response to new stringent regulations and environmental policies encouraging alternative eco-friendly treatments, anaerobic digestion (AD) turned an attractive solution in the perspective of sustainable and integrated management of tannery solid wastes and wastewaters. Besides AD, other alternative treatments have been proposed in order

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https://doi.org/10.1016/j.wri.2017.12.001
Received 6 April 2017; Received in revised form 31 October 2017; Accepted 7 December 2017

to divert fleshing and other leather wastes from final landfill disposal or incineration, in favour of energy and/or resource recovery. Some of the reported bioconversion treatments include: production of proteolytic enzymes from fleshing fermentation [18]; composting [10]; recovery of tanning agents [19]; biodiesel production [20]. Additionally, physico-chemical treatments have been applied to TF since more than 20 years for fat and protein recovery or glue production, generally in centralised industrial contexts, where the economy of scale makes it cost-effective to collect and treat TF for material recovery. For example, in the Tuscany tannery district, fleshing is collected from about 400 tanneries by a single company (SGS, Pisa, Italy), that separates fat and protein fractions and sells them on the market for cosmetic and fertilizer industries, respectively. Nevertheless, such a treatment requires a complex and energy-consuming processes and the market of final by-products is affected by strong price fluctuations. Thereby, the technological application of AD of TF has encountered the interest of medium or large size de-centralized tanneries, whose solid waste production is high enough to maintain stable process operation enabling the private investors to operate their own treatment solution, potentially self-sustained in terms of energy, saving in waste transportation and disposal costs, while allowing virtuous and profitable valorisation for the treated matrices. As reported by Priebe et al. [4], the first attempt to recover energy through anaerobic digestion of tannery wastes was conducted in 1982 by Cenni et al. [5]. Since then, various authors have investigated the feasibility of anaerobic processes for tannery wastes and sludge, especially in those countries where leather industry plays a prominent role within the national economy. Several available studies refer to tannery districts in: India [6–12], China [13,14], Latin America [4,15] as well as Italy [1,16]. As a general conclusion, studies regarding AD of leather solid wastes agree on process feasibility while warning against possible operational problems related to unbalanced C/N ratios and inhibitory conditions from ammonia, long chain fatty acids and sulphide. Moreover, biological treatments and, specifically, AD, proved to perform better for untanned wastes than for tanned ones, since tanning operations are aimed to stabilize leather collagen. Priebe et al. [4] tested anaerobic biodegradability of tannery wastes holding different chrome concentrations and concluded that the higher the chrome content, the lower the methane yield, mainly due to the low hydrolysis of tanned stabilized material. The same authors highlighted that proper selection of seed sludge and substrate hydrolysis pre-treatments, may be crucial to overcome low-performance drawbacks. Similarly, Dhayalan et al. [17] tested biodegradability of untanned, chrome-tanned and vegetable-tanned leather solid wastes; the highest methane yield was observed for untanned waste, albeit vegetable-tanned wastes proved more biodegradable than chrome-tanned ones. In the same study, the effect of detanning pretreatment was also evaluated and resulted in an increase in waste biodegradability.

Though, the majority of the reported studies refers to AD of a mixture of tannery wastes and other substrate either originated from tannery industry and not. The present work is intended to increase knowledge on the technological application of anaerobic digestion of sole Tannery Fleshing, in the perspective of its application as on-site treatment solution for decentralised tanneries. Particularly, critical conditions related to high organic load and ammonia concentrations have been studied in order to define applicability range and system robustness prior to overburden conditions. Co-digestion with tannery primary sludge has also been tested, with the only purpose of confirming its feasibility and effect as possible mitigation solution.

