Integrated Chemical Biochemical Technology to Reduce Ammonia Emission from Fermented Municipal Biowaste

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ABSTRACT: A new eco-friendly process is reported, the implementation of which at EU level could reduce ammonia and GHG emissions from fermented biowaste by over 1 Gt yr^{-1}. The present work reports the case study of municipal biowaste (MBW). The process is based on the use of soluble bioorganic substances (SBO) as auxiliaries in the anaerobic fermentation of MBW to produce biogas and digestate with reduce ammonia content. The SBO-assisted process enables a virtuous biowaste cycle, where MBW is sequentially fermented under anoxic conditions, the digestate is composted, the compost generated is hydrolysed yielding SBO, which is recycled to the anaerobic fermentation reactor at 0.2% concentration. The results show that, depending upon MBW source, fermentation inoculum and SBO concentration in the fermentation slurry, about 40% reduction of ammonium in the digestate is achieved, whereas the control fermentation without SBO exhibits up to 11% ammonia increase. The microbial community and biogas production are not significantly affected by SBO addition. The data are consistent with biological and chemical processes occurring in SBO assisted fermentation. These comprise ammonia production by protein hydrolysis catalysed by proteolytic bacteria and ammonia oxidation to N_2 catalysed by SBO. The results confirm the benefit provided by the use of SBO to reduce the environmental impact of biowaste. These encourage the implementation of SBO assisted fermentation in real operational environment.

KEYWORDS: *Municipal biowaste, Anaerobic fermentation, Soluble bioorganic substances, Ammonia oxidation, Microbial community composition*

■ INTRODUCTION

The management of MBW constitutes a well-known environmental and economic burden for society, due to increasing population, human urbanization and consumption habits. Anaerobic fermentation is widely practiced to treat both solid biowaste[1] and wastewater.[2] The specific technology produces biogas used as biofuel and solid digestate applicable as fertilizer. However, the fermentation process causes a secondary environmental problem associated to the biodegradation of protein matter, as well as the formation and accumulation of toxic ammonia (NH_3) in the digestate.[3] Several biochemical and chemical technologies can be employed as

secondary treatment of the digestate.[4] These imply additional running costs ranging between 1-13 $ kg^{-1} of nitrogen, which contribute a negative economic impact for waste management. A published study[5] has reported processing costs up to 156 € t^{-1} of MBW. This is paid for 57% from tipping fees and 43% from biogas sales.

Organic chemical reactions constitute a promising strategy for improvement of the current MBW plants' economy. Chemical hydrolysis and oxidation reactions applied on MBW allow producing value added SBO.[6] These products incorporate the same efficiency as commercial commodities derived from fossil sources for a number of applications in several sectors of agriculture and the chemical industry. The sustainability of MBW chemical processing is prospected by the SBO production cost of 0.2-1 € kg^{-1} against a potential market value of 1-800 € kg^{-1}, which depends on the SBO's production process and use.

With specific reference to the presence of NH_3 in the MBW anaerobic digestate, previous work[4] has reported that SBO, obtained by chemical hydrolysis of MBW compost and employed as additive at 0.05-0.2% concentration in the MBW feed of the anaerobic fermentation reactor, enables substantial reduction of the NH_3 content in the anaerobic digestate formed. The NH_3 reduction achieved in the SBO assisted fermentation, as opposed to the conventional process performed in the absence of added SBO, prospects a virtuous cost-effective biowaste cycle integrating chemical and biochemical reactions (Figure 1).

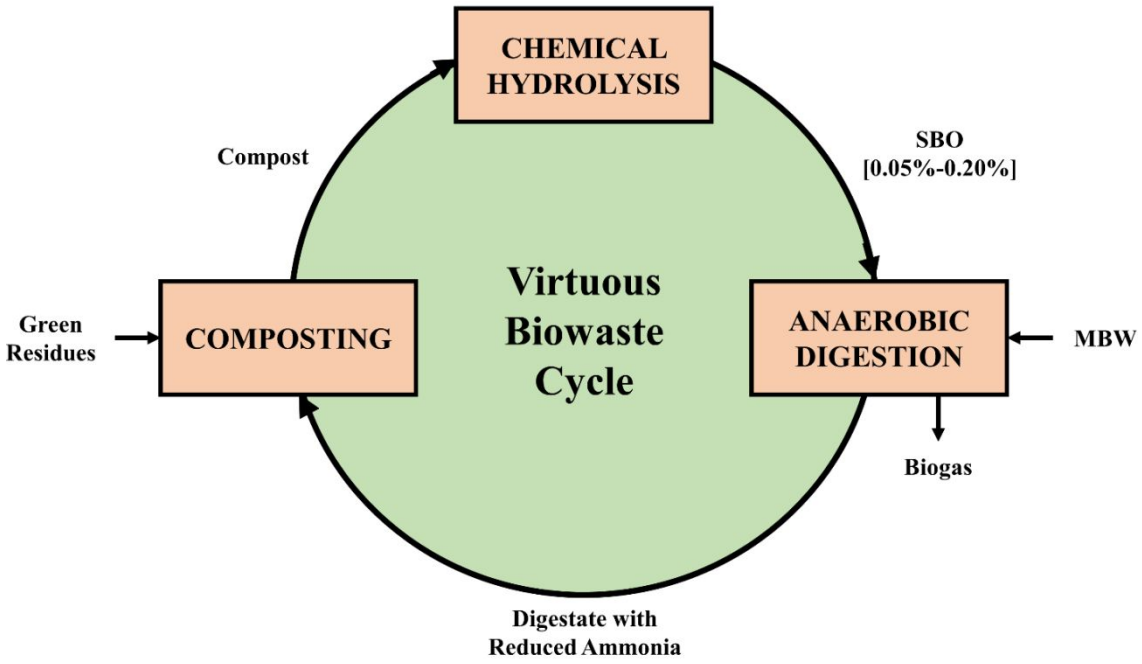

Figure 1. Virtuous cost-effective cycle based on the effect of SBO on the management of MBW.

The present work addresses two fundamental technological issues, which were not investigated in the previous work.[4] These are (i) the fate of NH_3 and (ii) the mechanism underlying the effect of SBO. Both issues are highly relevant for the anaerobic fermentation of biodegradable waste from any vegetable and animal source. A most recent paper[7] reports that the above SBO effect takes place also in the fermentation occurring in the animal intestine as well as in closed bioreactors dedicated to the production of biogas from manure. In the first case, the addition of 0.25% SBO in the animal diet causes animals to produce manure with 30% less ammonia and 42% less GHG emissions as compared to control animals fed with the diet that excluded SBO addition.[7] In the second case, the anaerobic fermentation of manure in the presence of added SBO, carried out in reactors producing biogas,[8] produces less ammonia as opposed to the control fermentation performed in the absence of SBO. The relevance of the above studies can be realised, considering that livestock breeding contributes about 15% of the global gaseous environmental impact caused by human activities.[7]

The above findings[4,7,8] point out that, although the scheme presented in Figure 1 prospects a virtual MBW cycle based on the new SBO assisted fermentation process,[9] the NH_3 abatement achieved by the use of SBO is relevant in connection to any environmental impact, which could be caused due to conversion of NH_4^+ to other N compounds. Particular concern arises from nitrates and nitrogen oxides, which could be present and/or formed in the condensed and gas phases of fermentation, respectively. Disposal of the anaerobic digestate is commonly practiced by spreading onto soil. Although this practice may contribute to soil fertilisation, inappropriate handling of the product containing excess nitrates and/or ammonia may cause acidification and eutrophication in ground water due to nitrates leaching through soil and/or emission of ammonia and GHG gases into the air, respectively. Manure is mostly responsible for these impacts.[7] Due to the relevance of these issues, it was highly worthwhile to undertake the work reported here in after. Knowledge of the mechanism responsible for the effect of SBO in the anaerobic fermentation of biowastes could allow identifying the reaction parameters required to optimize the process in different operational, regional and local environments, to assess its sustainability and to implement the product's use at commercial level.

To address the ammonia fate issue, it was necessary to perform a mass balance of the anaerobic fermentation process in the presence of added SBO, compared to the control process carried out

in the absence of SBO. Previous studies have focused on the mass balance of anaerobic fermentation processes for different waste materials[10] accounting both for total nitrogen (TN) and carbon (C) balance, but none reports the speciation of TN. However, anaerobic fermentation slurries may contain or generate during the process several N compounds, which could be potentially toxic depending on their concentration in the system. These comprise NO_3^- and NH_4^+ ions in the digestate condensed phase, as well as N oxides in the gas phase, which have been analyzed in the present study. The former molecules impose restrictions to the disposal of the digestate, in order to mitigate the risk of excessive NH_4^+ and NO_3^- pollution in soil and ground water. N oxides constitute GHG, which contribute to global warming. In the case of the scheme in Figure 1, it was imperative to assess whether the decrease of ammonia in the digestate caused by SBO addition[4] into the fermentation slurry generated nitrates and/or nitrogen oxides. This event would cause a tertiary pollution problem consequent to the reduction of the secondary pollution of ammonia generated by the conventional anaerobic fermentation process performed in the absence of SBO. To fully understand the reaction mechanism of SBO in anaerobic fermentation systems, a microbiological analysis[11] was also performed to decipher whether the reduction of NH_3 achieved in the presence of SBO was due to chemical reactions catalyzed and/or inhibited by SBO or due to biochemical pathways involving microorganisms, which were enriched[12] in the presence of SBO from the pool of strains that caused NH_4^+ production in the absence of SBO. Thus, the experimental work was focused on the investigation of the fermentation phase comprising the formation of ammonia and oxidation of inorganic N, more than on the reactions leading to methane formation.

■ EXPERIMENTAL SECTION

Substrate and Inoculum Preparation. SBO was available from a previous work.[4] It was obtained by alkaline hydrolysis of the compost produced in the waste treatment plant of Acea Pinerolese Industriale located in Pinerolo (Italy). The compost was obtained from a mix of urban gardening residues and the anaerobic digestate of unsorted urban food waste (FW). In the present work the SBO was used as additive for the anaerobic fermentation of FW, which were supplied by different hotels in the area of Limassol (Cyprus). Upon collection, FW were immediately transferred to the laboratory for further treatment (blending and mixing) to produce a homogenous mixture, which was subsequently stored at -20 °C before use. Fermentation inoculums comprised

the primary (P) and dewatered (D) sludge provided from the wastewater treatment plant of Sewerage Board of Limassol-Amathus (SBLA) in Moni (Cyprus). Primary sludge consisted of suspended solids and organics captured in the primary treatment process through gravitational sedimentation by a primary clarifier, while dewatered sludge was produced in anaerobic digestion bioreactors followed by a dewatering process. The inocula were incubated at 55 °C for 1 week for adaptation purposes. Food wastes (FW) were collect from restaurants in the Cyprus Limassol area. The contents of TS w/w % and VS w/w % referred to TS dry matter, respectively, were 2.01 ± 0.28 and 90.16 ± 2.74 for P, 19.24 ± 0.30 and 62.00 ± 0.24 for D, 36.47 ± 0.33 and 86.19 ± 3.12 for FW, 100 and 63.40 ± 0.42 for SBO.

Anaerobic Fermentation Plan. Lab-scale experiments were conducted using shake flasks as reactors with a working volume of 150 mL, which were operated under anaerobic conditions for FW fermentation. Eight treatments were tested: 1) Primary sludge (P) as control experiment; 2) P supplemented with SBO; 3) P supplemented with FW; 4) P supplemented with FW and SBO; 5) Dewatered sludge (D) as control experiment; 6) D supplemented with SBO; 7) D supplemented with FW; 8) D supplemented with FW and SBO. Each experiment was conducted in duplicate at 55 °C. The concentration of SBO in treatments 2, 4, 6, 8 was 0.2% (w/w). The FW/inoculum ratio (w/w of Volatile Solids) at the beginning of each experiment was 2. The reactors were fed at the beginning of the experiment with pretreated FW (mashed in a laboratory blender) and the volume of inoculum (P and D) required to form a total solids (TS) content of 3.14% (w/w). ~~The reactors were fed at the beginning of the experiment with pretreated FW (mashed in a laboratory blender) and the volume of inoculum (P and D) required to form a total solids (TS) content of 3.14% (w/w).~~ The reactors were maintained under batch conditions for 14 days and samples were withdrawn at ~~specific time~~the start and end of the fermentation ~~intervals~~ for total nitrogen by Kjeldahl, NH_4^+, NO_3^- and NO_2^- analysis. The fermentation was stopped at day 14 to allow comparing the data for the ammonia formation and fate with previous data obtained in the Acea anaerobic fermentation plant operating with the same hydraulic retention time. The Acea plant served as the reference plant in the LIFECAB project (LIFE16 ENV/IT/000179) co-funded by the EU (see Acknowledgements section). The specific plant constitutes a typical example of anaerobic fermentation plants processing municipal biowaste throughout Europe. The objective of the current work was to assess the replicability of the SBO effect on the control of the formation and fate of ammonia under similar conditions to the operation of ~~the same hydraulic retention time as~~

~~in~~ the Acea plant, taking into account changes of inocula and food wastes in different geographical sites, and of food consumption habits and social contexts.

DNA Extraction and Next-Generation Sequencing. 16S rRNA sequencing was performed to determine the composition of the microbial community at the beginning of the process and upon fermentation completion. Samples of 0.3 g each were withdrawn from the inoculums applied (P and D sludge) at the start of fermentation and from all slurries (treatments 1-6) at fermentation end. Total genomic DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals, USA) and the genome was sequenced by Novogene (Beijing, China). The V3–V5 region was targeted employing the primer set 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACCAGGGTATCTAAT-3′). PCR amplification was conducted as previously reported.[13] Sequencing was performed on an Ion S5TM XL (Thermo Fisher, USA) in Novogene (Beijing, China). Clean reads were assigned to operational taxonomic units (OTUs) at 97% sequence similarity and were classified with the SSUrRNA database using a confidence threshold of 80%.

Biogas Volume and Analyses. The biogas volumes were regularly measured using a wetted 50 ml glass syringe and reported at atmospheric pressure and a temperature of 35 ◦C. The volume was discharged after each measurement allowing the headspace pressure to equilibrate with the atmospheric pressure. The gas composition in the headspace was determined by withdrawing 1 ml from the headspace and analyzed using a gas chromatograph (Agilent Technologies, 7820OA, Santa Clara, CA, USA) fitted with a ShinCarbon ST 50/80 (2 m length, 2.2 mm ID) mesh column (Restek Corporation, Bellefonte, PA, USA) and thermal conductivity detector as previously described.[14, 15] Gas samples from each treatment were collected and analyzed for CH_4, CO_2, H_2, N_2, O_2, N_2O. Argon was used as the carrier gas at a constant pressure of 5 psi. A calibration curve was prepared for each gas to determine the concentration of gas samples' constituents. The samples were analyzed immediately upon collection, while 1 mL of injection volume and 160 °C column temperature was used. The coefficient of variation for 10 samples was ±2%. The composition of the gas standard used for biogas analysis were 60% CH_4 and 40% CO_2. The biogas composition of other gases such as H_2, O_2, N_2, and CO_2 was determined by 99.99% gas standards. The Limit of Blank, Limit of Detection and Limit of Quantitation (LoB/LoD/LoQ) values for the determination of H_2, O_2, N_2, CH_4, and CO_2 volumes were calculated at 0/0.4/8.4 mL, 0/0.4/3.4 mL, 0/1.2/10.5 mL, 0/0.2/7.0 mL, and 0/0.3/1.1 mL, respectively. NH_3 concentration was analyzed in gas samples using Draeger-tubes. The cumulative methane yield was calculated as previously

reported.[16] ~~Gas samples from each treatment were collected and analyzed for CH_4, CO_2, H_2, N_2, O_2, N_2O using gas chromatography (GC) (Agilent Technologies, 7820 A) equipped with a thermal conductivity detector (TCD) and a ShinCarbon ST packed column (Restek Corporation, 230 Bellefonte, PA, USA). Argon was used as the carrier gas at a constant pressure of 5 psi. A calibration curve was prepared for each gas to determine the concentration of gas samples' constituents. The samples were analyzed immediately upon collection, while 1 mL of injection volume and 160 °C column temperature was used. The coefficient of variation for 10 samples was ±2%. The cumulative methane yield was calculated as previously reported.[14] The Limit of Blank, Limit of Detection and Limit of Quantitation (LoB/LoD/LoQ) values for the determination of H_2, O_2, N_2, CH_4, and CO_2 volumes were calculated at 0/0.4/8.4 mL, 0/0.4/3.4 mL, 0/1.2/10.5 mL, 0/0.2/7.0 mL, and 0/0.3/1.1 mL, respectively. NH_3 concentration was analyzed in gas samples using Draeger-tubes.~~

Analysis of Total Nitrogen in the Condensed Phase. Total N was determined on 10 mL sample withdrawn from the fermentation slurry phase at the start and end of the fermentation, to which 40 mL deionized water, 2.5 g of potassium sulphate catalyst and 10 mL sulfuric acid in a glass tube were added. The mixture was heated at 150 °C for 30 min, 250 °C for 30 min and 420 °C for 1 h. The tube was then left to cool and 50 mL of water were added slowly under shaking. Subsequently, 30 mL of boric acid were added to the suspension and distillation was performed using a Kjeldahl apparatus (UDK 129, VELP Scientifica, Italy). The distillation process was completed upon collection of 100 mL condensate. Titration was performed using 0.01 M sulfuric acid and bromocresol green-methyl mixed indicator, which was added dropwise to the distillate until the solution was turned into a violet at endpoint.

Analyses of NH_4^+, NO_3^- and NO_2^- in the Condensed Phase. The concentration of these N species were determined using the UV/VIS spectrophotometer (JENWAY 7315, Staffordshire, UK).

NH_4^+ was determined on 1.0 mL as previously reported.[15-17] The sample was mixed thoroughly into the reaction cell (20-30 °C). A dose of the reagent NH_4-1K kit (Merck, Germany) was added to the cell, which was tightly sealed and vigorously shaken until the reagent was completely dissolved. After 15 min the sample absorbance at 667 nm was measured.