1.2. AD of protein-rich wastes

The potential methane yield of a given substrates strongly depends on the relative content in terms of carbohydrates, proteins and lipids, since the three macro-compounds have different degradation kinetics and specific methane yield. Lipids have the highest stoichiometric methane potential, due to the high number of C and H atoms in their molecular structure, although long chain fatty acids (LCFA) accumulation and lipids adsorption onto solids' surface may lead to inhibitory conditions and operational problems, e.g. sludge floatation and/or washout, especially in case of granular sludge [21–23]. Lipids as fats, oil and grease are the main component of some industrial wastewater such as slaughterhouse, dairy industry or fat refineries [24]. Carbohydrates are characterized by high C/N ratios, conductive for high degradation rate albeit unbalanced acidogenic and methanogenic reactions' rates may lead to volatile fatty acids (VFA) accumulation and system acidification [23]. They are the main component of the organic fraction of municipal solid wastes and, in general, cellulose is the most abundant in complex organic wastes [22]. Proteins' methane yield is comparable with carbohydrates' and protein-rich wastes are typically originated from farms, agro-industrial and meat-processing sectors. When dealing with solid wastes derived from animal tissues (e.g. TF), fibrous proteins are the main building material. Collagen and elastin are present in connective tissue, ligaments and tendons; keratin in skin, hair, feathers, horns and hoofs; and myosin in muscles [22]. Despite their high organic content and high biodegradability, anaerobic digestion of protein-rich wastes has experienced limited application worldwide. This is mainly due to the inhibition effect of ammonia accumulation (released during protein degradation) resulting in process instability or inhibitory steady state and eventual low biogas production. According to Kovács et al. [25], out of the more than 7800 AD plants operating in the EU in 2013, none was processing protein-rich waste as primary substrate, despite the relevant generation of such wastes at the European level. The interest to widen further AD application as sustainable organic waste treatment and the relevant entity of protein-rich waste generation have been pushing research and industry to tackle operational limitations due to ammonia inhibition. Kovács et al. [25] challenged the common belief that protein-rich feed as mono-substrate should be avoided due to process inhibition. In their study, a preliminary experimental phase regarded two feed-batch lab-scale reactors, fed by pig-blood and casein, respectively, at increasing input loads until process failure took place, due to ammonia inhibition. Results show that AD of protein-rich monosubstrates is feasible up to certain weekly protein load of 4–6 g_protein_VS/l and consequent total ammonia nitrogen (TAN) level up to 7–8 g/l, biomass acclimation being crucial.

1.3. Effect of ammonia and organic load

Ammonia inhibition is acknowledged as one of the primary causes of AD failure and several studies focused on the actual

mechanism of its toxic effect on the microbial activities in the last decades. Although several technics have been proposed and implemented for efficient and cost-effective ammonia control in full-scale digesters, AD implementation to nitrogen-rich substrates is still challenging [26–28]. In its review on AD ammonia inhibition, Rajagopal et al. [26] emphasized that research on parameters influencing ammonia inhibition need to go further and that particular focus is now given on the evolution of the methanogenic population at increasing ammonia levels.

The role of ammonia in anaerobic digesters is multiple. Optimal levels of ammonia (up to 200 mg/l) ensure adequate supply of nitrogen as nutrient substance for anaerobic biomass and increase system's buffer capacity, counteracting acidification lead by VFA production [26]. On the contrary, ammonia concentration exceeding certain critical thresholds is detrimental to the process due to its toxic effect. In aqueous systems, TAN accounts for both the unionized free ammonia (FA), NH_3, and the ionized form, NH_4^+, whose equilibrium is governed by pH and temperature according to Eq. (1) [26,27].

$$FA = TAN\left(1+\frac{10^{-pH}}{10^{-(0.09018+\frac{272.92}{T})}}\right)^{-1} \quad (1)$$

Authors widely agree that FA is the actual toxic form. The main inhibition mechanisms proposed are ascribed to: (i) direct inhibition on enzyme production by methanogenic biomass and (ii) effects of passive diffusion into bacterial cells, as FA can cross cellular membrane and interfere with internal cell pH, leading to proton unbalance and energy requirement increase [28]. However, the extent of FA diffusion and consequent inhibition depends on the physiology of the methanogenic biomass [14]. Reported toxic threshold of ammonia concentrations vary widely. Chen et al. [14] reported that values of ammonia concentration causing 50% of methane production reduction range from 1.7 to 14 g/l. Such a wide range is due to the differences in substrate, inoculum seed sludge, environmental conditions (temperature and pH) and biomass acclimation. Gallert et al. [29] highlighted that many studies reporting critical concentrations does not distinguish between FA and TAN levels, probably increasing the concentration range further. Prior to complete system failure, an inhibited steady state has been reported as the most commune consequence of severe ammonia inhibition. According to Chen et al. [14], such a steady-state condition is characterized by process stability at a lower methane production, resulting from the interaction between the concomitant effects of FA, VFA and pH. Numerous strategies have been explored in order to prevent or control ammonia inhibition. Co-digestion is among the most applied and successful solutions, allowing for C/N optimization and dilution of possible inhibitory compounds. Shanmugam and Horan [30] reported the positive effect of co-digesting tannery fleshing with municipal solid waste with respect of AD of sole tannery fleshing, due to C/N ratio re-balance together with pH control (Table 3).

On the other hand, in full-scale reactors, the maximization of biogas production is generally achieved by increasing organic loading rates, OLR [31]. High organic loads allow for high-rate kinetics, provided sufficient nutrients and buffering conditions and proper inoculum: substrate ratios. Though, overload conditions may unbalance kinetic rates in the pathways of AD reactions, leading to unstable or inefficient states. VFA accumulation is among the first consequence of process overloading. In case of low buffer capacity of the system, VFA accumulation causes fast drop in pH which, in turn, inhibits methanogenic microorganisms – generally more sensitive to low pH than acid-producers – leading to further VFA accumulation. When dealing with protein-rich substrates, high organic loads results in high levels of ammonia, making it difficult to discriminate between the negative effect of system overload or ammonia inhibition as reported by Moestedt et al. [31]. In the mentioned study, authors aimed to separate the effect of these two factors by running two semi-continuous reactors fed by protein-rich thin stillage. One reactor was operated at increasing OLR and the other at increasing loading of urea, in order to increase ammonia concentration in the reactor. The outcomes showed that the critical ammonia concentration for process stability was around 1 g/l in both reactors, irrespective of OLR.