NO_3^- was determined as previously reported.[16-18] The method is based on the reaction of NO_3^- ions with 2,6-dimethylphenol (DMP) to form 4-nitro-2,6-dimethylphenol, the concentration of which is determined photometrically. Specifically, 1.0 mL of sample was mixed at room temperature with 1.0 mL of reagent NO_3-1K (Merck, Germany) and the mixture was homogenized. The absorbance of each sample was determined at 340 nm.

NO_2^- was determined as previously reported.[17-19] The method is based on the reaction in acidic solution of NO_2^- with sulfanilic acid to form the diazonium salt. The latter reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red-violet azo dye that is quantified photometrically. Experimentally, 5.0 mL sample was placed into the reaction cell, closed and shaken vigorously to dissolve the sulfanilic acid reagent (Merck, Germany). Subsequently, the mixture was allowed reacting for 10 min and the absorbance was measured at 540 nm.

Statistical Analysis. Significant differences in the mean content values of the chemical species involved in the N mass balance conducted in this work were determined. The concentration values of all N species measured in the gas and condensed phases were compared through one-way analysis of variance (ANOVA) to assess significant differences at $p < 0.05$ level.

■ RESULTS AND DISCUSSION

N Mass Balance. P and D sludge were initially adapted to thermophilic culture conditions. Thus, both inoculums were maintained for 7 days at 55 ^oC prior addition of each sludge in the reactors. At the beginning of the experiments, flasks were flashed with CO_2 and the pH value was adjusted to 8, which was maintained between 6.8-8.5 until the end of fermentation. Total organic nitrogen (N-org) was calculated by subtracting the analyzed values for NH_4^+ and NO_3^- nitrogen from the total nitrogen measured in the condensed phase. No NO_2^- N was found. Table 1 reports the nitrogen weight values for the different N containing species present in the condensed and gas phases at the start (Ti) and end (Tf) of fermentation. The TN value in Table 1 constitutes the sum of all nitrogen species. For each treatment, Table 1 reports also the average TN values (TNavg), demonstrating that Ti and Tf values deviate by 5-29% of their TNavg, while the average % standard deviation is 15%. Obtaining the mass balance data in Table 1 required dealing with the heterogeneous solid-liquid slurry phase, which could negatively affect withdrawal of a representative sample for analysis, using four different analytical methods applied to measure each of the N species involved in the fermentation, as well as recovery and measurement of the total

256 weight and volume of the condensed and gas phases, respectively. Thus, a range of factors
257 associated to sampling and measurements could have affected the accuracy and/or reproducibility
258 of the analytical data obtained at the beginning and end of fermentation. Nevertheless, under these
259 complex circumstances, the data obtained demonstrate that the analytical and mass handling
260 protocols adopted in the present work allowed obtaining a satisfactory reliable mass balance that
261 accounts for the N species present at the start and end of fermentation.

Table 1. Nitrogen mass incorporated in different constituents, including total organic nitrogen (N-org), total nitrogen (TN), nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), nitrogen (N_2) and nitrous oxide (N_2O), contained in the experiments at the beginning (Ti, 0 d) and at the end (Tf, 14 d) of fermentations.

Test		Ti [mg]						Tf [mg]						TNavg[d]
		N-org	$N\text{-}NO_3$	$N\text{-}NH_4^+$	TN	N_2	N_2O	N-org	$N\text{-}NO_3$	$N\text{-}NH_4^+$	TN	N_2	N_2O	
P[c]	mean[a]	75.2	0.00	49.4	124.6	0.000	0.00	27.4	0.00	65.0	105.6	13.0	0.03	115
	std[b]	3.9	0.0	3.0	8.9	.000	0.0	5.9	0.0	4.9	17.8	0.0	0.00	11.8
P.SBO[c]	mean[a]	131.4	4.90	66.3	202.7	0.000	0.00	101.2	2.10	67.6	177.0	6.10	0.01	190
	std[b]	0.0	0.0	2.1	0.0	.000	0.0	39.2	0.0	2.4	47.5	0.1	0.00	9.5
P.FW[c]	mean[a]	304.4	9.00	61.7	375.2	0.000	0.00	235.2	7.10	66.9	316.3	6.50	0.52	346
	std[b]	2.1	0.1	1.0	2.4	.000	0.0	21.5	0.1	8.1	39.7	0.2	0.30	12
P.FW.SBO[c]	mean[a]	321.0	8.90	64.9	394.9	0.00	0.00	259.7	8.40	62.7	343.9	12.9	0.19	369.4
	std[b]	5.3	0.0	5.9	9.7	.0	0.0	36.2	0.1	0.3	50.4	5.7	0.01	9,8

D[c]	mean[a]	302.3	21.1	118.9	442.4	0.00	0.00	268.4	3.3	187.2	474.2	14.7	0.33	458
	std[b]	51.0	0.2	12.8	81.9	0.00	0.00	46.3	0.1	12.8	65.3	1.9	0.01	4.9
D.SBO[c]	mean[a]	511.9	25.2	143.0	680.2	0.00	0.00	300.9	3.8	139.0	522.6	5.1	0.20	565
	std[b]	34.1	0.8	18.9	14.8	0.00	0.00	1.8	0.0	10.7	2.9	1.0	0.01	28.9
D.FW[c]	mean[a]	691.1	33.0	109.5	833.7	0.00	0.00	477.4	12.0	121.9	616.5	5.0	0.08	725
	std[b]	0.0	0.0	30.9	0.0	0.00	0.00	67.2	0.0	1.9	78.3	0.3	0.00	21.2
D.FW.SBO[c]	mean[a]	422.0	28.5	142.4	593.0	0.00	0.00	310.1	5.8	86.3	407.2	4.3	0.14	500
	std[b]	14.1	0.0	17.6	7.2	0.00	0.00	33.8	0.0	13.6	72.4	0.5	0.07	26.3

[a]Values comprise sum of the mass measured in the solid, liquid and gas phases and are given as average (mean) contents calculated over duplicates. [b]Values comprise sum of the standard deviation (std) values calculated for the condensed and gas phases. [c]Abbreviations: P (Primary sludge), P.SBO (Primary sludge with added SBO), P.FW (Primary sludge with FW, P.FW.SBO (Primary sludge with FW and added SBO), D (Dewatered sludge), D.SBO (Dewatered sludge with added SBO), D.FW (Dewatered sludge with FW), D.FW.SBO (Dewatered sludge with FW and added SBO). [d]Average of the TN values at Ti and Tf, and std calculated as % of TNavg.

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Regarding each N species, Table 2 also reports the % change values calculated using the corresponding weight values according to the following equation:

$$\Delta N\ \% = 100 * \left(\frac{N(Tf) - N(Ti)}{N(Ti)}\right) \quad (1),$$

where N(Tf) and N(Ti) comprise the mass values of the different N species, i.e. for N standing for N-org, N-NO_3^-, N-NH_4^+, N-N_2, N-N_2O, as listed in Table 1 and 2, at the end and start of the fermentation. For the calculation of ΔN_2 and ΔN_2O, the data at Ti are the values measured at day 3, since only after 3 days from the start of the fermentation these N species became measurable. Moreover, the concentration of NH_3 in the gas phase was below the detection limit at all times. N_2O was rarely measurable and overall negligible during the 14 days of fermentation. The data shows large significant changes of all N species content during the fermentation. In all trials, the content of N-org was the end of fermentation was lower that at the start of the fermentation. The maximum N-org decrease at the end of the fermentation occurred in the P reactor (-63.5%), while the lowest decreases were obtained in the P.FW.SBO (-18.7%) and D (-15.9%) experiments. Also the NO_3^- content was always lower at fermentation end as compared to the onset of the trials. ΔNO_3^- ranged from –85% in the D.SBO to –5% in the P.FW.SBO fermentations, while the N_2O quantities measured were negligible at all times. ΔNH_4^+ ranged from 58 % in the D to –39% in the D.FW.SBO and ΔN_2 varied between 541% in the D and 23% in the P.FW.SBO fermentations.

Table 2. ΔN % values calculated according to equation 1 from the corresponding weight values for each nitrogen species at fermentation start (Ti) and end (Tf).

Test		Weight values (mg) at Ti[d]					Weight values (mg) at Tf					ΔN values (% w/w)				
		N-org	N-NO_3	N-NH_4^+	N_2	N_2O	N-org	N-NO_3	N-NH_4^+	N_2	N_2O	ΔN-org	ΔNO_3	ΔN-NH_4^+	ΔN_2[d]	ΔN_2O[d]
P[c]	mean[a]	75.2	0.0	49.4	8.6	0.00	27.4	0.0	65.0	13.0	0.03	-63.5	0.00	31.67	53.0	-
	std[b]	3.9	0.0	3.0	0.0	0.00	5.9	0.0	4.9	0.0	0.00	0.50	0.00	1.89	0.00	-
P.SBO[c]	mean[a]	131.4	4.9	66.3	2.1	0.00	101.2	2.1	67.6	6.1	0.01	-24.2	-57.93	1.91	188.4	-
	std[b]	0.0	0.0	2.1	0.0	0.00	39.2	0.0	2.4	0.1	0.00	0.04	0.00	0.42	0.29	-
P.FW[c]	mean[a]	304.4	9.0	61.7	3.4	0.52	235.2	7.1	66.9	6.5	0.52	-22.7	-21.91	8.34	96.1	0.0
	std[b]	2.1	0.1	1.0	0.6	0.00	21.5	0.1	8.1	0.2	0.00	0.06	1.25	1.43	0.54	0.0
P.FW.SBO[c]	mean[a]	321.0	8.9	64.9	10.5	6.42	259.7	8.4	62.7	12.9	0.19	-18.7	-5.22	-3.10	23.5	4.03
	std[b]	5.3	0.0	5.9	5.0	2.81	36.2	0.1	0.3	5.7	0.01	0.19	3.50	1.28	2.68	0.45
D[c]	mean[a]	302.3	21.1	118.9	2.3	0.28	268.4	3.3	187.2	14.7	0.33	-15.9	-84.01	57.81	540.8	18.6
	std[b]	51.0	0.2	12.8	0.0	0.00	46.3	0.1	12.8	1.9	0.01	1.25	0.39	6.21	4.39	0.08
D.SBO[c]	mean[a]	511.9	25.2	143.0	2.3	0.09	300.9	3.8	139.0	5.1	0.20	-43.2	-84.63	-2.43	128.5	140.1
	std[b]	34.1	0.8	18.9	0.0	0.00	1.8	0.0	10.7	1.0	0.01	5.00	1.33	1.41	1.00	6.69
D.FW[c]	mean[a]	691.1	33.0	109.5	2.3	0.00	477.4	12.0	121.9	5.0	0.08	-32.4	-63.52	11.62	115.7	-
	std[b]	0.0	0.0	30.9	0.2	0.00	67.2	0.0	1.9	0.3	0.00	0.70	0.00	9.54	6.60	-
D.FW.SBO[c]	mean[a]	422.0	28.5	142.4	1.1	0.00	310.1	5.8	86.3	4.3	0.14	-29.8	-79.66	-39.52	284.1	-
	std[b]	14.1	0.0	17.6	0.6	0.00	33.8	0.0	13.6	0.5	0.07	0.10	0.00	2.09	1.01	-

[a]Values comprise sum of the mass measured in the solid, liquid and gas phases and are given as average (mean) contents calculated over duplicates. [b]Values comprise sum of the standard deviation (std) values calculated for the condensed and gas phases. [c]Abbreviations: P (Primary sludge), P.SBO (Primary sludge with added SBO), P.FW (Primary sludge with FW, P.FW.SBO (Primary sludge with FW and added SBO), D (Dewatered sludge), D.SBO (Dewatered sludge with added SBO), D.FW (Dewatered sludge with FW), D.FW.SBO (Dewatered sludge with FW and added SBO).[d]Values at Ti are the same as in Table 1, except for N_2 and N_2O measured at day 3.

The ΔN-org % values in Table 2 indicated consumption of organic nitrogen in fermentation. This was expected to produce mineral N, as indeed confirmed by the large changes observed for the NH_4^+ and N_2 content obtained upon fermentation completion. On the other hand, the consumption of nitrate ions indicated by the large negative ΔNO_3^- values implied significant participation of nitrate ions in the mineralization of organic nitrogen. In an attempt to generate hypothesis about the mechanism potentially triggered for the mineralization of organic nitrogen, the relationship between ΔN % values of the different N species involved was analyzed. The only significant relationship (Figure 2) was determined between ΔN_2 % and ΔNH_4^+ %. Figure 2 exhibits that N_2 is produced at the expense of ammonia N. The values for D and P.FW.SBO were omitted from the plot of Figure 2, due to large deviation from the trend obtained by the remaining 6 experiments. The aforementioned values fit well equation 2,

$$\Delta N_2 = -3.28\ \Delta NH_4^+ + 150 \quad (2),$$

with 0.94 correlation coefficient.

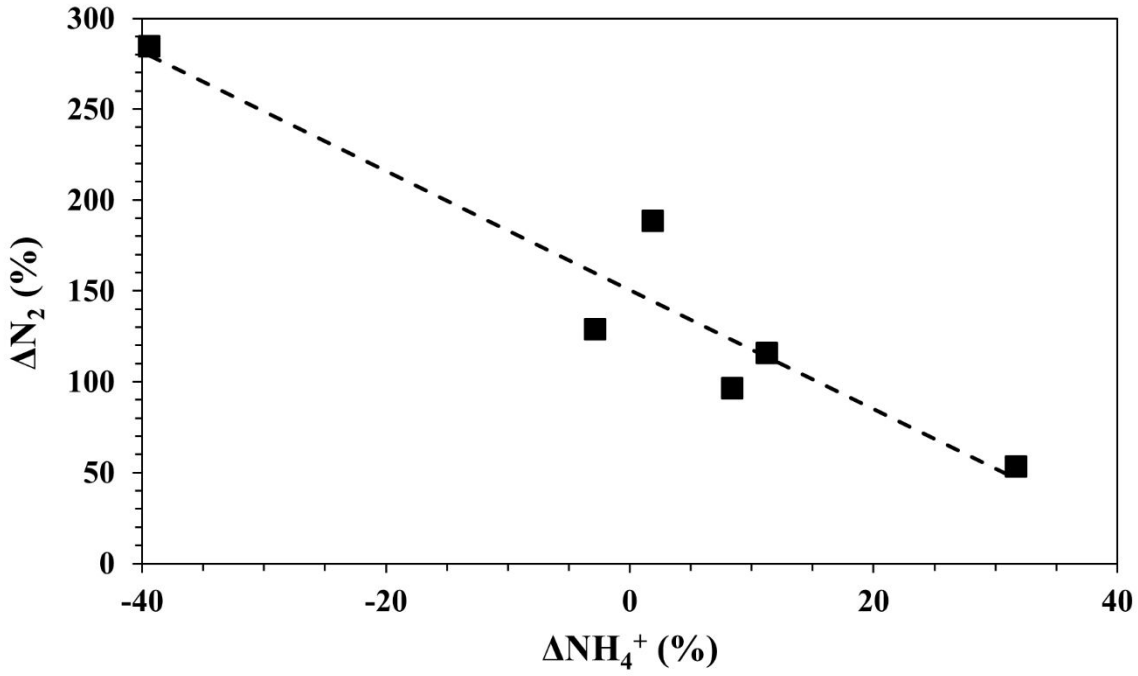


Figure 2. Plots of ΔN_2 % vs. ΔNH_4^+ % experimental values for P, P.FW, P.SBO, D.FW, D.SBO and D.FW.SBO listed on Table 2.

SBO effect on ammonia production in anaerobic fermentation under different conditions. Figure 3 reports the results of the statistical comparison of ΔNH_4^+ % values in Table 2 for all fermentations.