In the present study, the role of ammonia and organic load on AD of sole tannery fleshing was investigated through parallel batch tests at increasing initial organic loads. Co-digestion of TF with tannery primary sludge (TPS) was also tested.

2. Material and methods

2.1. Experimental set-up

Wastes were collected from the tannery district of León (Guanajuato, Mexico), which represents the largest one in Mexico [32]. Tannery fleshing was provided by a commercial tannery, after liming process. Tannery primary sludge was collected from the

Table 1
Experimental initial conditions.

	Inoculum/substrate ($VS_{biomass}/VS_{substrate}$)	Inoculum (gVS/l)	Tannery fleshing (gVS/l)	Tannery primary sludge (gVS/l)	Initial organic load (gVS/l)
Test A	1	5	5	–	5
Test B	0.5	5	10	–	10
Test C	0.25	5	20	–	20
Test D	2.5	5	–	2	2
Test E	0.42	5	10	2	12
Test F (Acetate control)	–	5	–	–	–
Test G (Blank control)	–	5	–	–	–

Table 2
Average substrates and inoculum composition.

	TF		TPS		Inoculum	
TS	170 ± 3	g/g[a]	190 ± 2	g/l	141 ± 1	mg/g[a]
VS	85.5 ± 0.6	%	53. ± 0.3	%	87.7 ± 0.1	%
COD_tot	0.33 ± 0.08	g/g[a]	19.4 ± 0.5	g/l		
COD_sol	–	g/g	2.6 ± 0.3	g/l		
pH	11–12	–	7	–	6.7	

[a] on wet weight base.

Table 3
Methane production values reported in literature for TF anaerobic digestion and co-digestion, at batch laboratory-scale.

Substrate	Organic load (gVS/l)	Methane production ($Nl\_CH_4/gVS_{\_add}$)	Temperature (°C)	Working volume (ml)	Reference
TF + municipal solid waste	–	0.327–0.699	35	400	[30]
TF + Tannery Primary Sludge	17.2–27.7	0.263–0.483	30	70–130	[8]
TF	0.1	0.552	37	500	[3]
TF + Tannery Primary Sludge + Tannery	11.5 l	0.1–0.274	–	650	[11]
Secondary Sludge	7.7–18.5	0.172–0.269	–	650	[12]
Hydrolysed (untanned) collagen	4.2	0.180	35	350	[4]
Crhome wet-blue shavings	2.5 gVS/l	0.103	35	350	

chemical primary sedimentation tank in the tannery WWTP of León. In the plant, polyelectrolytes are added to enhance solid precipitation together with sulfuric acid for pH adjustment. All samples were stored at 4 °C before the experiment. Granular seed sludge was collected from a UASB digester treating tequila vinasses (Casa Herradura, Amatitan, Jalisco, México) and stored at 4 °C. Prior to its utilization as inoculum in the tests, it was re-activated at room temperature with the injection of acetate and no nutrient addition. The acetate consumption was monitored by analysing the supernatant liquor and the inoculum used after acetate's complete degradation (around three days after injection).

Multiple parallel tests were conducted in serum bottle of 120 ml volume. Initial conditions for the seven tests, namely Test A to G, are summarised in Table 1. According to previous results [39], the not-acclimated inoculum proved suitable for AD of tannery wastes. All the tests had the same initial inoculum concentration of 5 gVS/l and different initial substrate concentrations. Such an experimental condition was intended to assess the substrate effect on the given biomass, by substrate-dependent variation of the I/S ratio. Tests A, B and C had increasing initial TF concentration of 5, 10 and 20 gVS/l, respectively. Test D was performed with TPS at initial substrate concentration of 2 gVS/l and test E was conducted to assess substrates' co-digestion with initial fleshing concentration of 10 gVS/l and primary sludge of 2 gVS/l. Finally, Test F was run as positive control (with acetate addition) and test G as blank control (inoculum only). Seed sludge granules were rinsed in order to remove residual organic matter, prior to be placed in the serum bottles. Size reduction of TF was achieved by mincing it with a commercial meat miller. Mineral medium was prepared according to [33] and added in order to provide optimum conditions for methanogenic activity. Yeast extract was added in the proportion of 100 mg per liter of final volume solution. The serum bottles were filled to obtain a working volume of 70 ml, sealed with rubber stoppers and crimped with aluminium ring. The headspace was flushed with a mixed gas of 80% CO_2 and 20% N_2 for 5 min. Initial pH was adjusted at 7 with addition of 1 M HCl solution. An incubator with rotating plate ensured continuously stirred conditions and mesophilic temperature of 32,5 °C. Biogas production was measured regularly through water displacement; water was saturated with commercial sodium chloride, to minimize CO_2 dissolution. The frequency of biogas sampling was decided according to the observed biogas production in order to limit excessive overpressure interference. On average, gas in the bottle headspace was purged (until pressure equilibrium) every 12 or 24 h in the first exponential production phase and every 2–3 days in the low-rate production phase. The experiments were run until biogas production rates levelled off to the endogenous levels observed in the blank control (biogas production below 1 ml per day). During the duration of the experiment, 2-ml samples were withdrawn from the serum bottles in order to analyse: $N\text{-}NH_4^+$; VFA and pH.