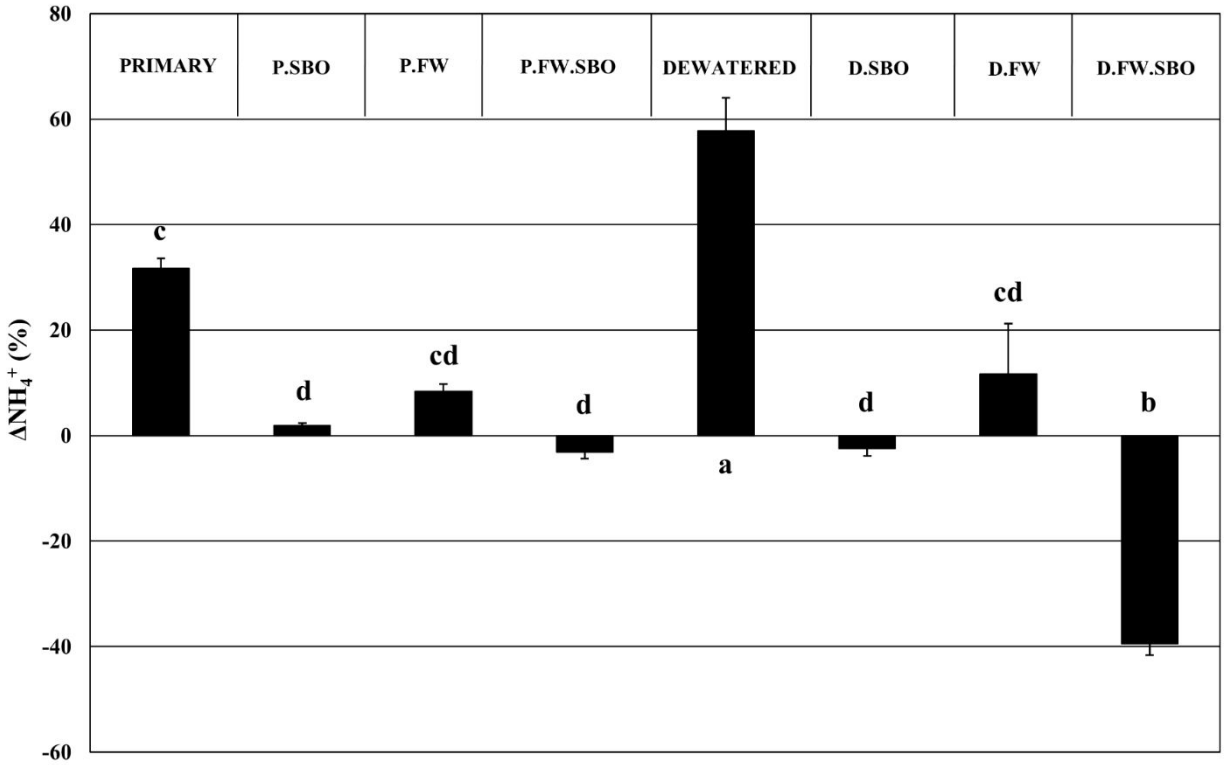


Figure 3. Results from the statistical comparison of ΔNH_4^+ % values for all treatments (Primary: Primary sludge; P.SBO: Primary sludge with SBO; P.FW: Primary sludge with FW; P.FW.SBO: Primary sludge with FW and SBO; Dewatered: Dewatered sludge; D.SBO: Dewatered sludge with SBO; D.FW: Dewatered sludge with FW; D.FW.SBO: Dewatered sludge with FW and SBO) at the beginning and end of fermentation runs. Values are given as mean and standard deviation (error bars) calculated over triplicates. Values with no letters in common differ significantly ($P < 0.05$).

The positive ΔNH_4^+ values in Figure 3 indicate production of ammonia during the fermentation. The negative ΔNH_4^+ values indicate that the ammonia present in the slurry phase at day zero was consumed during the 14 days of fermentation. According to Figure 3, production of ammonia occurs in 5 cases. However, consumption of ammonia occurs in three cases, all containing SBO. Even in the P.SBO experiment, which exhibited a small production of ammonia, the apparent ammonia production was substantially lower as compared to the corresponding P control slurry. The statistical analysis applied confirms that, in all cases, the amount of ammonia present in the slurries containing SBO at fermentation end is significantly less than the content of the

corresponding control slurry. Particularly remarkable is the negative ΔNH_4^+ % obtained in the D.FW.SBO slurry, demonstrating that at the end of fermentation the ammonia content was 39.5% lower as opposed to the beginning of the experiment. As shown in the fermentation of all other slurries (Table 2), the large consumption of the initial ammonia content observed in the D.FW.SBO experiment is accompanied by production of N_2. However, in the aforementioned trial, a high ΔN_2 value of 284 was obtained, which was only lower to the value observed for D fermentation.

The ammonia data confirm previous findings on the positive effect of SBO in the anaerobic fermentation of biowaste.[4,18–20] Moreover, further knowledge is contributed pertinent to the parameters affecting SBO performance as auxiliary chemical for anaerobic fermentation processes. The data of Figure 3 exhibit that the level of the SBO effect depends clearly on the inoculum. Previous work has been carried out using FW collected and inoculum produced by the Italian Acea plant. Two papers have been published. The first study[4] reports the use of the same SBO as the one employed in the present work, here in after named as CVD SBO, but applied under different experimental conditions. CVD SBO was used at 0.05% and 0.2% (w/v) concentration in the fermentation slurry, which contained 2.5% (w/v) total solids (TS). The second ~~paper[18]~~ paper[20] reports the use of a different type of SBO obtained by alkaline hydrolysis of gardening residues, hereafter referred to as CV SBO. This was used at 0.05% and 0.2% (w/v) concentration in the fermentation slurry, which was supplemented with 1.4% and 4.1% (w/v) TS. Table 3 summarizes the different experimental conditions and the ammonia % change calculated according to the above general equation 1 where N stands for NH_4^+, ~~ammonia % change (ΔNH_4^+ %) calculated according to equation 1,~~ which were obtained both in the present and previous work. The data show the level of the SBO effect on the ammonia content at fermentation end depending on: i) the type and concentration of SBO, ii) the type of FW and inoculum, as well as iii) the concentration of total (TS) and volatile (VS) solids in the fermentation slurry. In 4 cases, the negative ΔNH_4^+ % value calculated demonstrates that the ammonia content at fermentation end was lower as compared to the content at the start of the fermentation. In the rest of the experiments presented, the positive ΔNH_4^+ % value shows that ammonia was produced during the fermentation. In all experiments, the control fermentation performed in the absence of SBO, the ΔNH_4^+ % of which are not included in Table 3, produced more ammonia as opposed to the SBO assisted fermentation.

Table 3. Variation (%) of ammonia nitrogen (ΔNH_4^+ %) in the fermentation of FW performed in the presence of SBO under different experimental conditions.

Reference	Country[a]	Inoculum	FW Source	TS, %[f]	VS, %[f]	SBO	SBO, %[g]	ΔNH_4^+, %
This work	Cyprus	P SBLA[b]	Hotels[d]	3.1	3.03	CVD	0.2	-3.10
This work	Cyprus	D SBLA[b]	Hotels[d]	3.1	3.03	CVD	0.2	-39.5
4	Italy	Acea[c]	MBW[e]	2.5	1.3	CVD	0.2	-5.60
4	Italy	Acea[c]	MBW[e]	2.5	1.3	CVD	0.05	2.32
1820	Italy	Acea[c]	MBW[e]	1.6	0.67	CV	0.2	-0.01
1820	Italy	Acea[c]	MBW[e]	1.6	0.67	CV	0.05	11.7
1820	Italy	Acea[c]	MBW[e]	4.1	1.6	CV	0.2	15.1
1820	Italy	Acea[c]	MBW[e]	4.1	1.6	CV	0.05	27.0

[a]Country were inocula and FW were collected, and experiments were performed. [b]Primary (P) and dewatered (D) sludge obtained from SBLA wastewater treatment plant. [c]Recirculation digestate recovered from Acea plant biogas reactors. [d]Hotels' restaurants in Limassol (CY) area. [e]Humid organic fraction of municipal solid waste from separate source collection in Pinerolo (I) area. [f]Initial ~~Total~~ total (TS) and volatile (VS) solids w/w % content in fermentation slurry~~.~~; final VS content at the end of the biogas fuel exponential phase was about 50 % of the initial Vs content. [g]Added SBO w/w % concentration in fermentation slurry.

Methane Production in Fermentation Trials. Biogas was produced in all fermentation trials. The increase of the biogas cumulative volume upon increase of fermentation time exhibited the same pattern as that reported for Acea fermentations trials[4,18-20] in Table 2. ~~The results showed the typical lag, log and stationary phases corresponding to microbial growth.~~ Figure 4 reports the cumulative methane production pattern for the P.FW.SBO and D.FW.SBO fermentations, which were characterised (Table 2) by the highest SBO effect reducing ammonia below the content measured at fermentation start, as well as for the respective control fermentations. The plot shows the typical lag phase, followed by the log phase associable to microbial growth starting at day 5 with its slope tapering off at day 9 toward the expectable stationary phase. The volume of CH_4

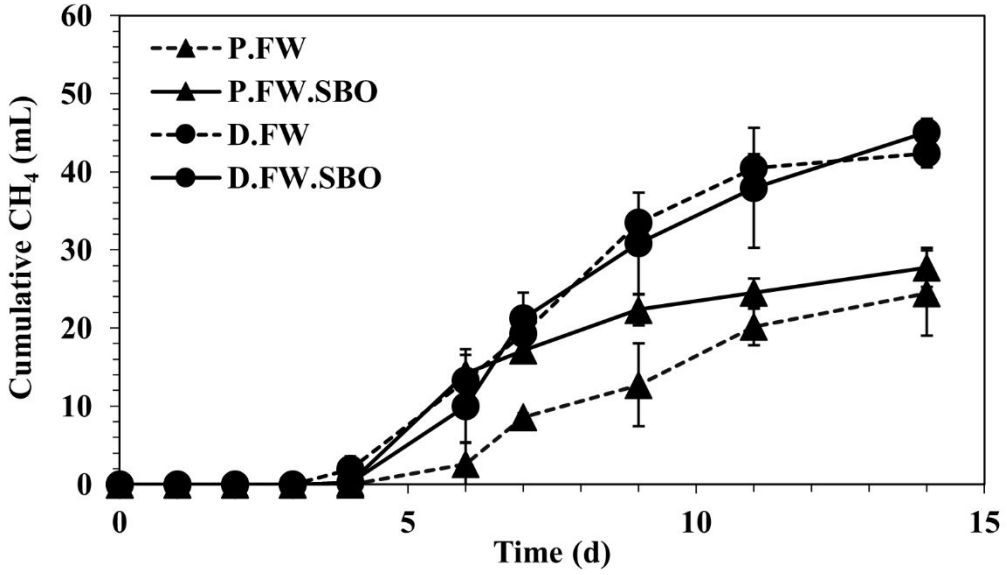


Figure 4. Cumulative methane production in P.FW, P.FW.SBO, D.FW and D.FW.SBO fermentations.

~~The production of CH_4 was initiated following 4 d of cultivation and continued for the remaining part of the experiments. The volume of CH_4~~ formed was higher in fermentations inoculated with dewatered sludge (D.FW and D.FW.SBO) as compared to the production achieved in experiments inoculated with primary sludge (P.FW and P.FW.SBO). The data show the significant effect of the inoculum on the methane production. On the other hand, the data for the fermentations carried out with the same inoculum in the presence and absence of SBO indicate that the presence of SBO does not affect much the methane production, ~~sbo did not exhibit any evident influence on the production of the biofuel as compared to the corresponding control fermentation. The control trials~~

~~conducted using primary (P) and dewatered (D) sludges only did not produce any CH_4,~~ whereas it has been previously stablished that the biogas potential of SBO is negligible.[4]

Microbial Community Dynamics During the Anaerobic Process. The composition of the microbial communities formed in the fermentation slurries investigated was determined at the beginning of each culture, using the microbial inoculums applied, and at fermentation end (Figure 5).

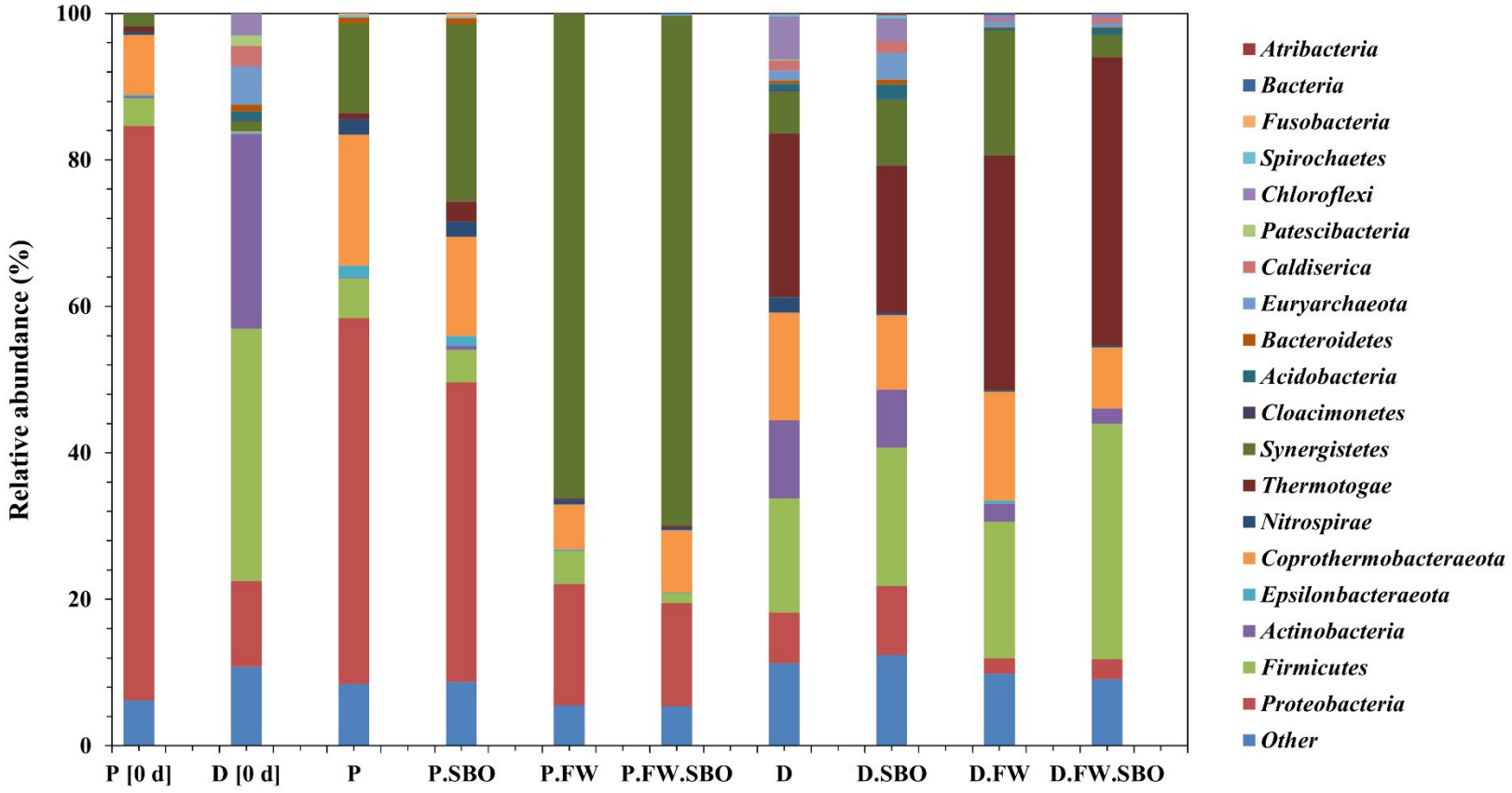


Figure 5. Phylum level relative abundance of bacterial communities present in the experiments. The samples analysed correspond to the inoculums at the beginning of fermentation P (0 d) and D (0 d) as well as the final communities formed following 14 d of cultivation (P, P.SBO, P.FW, P.FW.SBO, D, D.SBO, D.FW and D.FW.SBO). Phylogenetic groups accounting for ≤1% in at least one sample are denoted as other.

The results reported in Table 2 and Figure 2 evidence that the mineralization of organic nitrogen implies two main reactions. These comprise formation of ammonia and the oxidation of ammonia yielding mainly N_2. The issue posed by the findings of microbial analysis was to assess the potential function of the microbial community and that of SBO in the system. Thus, the aim of the present work was to assess whether SBO stimulated a selection of bacteria in the microbial community, which acted as biocatalyst for the oxidation of ammonia, or whether SBO acted as chemical catalyst driving the oxidation of ammonia without participation of the microbial community.

The fate of NH_4^+ in anaerobic bioprocesses is defined by anammox bacteria directing oxidation of the pollutant into N_2 gas through use of NO_2^- as electron acceptor under anoxic conditions.[~~19~~ 21] Bacteria performing the anammox process comprise genera that belong to the *Planctomycetes* phylum.[~~20~~–22] Anammox bacteria incorporate slow growth rate, low cell yield and can be significantly affected by environmental conditions.[~~21~~23,~~22~~–24] They need prolonged cultivation to achieve elevated relevant abundance with high activity.[~~23~~25,~~24~~–26] The results show that anammox bacteria were not detected in the microbial community analysis performed (Figure 5). These exclude anammox bacteria acclimatization in the cultures formed during the 14 d of cultivation, which could potentially drive NH_4^+ oxidation into N_2 gas.

Coprothermobacter comprise gram negative anaerobic thermophilic ~~bacteria~~[~~25~~]bacteria[27,~~26~~–28] associated with protein degradation into organic acids, NH_3 and CO_2,[~~27~~–29] but also with the degree of protein solubilization.[~~25~~27,~~26~~–28] Members of this genus were detected at high abundance in both P and D inoculums at the end of fermentation trials. At the beginning of the experiments employing P sludge *Proteobacteria* was the dominant phylum, while *Coprothermobacter, Firmicutes* and *Synergistetes* were also included in significant contents. The cultures conducted using D sludge included at fermentation start *Firmicutes, Actinobacteria, Proteobacteria* and *Euryarchaeota* as dominant phyla. Comparing the content of the most relevant bacteria in all reactors as opposed to the corresponding control did not exhibit significant variations between SBO-assisted and the corresponding control fermentations. *Proteobacteria* (particularly the *Gammaproteobacteria* class) are considered as active protein degraders holding multiple activities in anaerobic fermentation systems.[~~28~~–30] These strains can be often detected in waste treatment processes (e.g. activated sludge reactors) converting NO_3^- to N_2, through the metabolism of organic acids, carbohydrates and protein soluble fractions of FW.[~~29~~31,~~30~~–32] Evaluating the microbial composition

of both inoculums (D and P) confirmed the presence of *Proteobacteria*. However, no meaningful relationship can be established by plotting the relative abundance values of each of the bacteria depicted on Figure 5 and the ΔNH_4^+ or ΔN-org % values at fermentation end reported in Table 2.

The Function of SBO in Fermentation Trials. One positive result established in the present work is the confirmation that the effect of SBO also occurs in other experimental conditions as compared to those adopted in previous work (Table 2). The specific study validates indirectly the previous work conducted and supports the replicability of the SBO effect over different experimental conditions, which constitutes an essential requirement for the scale up of SBO assisted fermentations to commercial level.

An additional positive aspect of the present work is the confirmation that the decrease of ammonia in the fermentation occurs by eco-friendly oxidation to N_2, incorporating no or negligible production of nitrogen oxides. This alleviates any concerns that although the SBO assisted fermentation could reduce the environmental problem of ammonia formation, its application could cause an additional tertiary pollution problem in the biowaste production-collection-fermentation-product use/disposal chain arising from the formation of GHG. The absence of potential pollution generated by GHG formation caused through use of SBO is in agreement with Biagini et al.[7] findings. The specific study reported the use and effects of SBO as animal diet supplement. It demonstrated that the manure produced from rabbits fed with a standard protein diet containing 0.25 % of added SBO emits significantly less ammonia and GHG gases as opposed to manure produced by control animals fed with the same standard diet without addition of SBO. Moreover, these authors have also estimated that the implementation of the use of SBO to control anaerobic fermentation in animals' intestine, as well as the anaerobic fermentation of animal manure in closed bioreactors, is worth global yearly reductions at European level of 1.1 Mt NH_3 and approximately 1 Gt GHG.