Process performance was assessed on the base of cumulative methane production, specific methane production (SPM, $Nl\_CH_4/gVS_{\_add}$), methane content and VFA accumulation.

2.2. Analytical methods

Analysis of COD, TS, VS were performed according to methods APHA standard method [34]; $N\text{-}NH_4^+$ was measured according to 4500-NH3 F phenate method [34]. Tannery fleshing was characterized in terms of total proteins [35], total carbohydrates [36] and total lipids [37]. Representative homogeneous sample of TF was achieved by mincing around 5 kilos of fleshing. Acetate, Propionate and Butyrate were analysed through capillary electrophoresis (Agilent G1600A), prior to sample dilution, filtration and centrifugation at 3000 rpm. After purging headspace biogas into the inverted burette, biogas samples were withdrawn with a lock syringe and analysed though gas-chromatography to assess CH_4 and CO_2 content. The gas chromatograph (Agilent 6850) was

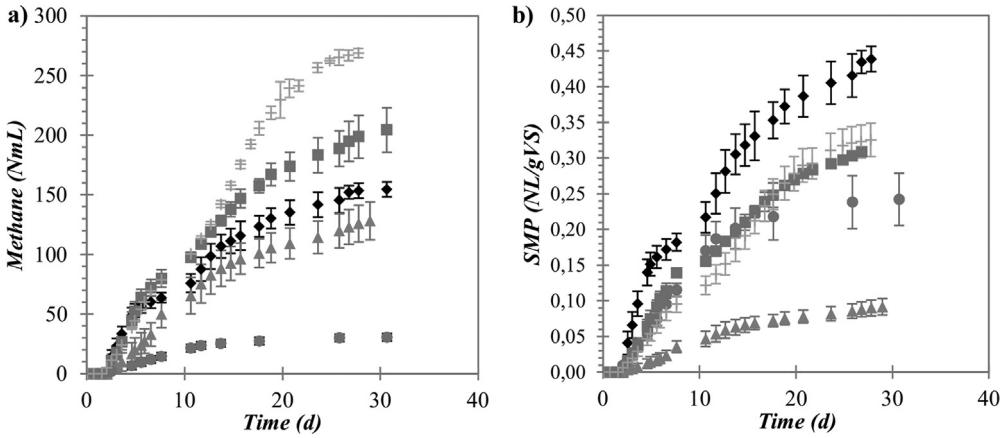

Fig. 1. Methane production curves: a) cumulative and b) specific methane production, SMP (◆ Test A, 5 gVS_TF/l; ■ Test B, 10 gVS_TF/l; ▲ Test C, 20 gVS_TF/l; ● Test D, 2 gVS_TPS/l; + Test E, 10 gVS_TF/l + 2 gVS_TPS/l).

equipped with thermal conductivity detector (TCD) and HT PLOT Q packed column, using nitrogen as carrier gas. The injector, oven and detector temperature were: 250, 70, and 250 °C.

3. Results and discussion

3.1. Substrate characteristics

Results on substrate characterization in terms of TS, VS, COD and pH are reported in Table 2. Tannery primary sludge showed moderate solid content of 2%, ammonia concentration of 403 ± 5 mgN-NH_4^+/l and neutral pH. However, it should be noted that such a waste is characterized by strong variations in terms of solid and pollutant content, due to the relevant fluctuations in tannery wastewater characteristics. Indeed, tanneries' industrial activity varies significantly over the week as well as over the year.

The protein and fat content on a dry-weight base of tannery fleshing were 71.3% and 12.7%, respectively; the remaining 16% was ascribed to inorganic material. These values are comparable with those reported by: Zerdani et al. [38] (protein 79%; fat 7.57%) and Thangamani et al. [8] (protein 56.5%; fat 4.79%).