The impact of the present work is further enhanced by the provision of further insight into the understanding of the role of SBO in fermentation. Analysis of the microbial composition in the absence and presence of SBO (Figure 5) does not provide evidence for the presence of a biochemical reaction that involves changes in the abundance of bacteria correlated to changes in the content of organic and inorganic N species observed in the SBO assisted fermentation, as compared to control trials. Under this circumstance an alternative explanation, that could be used to understand the action of SBO, relies in the chemical interaction of the product with the organic

matter degraded and/or with the microorganisms incorporated, which could potentially change the course of organic N mineralisation. Thus, although the fermentation conducted in the absence of SBO is driven by biochemical reactions associated with the metabolism of the bacterial population listed in Figure 5, the specific mineralisation of organic N in the presence of SBO could mainly comprise a chemical reaction ~~which~~ that does not depend on microbial bioreactions and/or effects resulting from alterations in the bacterial metabolism that occur due to the interplay of SBO with the microorganism.

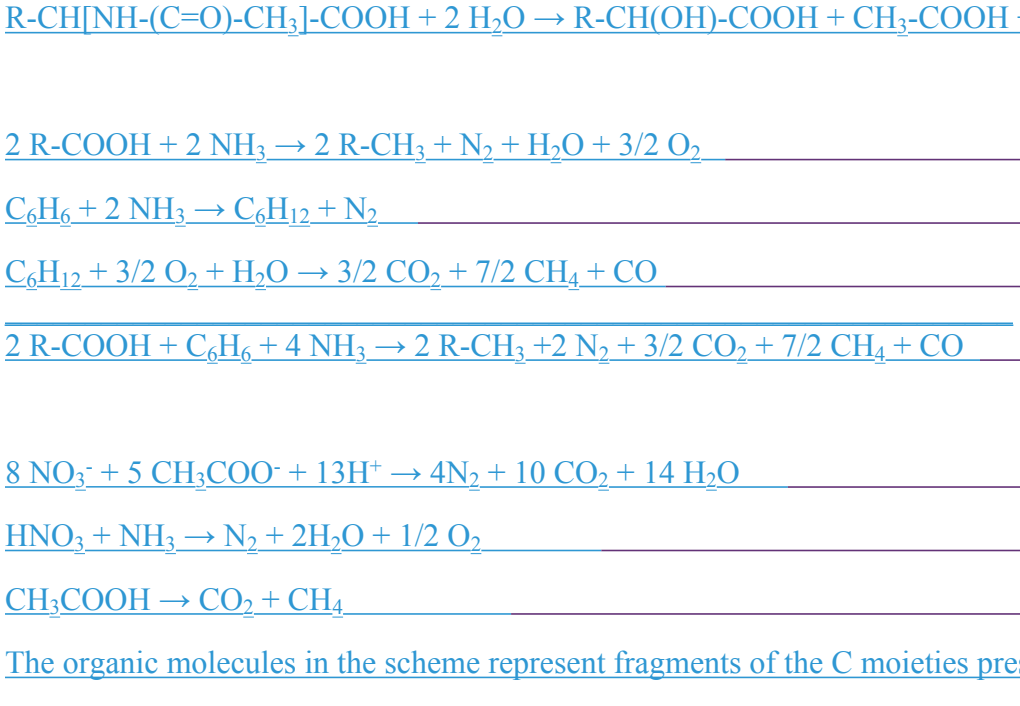
The SBO used in the present work constitutes a mixture of substances with 5 to over 750 kDa molecular weight containing several acid and basic functional groups bonded to Si, Fe, Al, Mg, Ca, K. These chemical features are inherited from the pristine lignocellulosic composted matter from which SBO were obtained. The SBO incorporate multiple properties. Thanks to their water solubility, ionic and complexing functional groups of variable strength, and molecular conformation,[6] they bond organic molecules and mineral elements, and allow their controlled release in aqueous media. This property has been shown to incorporate useful applications for the manufacture of controlled release ~~fertilisers~~[31]fertilisers,[33]; ~~detergents~~[32] detergents[34] and dyeing ~~baths~~[33] baths[35] formulates. Particularly interesting in the context of the present work is a ~~paper~~[34] paper[36] reporting the property of SBO to catalyse oxidation reactions in water, in the absence of any microorganism, through a photo-Fenton process. This is possible thanks to SBO Fe ion content, as well as the water solubility and solution conformation of the product, which keeps the Fe ions in solution under circumneutral or alkaline pH conditions.

On the other hand previous ~~work~~[35]work,[37]; which investigated the effect of SBO in the pig's cecal fermentation of a protein diet, has shown that the SBO causing the highest 17% decrease in ammonia production, relatively to the control fermentation conducted in absence of SBO, additionally resulted in the largest decreases of C4 (11-18%) and C5 (25-31%) carboxylic acids, and more evidently of C4i (15-23%) and C5i (32-36%) isoacids' production. The lower C4i and C5i to C4 and C5 ratio, coupled to the decreased ammonia production achieved, indicates lower proteolitic activity of the bacterial population in the fermentation slurry. Similar effects have been reported with pediocin A, a protein bacteriocin known to modulate the intestinal microflora metabolism in pigs by clostridia inhibition.[36][38]

The collected experimental data (Figures 2-4), suggest that the mineralisation of organic N occurred in two steps. These first involve the hydrolysis of protein matter to amino acids and

deamination of amino acids to produce carboxylic acids and ammonia. The second step is the oxidation of ammonia. The present data (Figures 2-4), coupled to the findings reported for the effects of SBO in the fermentation occurring in the animal intestine,[35-37] suggest that SBO could potentially participate in both steps. The first step is most likely catalysed by the microflora listed in Figure 5, as several microorganisms holding proteolytic activity were present in all slurries. Although the abundance of proteolytic bacteria in Figure 5 is not correlated with ΔN-org % values in Table 1, the participation of proteolytic species cannot be excluded. The second step proceeds most likely due to the activity of SBO as chemical catalysts, since anammox bacteria were not detected in fermentation slurries. Based on the capacity of SBO to promote photo-Fenton oxidation mechanism,[34-36] the activity of SBO as chemical catalyst could be due to the Fe ions bonded to the organic matter. Other workers[37-9] report that: i) several mineral elements can reduce nitrate ions to NH_3, N_2O and N_2, ii) the effect of these elements is higher if they are present in soluble form in the system, and iii) under proper experimental conditions in the presence of Fe, nitrate ions are completely reduced to nitrogen gas, with no formation of NH_3, N_2O or NO_2. The mineral elements reported by these authors comprise the same molecules included in the chemical composition of SBO. The above mineral elements, which are bonded to the soluble SBO organic matter, are present in soluble form in the investigated fermentation slurries containing SBO.

The following plausible reactions could ~~may~~ occur during the fermentation:



$$\text{R-CH[NH-(C=O)-CH}_3\text{]-COOH} + 2\ H_2O \rightarrow \text{R-CH(OH)-COOH} + \text{CH}_3\text{-COOH} + NH_3 \quad (1),$$

$$2\ \text{R-COOH} + 2\ NH_3 \rightarrow 2\ \text{R-CH}_3 + N_2 + H_2O + 3/2\ O_2 \quad (2)$$

$$C_6H_6 + 2\ NH_3 \rightarrow C_6H_{12} + N_2 \quad (3)$$

$$C_6H_{12} + 3/2\ O_2 + H_2O \rightarrow 3/2\ CO_2 + 7/2\ CH_4 + CO \quad (4)$$

$$2\ \text{R-COOH} + C_6H_6 + 4\ NH_3 \rightarrow 2\ \text{R-CH}_3 + 2\ N_2 + 3/2\ CO_2 + 7/2\ CH_4 + CO \quad (2\text{-}4)$$

$$8\ NO_3^- + 5\ CH_3COO^- + 13H^+ \rightarrow 4N_2 + 10\ CO_2 + 14\ H_2O \quad (5)$$

$$HNO_3 + NH_3 \rightarrow N_2 + 2H_2O + 1/2\ O_2 \quad (6)$$

$$CH_3COOH \rightarrow CO_2 + CH_4 \quad (7)$$

The organic molecules in the scheme represent fragments of the C moieties present in

lignocellulosic proximates composing FW. Reaction 1 accounts for the mineralisation of organic N to ammonia N. The global reaction (2-4), resulting from the sum of reactions 2, 3 and 4, describes the fate of ammonia formed in reaction 1. Reactions 5 and 6 account for the consumption of nitrates confirmed by the data of Table 1. Reaction 7 accounts for the formation of the main biogas components. Other more complex redox pathways certainly operate, which include the participation of the mineral elements, particularly the Fe^{3+}/Fe^{2+} couple. These ions are bonded to SBO and their half reaction

$$(SBO)Fe^{3+} + e^- \leftrightharpoons (SBO)Fe^{2+} \quad (8)$$

is reversibly shifted forward and backword during the process. In this fashion the ions' couple present in the system increases the rates of reaction (2) through (6) without getting consumed in the process.

The Gibbs free energy, calculated according to the following references, is - 123 kJ/N_2 mole for the global reaction (2-4),[40] -1132 kJ/N_2 mole for reaction (5),[41] -360 kJ-/N_2 mole for reaction (6),[42] - 213 kJ/C mole,[42] –143 kJ/N mole for reaction (1).[42] ~~The following plausible reactions could explain the oxidation of NH_3 to N_2 in the present work:~~

~~$2\ R\text{-}COOH + 2\ NH_3 \rightarrow 2\ R\text{-}CH_3 + N_2 + H_2O + 3/2\ O_2$ (1)~~

~~$C_6H_6 + 2\ NH_3 \rightarrow C_6H_{12} + N_2$ (2)~~

~~$C_6H_{12} + 3/2\ O_2 + H_2O \rightarrow 3/2\ CO_2 + 7/2\ CH_4 + CO$ (3)~~

~~$2\ R\text{-}COOH + C_6H_6 + 4\ NH_3 \rightarrow 2\ R\text{-}CH_3 + 2\ N_2 + 3/2\ CO_2 + 7/2\ CH_4 + CO$ (1-3)~~

~~$8\ NO_3^- + 5\ CH_3COO^- + 13H^+ \rightarrow 4N_2 + 10\ CO_2 + 14\ H_2O$ (4)~~

~~$HNO_3 + NH_3 \rightarrow N_2 + 2H_2O + 1/2\ O_2$ (5)~~

~~The organic molecules in the scheme represent fragments of the C moieties present in lignocellulosic proximates composing FW. Reaction (1-3) comprises the global reaction of partial reactions (1), (2) and (3). Reactions (4) and (5) account for the consumption of nitrates confirmed by the data of Table 1. Other more complex redox pathways certainly operate, which include the participation of the mineral elements, particularly the Fe^{3+}/Fe^{2+} couple. These ions are bonded to SBO and their half reaction~~

~~$(SBO)Fe^{3+} + e^- \leftrightharpoons (SBO)Fe^{2+}$ (6)~~

~~is reversibly shifted forward and backword during the process. In this fashion the ions' couple present in the system increases the rates of reaction (1) through (5) without getting consumed in the process.~~

~~The Gibbs free energy per mole of N_2, calculated according to the following references, is - 123 kJ for the global reaction (1-3),[38] -1132 kJ for reaction (4),[39] -360,25 kJ for reaction (5).[40]~~

The oxidation of ammonia to nitrogen gas is favoured in all aforementioned reactions. Indeed, all fermentations produce N_2. Figure 2 shows that in most cases, N_2 production is inversely related to NH_4 production, apart from the data of D and P.FW.SBO fermentations. The values obtained, which nicely fit the linear regression equation (2), show that the slurries containing SBO produce more N_2 as compared to other experiments conducted without SBO, although the former trials contain less ammonia. The D.FW.SBO fermentation produced more N_2 coupled to the highest consumption of ammonia. This experiment lowers the ammonia in the digestate at fermentation end largely below the amount present at the start of the trial. Whereas these findings support the role of SBO as chemical catalyst, the deviation of the data for D and P.FW.SBO fermentations from the linear regression equation (2) could be caused by a different mechanism involving the interaction of SBO with microorganisms present in the D and P.FW.SBO slurries.

The above reactions 1-7 comprise~~are~~ only examples~~. They~~ indicating~~e~~ that all reactions are thermodynamically favored and each one contributes to the energy reduction of the whole fermentation process. The energy reduction due to the mineralisation of organic C, 213 kJ/C mole for reaction (7), is ~~more than that for~~higher as compared to the mineralisation of organic N, 143 kJ/N mole for reaction (1). The highest specific contribution comes from the oxidation of organic C by nitrate N (reactions 5). The data in Figure 4 and Table 1 show that the total consumption of organic C in the slurry phase yielding methane at day 14 is 23 mg for D.FW, 24 mg D.FW.SBO, 13 mg for P.FW and 15 mg for P.FW.SBO, against a total consumption of organic N of 214 mg for D.FW, 112 mg for D.FW.SBO, 69.2 mg for P.FW and 61.3 mg for P.FW.SBO. The higher consumption of organic N, compared to the methane C production, suggests that most of carboxylic acids and hydroxycarboxylic acids formed in reaction 1 do not undergo further degradation to yield CH_4 and accumulate in the digestate slurry phase. This implies that in the first 14 days of the fermentation the reactions (1) through (6) leading to the formation and oxidation of inorganic N occur before reaction (7) or others yielding methane. It is the likely results of the exemplified reactions (1-6) involving organic and inorganic N being thermodynamically more

favoured and/or faster than the exemplified reaction (7) or others yielding biogas from carboxylic and hydroxyl-carboxylic acids formed in reaction (1). Under these circumstances, the first 14 days of the fermentation trials represent the phase where most of the reactions involving N species occur, while the exponential phase of the fermentation comprising the main reactions yielding the methane biofuel has not yet started.

Based on the above findings, the microbiological analysis of the present experimental work was focused on bacteria existing in the microbial community which can act as biocatalysts holding the capacity to metabolize nitrates and ammonia. The analysis was performed employing a primer set used for bacteria detection, since the main objective of the work was to assess the biological processes occurring in SBO assisted fermentations by proteolytic bacteria, as opposed to the establishment of correlations with CH_4 and CO_2 production. Indeed, the results of the microbiological analysis reported in Figure 5 and the low relative abundance of the methanogenic *Euryarchaeota* bacteria detected, compared to the most abundant bacteria, is consistent with the interpretation given above based on Figure 4 and Table 1 data.~~given thatsignificantly~~

~~The~~~~Although~~The analysis of the energy reductions for the above reaction examples may sound as a speculation that well defines a hypothesis,~~.~~ since ~~T~~the experimental data obtained are not enough to corroborate definitely the hypothesis yet. In~~A~~~~t~~ this regard it should be acknowledged that, due to the chemical complexity of the investigated system, it is likely impossible to assess experimentally ~~experimentally~~ which specific molecules participate in the fermentation process ~~experimentally~~. Nevertheless, the acquired data ~~acquired~~based on the chemical analysis of the different N containing species and N mass balance at the start and end of ~~the~~ fermentation are consistent with the free energy reduction values of the above hypothetical reactions (1) through (7). Specifically, the above thermodynamic analysis supports the experimental confirmation of the main objective of the present work to demonstrate the eco-friendly fate of the diminished ammonia N content in the fermentation carried out in the presence of SBO.

The experimental data in Tables 1-3 and Figures 2-5, as well as the information acquired from the above cited literature suggest that combined chemical and biological processes occur in the present work. However, the results obtained do not allow assessing the individual levels of contribution of the two type of processes tested. Most likely, synergy occurs between chemical and biochemical reactions. In such complex systems, which involve mixes of microorganisms and heterogeneous chemical compounds, understanding of the underlying mechanisms and

interactions between the multitude of living organisms and chemical substances incorporated is not easy.

■ CONCLUSIONS

The results of the present work confirm that the SBO assisted fermentation of FW is undoubtedly ecofriendly, since decrease of NH_4^+ and NO_3^- occurs in the condensed phase, while N_2 is produced in the gas phase, and no or negligible formation of gaseous nitrogen oxides is detected. Also, the study demonstrates that the effects of SBO are well reproducible for the SBO assisted fermentation carried out both employing inocula and FW ~~collected~~ produced in Cyprus ~~and fermented under different operational conditions~~ as well as using the inoculum and ~~the~~ MBW ~~processed~~ produced and processed in the Italian Acea plant. The reproducibility of the SBO effects on the ammonia production and fate has been attained in spite of the fact that, due to the different inocula and FW used in the present work, the methane production has resulted delayed compared to that in the Acea plant. ~~Finally~~Nonetheless~~,~~ the present work ~~has allowed assessing~~contributes new important knowledge, i.e. that that both biochemical and chemical ~~catalyses~~ catalysis operate in the SBO assisted fermentation.

Whereas further research could certainly lead to enhanced understanding of the prevailing mechanisms involved in the biological and chemical processes that take place, the confirmation provided that the effects of the SBO assisted anaerobic fermentation are undoubtedly ecofriendly and reproducible under different operational conditions is certainly an argument favoring implementation of the technology to commercial level. Realization of this perspective at full level could provide major environmental and social benefits worldwide stemming from the decrease of ammonia and GHG emissions, which accounts for over 1 Gt yr^{-1} reduction in Europe alone.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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■ ABBREVIATIONS

CV, composted green wastes; CVD, composted mix of green and food wastes; FW, urban food wastes; MBW, municipal biowaste; P, primary sludge; P.SBO, primary sludge with added SBO; P.FW, primary sludge with FW; P.FW.SBO, primary sludge with FW and added SBO; D, dewatered sludge; D.SBO, dewatered sludge with added SBO; D.FW, dewatered sludge with FW; D.FW.SBO, dewatered sludge with FW and added SBO; SBO, soluble bioorganic substances.

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