3.2. Methane production

Fig. 1 shows the cumulative and specific methane production curves for tests A to E. Blank control exhibited very low biogas production (< 2 ml/d), ascribed to endogenous metabolism only, whereas the positive control with acetate presented a maximum methane production rate of 9.45 ± 1.42 Nml_CH_4/d, during the exponential phase.

All the tests exhibited an initial lag phase of three days. Tests A and D at low initial concentrations - sole TF and sole TPS, respectively - showed stable behaviour and no detectable inhibition. Their final SMP of 0.44 ± 0.02 Nl_CH_4/gVS_{add} for test A and 0.24 ± 0.04 Nl_CH_4/gVS_{add} for test D are comparable with results obtained in a previous study [39], where two experiments were performed using the same sourced TF and TPS as sole substrates, at low input concentrations (organic loads of 1–2.5 gVS/l) and different I/S ratios. In the mentioned work, average SPM values of 0.47 ± 0.05 and 0.26 ± 0.06 Nl_CH_4/gVS_{add} were obtained for TF and TPS, respectively, and no noticeable inhibition observed. It should be noted that, in the tannery district of León, tannery wastewaters undergo a rough primary sedimentation in a specific tank within each tannery, before being discharged in the industrial sewer pipelines. Thus, part of the particulate organic content is removed before reaching the tannery WWTP and this might explain to some extent the moderate methane production obtained from TPS.

Methane yield reductions of 20% and 80% were observed for test B and C, respectively (Fig. 1b), indicating that inhibitory conditions occurred. The final SMP of co-digestion test E almost equals the sum of SMP values obtained in test B and D, suggesting that the main advantage of waste blending was the additive effect of TPS organic load contribution and, possibly, a dilution effect of inhibitory compounds. Table 3 reports the main findings available in literature about methane production from tannery solid wastes as sole or mixed substrate, under batch laboratory-scale conditions. Our results on SMP are in line with those reported by the studies reporting AD of TF as sole or blended substrate.

On the basis of chemical elemental analysis, Shanmugam and Horan [40] and Kameswari et al. [12] reported the following empirical formulas for TF: $C_4H_{11}NO_2$ and $C_{172}H_{295}N_{43}SO_{132}$, the corresponding C/N ratios ranging from 3.2 and 4. The high methane yield obtained by Shanmugam and Horan [30] was obtained by optimizing the blend of TF with municipal solid waste in order to obtain optimal C/N value of 15 and by pH correction to the optimum value of 6.5. Optimal C/N values for anaerobic digestion are generally indicated between 20 and 30 [23], though some studies reported successful applications at C/N ratios far outside the optimal range [41]. Results from the present study highlight process feasibility even at that very low C/N ratios (< 10), as in the case of sole tannery fleshing in test A, although process showed quite sensible to operational conditions such as high organic load and pH.

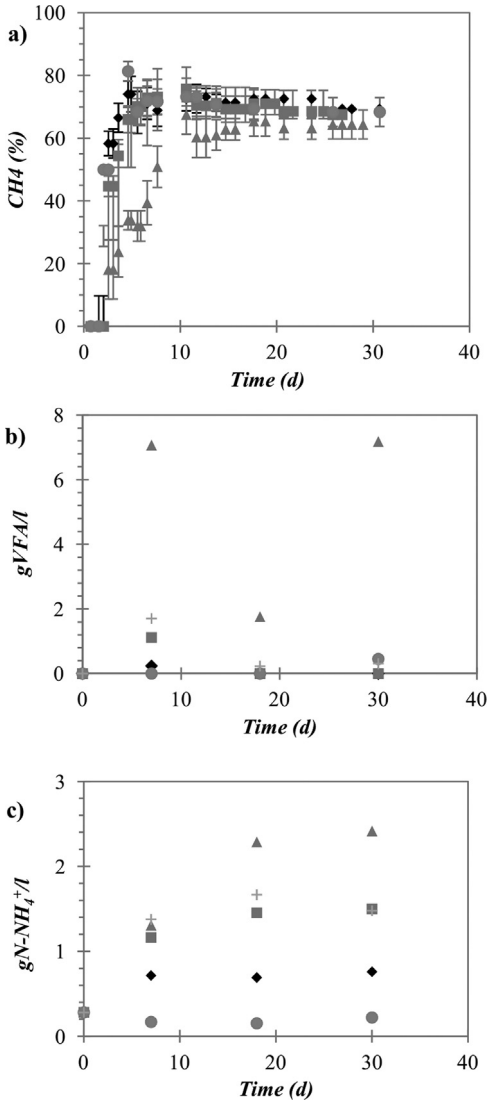

Fig. 2. a) Methane content in the biogas, b) VFA concentration and c) Ammonia concentration (◆ Test A, 5 gVS_TF/l; ■ Test B, 10 gVS_TF/l; ▲ Test C, 20 gVS_TF/l; ● Test D, 2 gVS_TPS/l; + Test E, 10 gVS_TF/l + 2 gVS_TPS/l).

Although co-digestion is widely encouraged in order to re-balance C/N ratios, also the co-substrate used in this study, TPS, exhibited a low COD/N ratio around 9. Nevertheless, co-digestion test E indicates a no-detrimental effect of wastes blending albeit it is likely related to dilution. The actual feasibility of TPS and TF co-digestion was already studied in previous works reported in [16]. Particularly, 150-liter pilot reactors were operated for more than six months, testing different mixtures of the two wastes, although the tannery primary sludge was kept as the main substrate in terms of VS mass contribution. On the contrary and according with the purpose of the present study, the fleshing was selected as the main substrate in the co-digestion test E. Other studies [8,12] also confirmed the technological feasibility of co-digestiong TF and tannery sludge. Zupančič et al. [3] reports a methane production of 0.552 Nl_CH_4/gVS$_{add}$, higher – though comparable – than the maximum obtained in test A (0.44 ± 0.02 Nl_CH_4/gVS$_{add}$); this is likely due to the significantly lower input organic load as well as the higher operating temperature. To the best of our knowledge, there are no studies reporting of batch AD of sole TF at high initial substrate concentration.

3.3. Organic load and ammonia influence on process performance

Fig. 2 presents data on methane percentage together with ammonia and VFA concentrations, throughout the experiment. The final ammonia concentrations in tests with sole TF as substrate were: 0.76 ± 0.03; 1.50 ± 0.08 and 2.42 ± 0.10 gN-NH_4^+/l, for test A, B and C, respectively. The increasing concentrations observed reflected the increasing proteinaceous input almost proportionally. In test D, ammonia was 0.22 ± 0.06 gN-NH_4^+/l, and the co-digestion Test E showed an additive effect of the two substrates degradation (observed value: 1.48. ± 0.20 gN-NH_4^+/l), as can be observed by matching results from tests B and D. Almost all the tests presented

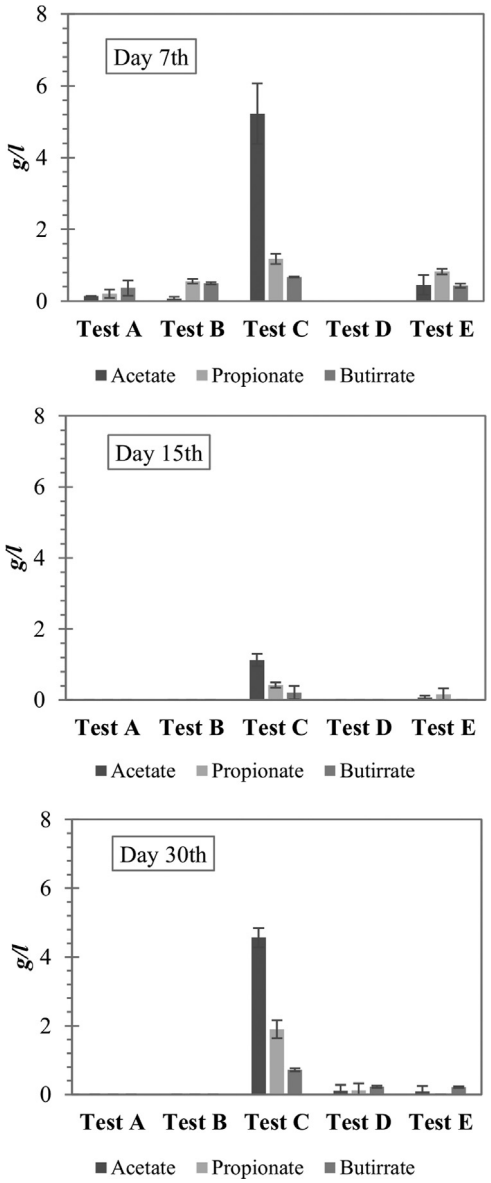

Fig. 3. Acetate, propionate and butirrate concentrations observed during the experimental time course.

VFA accumulation within the first week of the experiment (Fig. 2b). Though, in test A and D, the maximum VFA concentration kept at a minimal concentration below 0.5 g/l.

The adverse effect of high ammonia and organic load levels turned visible in tests B and, dramatically, in test C. As mentioned above, Test B experienced 20% decrease in specific methane yield compared to test A, but the overall behaviour throughout the test showed almost stable conditions (Fig. 1). Such a behaviour reasonably depicts an inhibited steady state, i.e. the process performed at a lower production rate while enduring stress conditions of 1.50 ± 0.08 gN-NH_4^+/l. A temporary VFA accumulation of 1.11 ± 0.09 g/l was observed in the first week. Conversely, test C proved strikingly unstable, with a final 80% reduction in specific methane yield. The final ammonia concentration reached 2.42 ± 0.10 gN-NH_4^+/l. Process instability is observed on the profile of methane production curve (linear production phases followed by almost zero-production plateau (Fig. 1), and the behaviour of VFA accumulation throughout the test, as presented in Figs. 2 and 3. At day 7th, the highest VFA concentration of 7.07 ± 0.95 g/l was detected and reflected by a low methane percentage of less than 40%, whereas the other tests reached quickly stable level of 60–70% (Fig. 2a); at day 15th, VFA fell below 1 g/l whereas increased again up to 7.1 ± 0.33 g/l at day 30th. Looking at ammonia (Fig. 2c), in all the tests except Test C, ammonia rose and stabilized from day 7th on, suggesting that protein hydrolysis and amino acid breakdown took place within this first time lapse. Conversely, in test C, ammonia concentration increased from day 7th to day 15th and then kept stable in day 30th. This pattern might indicate that even protein hydrolysis might have been affected and slowed down by severe inhibitory conditions. This hypothesis is in line with the study reported in [29], where authors investigated the role of

ammonia inhibition at increasing concentrations of peptone as mono-substrate and increasing addition of external ammonia source. Their findings report that, under mesophilic conditions, the rate of protein deamination (i.e. the release of amine groups due to amino-acid molecule breakdown) turned slower as ammonia concentration increased (TAN ranging from less than 1 g/l up to 6 g/l). However, Fernandes et al. [42] evaluated ammonia inhibition on hydrolysis of carbohydrates (as crystalline cellulose) and lipids (as tributyrin) at different induced ammonia concentration and fixed pH of 8. Their outcomes showed weak correlation between hydrolysis rates and ammonia concentrations, questioning the actual influence of ammonia on hydrolysis. The authors highlight that research on hydrolysis inhibition from ammonia are still limited, in contrast with the well-established knowledge on ammonia inhibition on methanogenesis and despite the fact that hydrolysis is commonly considered the rate-limiting step when dealing with solid-rich substrates.

Among the different microbial groups involved in AD, methanogens are generally considered the most sensitive to ammonia inhibition. Although conflicting positions are reported in literature, several studies have reported hydrogenotrophic methanogens to be more tolerant to high-ammonia exposure than acetotrophic ones, promoting a shift of methane production pathway towards syntrophic acetate oxidation and hydrogenotrophic methanogenesis, SAO-HM [13,27,39,43].

More in the detail, Fig. 3 reports the observed concentrations of acetic, propionic and butyric acids at days 7th, 15th and 28th, Tests B and E exhibited a temporary accumulation of VFA in the first week, depleted until not detectable levels in the subsequent analyses. Moreover, no significant reduction of CH_4 percentage in the biogas was observed (Fig. 2a), suggesting that no strong inhibition occurred for the methanogenic population. An unstable behaviour was detected for acetate, propionate and butyrate concentrations in test C. Based on acetate concentration observed in test C, it can be suggested that a first acetotrophics' inhibition may have occurred by the high acetate concentration of 4.56 ± 0.28 g/l at day 7th, whose corresponding CH_4 percentage is around 40%, in fact much lower than the other tests; a temporal acetoclastic biomass acclimation and/or the SAO-HM might have occurred in the second week, prior to further system failure in the last days (Fig. 2b). Also propionate accumulation was noticeable and reached 1.90 ± 0.26 g/l, suggesting propionate-degraders inhibition (Fig. 3). Some authors indicate the ratio of propionate and acetate concentration as process stability indicator. Specifically, values higher than 1.4 are reported to be representative of strong instability [44]. Based on the results observed in test C, the ratio was below this critical value, though process proved instable. Gallert et al. [29] observed propionate accumulation at ammonia concentration higher than 1 g/l, whereas Chen et al. [27] at ammonia concentration higher than 2 g/l, but performed under acid pH. Similarly, Moestedt et al. [31] detected propionate accumulation at TAN concentration higher than 1 g/l, reporting that this might be due either to inhibition of propionate oxidizers or to a shift of acidogenesis towards more propionate production, as the increase in hydrogen pressure in the system may direct this degradation phase towards the production of acids longer than acetic acid [45]. In the present study, it seems that propionate accumulation is likely due to propionate degraders inhibition more than other factors.

According to the results, only the lowest organic load of 5 gVS/l proved suitable for the experimental conditions tested. As already discussed, organic load and ammonia accumulation are strictly connected when processing protein-rich substrates, so that it is not easy to ascribe system failure to one rather than to the other factor, especially in this latter case of Test C, where both organic load and ammonia nitrogen are critically high. Based on Eq. (1), a first assessment of FAN concentration can be done, in order to evaluate its potential inhibitory effect. During the experimental time course, pH raised at levels around 8–8.5, in all the test treating TF. This is likely due to the alkaline pH of limed fleshing as well as to ammonia release. Despite the significant VFA accumulation exhibited in test C, acidification never occurred, confirming, in fact, the strong buffer capacity of the system. In this study, pH correction was not deemed an option as the aim was to push stressing conditions up to system failure. The maximum ammonium concentration of 2.42 ± 0.10 gN-NH_4^+/l experienced at the maximum organic load of 20gVS/l (Test C), is far below some of the toxic levels reported in literature, although toxicity ranges vary widely, as already discussed. The evident detrimental effect observed is very likely to be caused by FA. Depending on pH, FA might account for less than 1% of total TAN at pH 7, to almost 10% at pH 8 and up to 48% at pH 9. According to the calculated values, FA ranges from 0.16 (p 8), to 0.42 g/l (pH 8.5) in test B and from 0.25 g/l (pH 8) to 0.67 g/l (pH 8.5) in test C. These values fall within the concentration range discussed in the review conducted by Rajagopal et al. [26]. The reported FA inhibitory concentrations are: 316 mg/l at pH 8, 37 °C for AD of swine manure [46]; 750 mg/l at pH 8 and 37 °C during digestion of swine manure [47]; 1450 mg/l at pH > 7.6 and 51 °C for co-digestion of swine manure and other solid organic fractions [48]. It has to be noted that results from batch conditions can be far from being representative of methane production under continuous operation. On the one hand, the long-time course of the experiment and optimum initial conditions in terms of nutrient supply is conductive to complete organic matter degradation, including slow degradable compounds, that might not be degraded in continuous systems, unless very long sludge retention time are provided. On the other hand, batch systems are more sensitive to overload conditions and accumulation of different toxic compounds into a reduced working volume. Thereby, it may be that both overloading and ammonia inhibition adversely contributed to the low performance of test C, whereas test B is likely to have been affected mainly by ammonia inhibition, as organic load of 10 gVS/l have shown to be feasible for AD of tannery wastes at comparable experimental set-up (Table 2).

In the perspective of a technological application for onsite treatment of TF in medium or large-size de-centralised tanneries, organic load confirmed to be crucial for successful process stability. In the simplest scenario of no ammonia mitigation measures, even a low-performance stable state, as the one indicated in Test B, can be an attractive solution, as it would achieve solids reduction and waste stabilization, facilitating final disposal management. Conversely, in case the AD of TF is adopted within an integrated tannery and wastewater treatment train, the maximization of biogas production and methane content becomes of prominent interest, from an economic point of view. In this case, high organic loading rates would probably request operational strategies to minimize the toxic effect of high ammonia concentrations and co-digestion with the tannery sludge could be a cost-effective solution. Besides co-digestion and pH and control, biomass acclimation to high ammonia levels is a useful and economic solution, even though

acclimation periods might last some months [26]. Gao et al. [13], claimed that only few studies have attempted to use bacterial acclimation to attenuate ammonia inhibition in N-rich waste AD and their work successfully adopted in situ biomass adaptation under step-wise ammonia exposure up to TAN concentration higher than 4 g/l, during AD of protein-rich kitchen wastes. Together with ammonia control solutions (such as biogas recirculation [49] or struvite precipitation [50]), waste pre-treatment such as preliminary TF hydrolysation could also be valuable in order to prompt organic matter degradation as well as ease operational reactor management.

4. Conclusions

Results of the present work indicate that anaerobic digestion of TF is technically feasible at low organic load of 5 gVS/l; at higher organic loads of 10 and 20 gVS/l the system started to suffer from ammonia inhibition, due to TAN level of 1.50 ± 0.08 gN-NH_4^+/l and 2.42 ± 0.10 gN-NH_4^+/l that brought to inhibited steady state and system failure, respectively. The detrimental effect of ammonia exposure was exacerbated by relatively high pH values that never fell below 8, due to the alkaline pH of limed TF and the increase in buffering capacity brought by ammonia release. Overloading condition is likely to be occurred at the highest organic load testes, contributing to the low performance observed. In the perspective of full-scale implementation, organic loading rate is crucial for successful process stability. A steady-state process, even at a moderate methane production rate, such as the one indicated in the test with 10 gVS/l, could be still attractive as TF treatment solution since it would achieve on-site waste stabilization and stable process conditions. In order to optimize methane production, high organic loading rates would probably require ammonia control strategy and TF pre-treatments. Co-digestion with TPS proved a suitable solution since no adverse effect was observed and the final methane production accounted for an almost additive effect of the two substrates degradation and might represent a cost-effective solution towards an integrated tannery waste and wastewater treatment train.

Acknowledgements

The study was supported by European Union in the frame of the Marie Curie IRSES program (Carbala project 295176).

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