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Yadira Bajón Fernández, Ana Jacinta Soares, Peter Vale, Konrad Koch, Anne Laure Masse, Elise Cartmell

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Bajón Fernández, Yadira[a*], Soares, Ana[a], Vale, Peter[b], Koch, Konrad[c], Masse, Anne Laure[d] and Cartmell, Elise[a, e]

[a] Cranfield Water Science Institute, School of Water, Energy and Environment, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK

[b] Severn Trent Water, 2 St John's Street, Coventry, CV1 2LZ, UK

[c] Chair of Urban Water Systems Engineering, Technical University of Munich, Am Coulombwall 3, 85748 Garching, Germany

[d] Ecole Nationale Supérieure de Chimie de Rennes, 11 Allée de Beaulieu, 35708 Rennes Cedex 7, France

[e] Current address: Scottish Water, Juniper House, Heriot Watt Research Park, Edinburgh, EH14 4AP, UK

*Corresponding author; Tel. +44(0)7407390497; y.bajonfernandez@cranfield.ac.uk

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Abstract

Carbon dioxide (CO_2) enrichment of anaerobic digesters (ADs) without hydrogen addition has been demonstrated to provide a potential solution to manage CO_2 streams generated in the water and organic waste sectors, with concomitant increases in methane (CH_4)

production. This study investigates the CO_2 utilisation mechanisms, by considering chemical and biological pathways in food waste and sewage sludge ADs. *Methanosaetaceae* was observed to be the dominant methanogen in sewage sludge ADs (Abundance of 83.8 – 98.8%) but scarce in food waste units (3.5 – 5.8%). *Methanosarcinaceae* was dominant in food waste (14.3 – 32.4%), likely due to a higher tolerance to the free ammonia nitrogen concentration recorded (885 $mg \cdot L^{-1}$). ~~Ratios of~~ $R_{Methanosaetaceae}$ (ratio of *Methanosaetaceae* fluorescence signal between test and control) of 1.45 and 1.79 were observed for sludge ADs enriched once and periodically with CO_2, respectively (p-value <0.05), suggesting a higher *Methanosaetaceae* activity associated with CO_2 enrichment. Reduction of CO_2 by homoacetogenesis followed by acetoclastic methanogenesis was proposed as a CO_2 utilisation mechanism, which requires validation by radiolabelling or carbon isotope analysis.

Keywords

Anaerobic digestion, CO_2 valorisation, food waste, mechanisms, sewage sludge.

Highlights

- CO_2 utilisation mechanisms were investigated in food waste and sewage sludge ADs
- *Methanosaetaceae* was found dominant in sewage sludge ADs (83.8 – 98.8%)
- *Methanosarcinaceae* was dominant in food waste ADs (14.3 – 32.4%)
- $R_{Methanosaetaceae}$ was 1.45 and 1.79 in sludge ADs enriched once and periodically with CO_2
- CO_2 enrichment has been suggested to boost Acetoclastic methanogenesis

1. Introduction

Anaerobic digestion is a biological process that stabilizes organic wastes while generating biogas with a 50-75% methane (CH_4) and a 50-25% carbon dioxide (CO_2) content. Biogas has traditionally been utilised by combusting it in combined heat and power (CHP) engines or more recently by upgrading it to biomethane for this to be incorporated into the gas grid.

In both cases, these processes aim for the CH_4 calorific value to be utilised, without offering a route for utilisation of produced CO_2. Biogenic CO_2 emitted with biogas in anaerobic digesters (ADs) has been estimated at over 0.27 megatons CO_2 ($MtCO_2$) per annum for the UK water sector [1] and at 0.75 $MtCO_2$ per annum for the UK organic waste sector [2], suggesting that valorisation of this stream can further reduce the process carbon footprint and significantly contribute towards energy supply decarbonisation [1]. Bioconversion of CO_2 to CH_4 in anaerobic processes has been identified as one of the most economically feasible options for on-site carbon management [1], with previous investigations considering CO_2 enrichment of ADs with and without supplementation of H_2 as a reducing agent. Injection of CO_2 into ADs for its bioconversion to CH_4 without addition of H_2 has been proved to increase CH_4 production and reduce process carbon footprint for different anaerobic systems (upflow anaerobic sludge blanket (UASB) reactors, two phase ADs, single phase AD) and for different feedstocks (wastewater, food waste, sewage sludge, synthetic solutions, etc.) [3–10]. Reported increases in CH_4 production after CO_2 enrichment range between 13% to over 100% for different studies [2].

Although the capacity of anaerobic processes to utilise exogenous CO_2 without addition of H_2 has been demonstrated, previous studies have focused on quantifying the associated increase in CH_4 production, without an understanding of the mechanism by which the additional CO_2 is utilised. Uptake of CO_2 has been reported as an overall term involving the contribution of all the reactions in which it may be consumed or produced [4–6,9], which enables a comparison between units with and without CO_2 enrichment, but fails to elucidate the mechanism behind CO_2 utilisation. Besides, the scarce literature available regarding CO_2 utilisation routes (without H_2 supplementation) presents conflicting information in relation to its boost of the hydrogenotrophic [4,11] or

acetoclastic [5,6,9,10] pathways for CH_4 formation and is based on hypotheses rather than on experimental evidence. Hence, a better understanding of the fate of additional CO_2 injected in ADs is required to optimise the process and verify that CO_2 is being biconverted rather than captured as instable forms with the risk of re-release.

The present study aims to provide insights into the pathways of utilisation of CO_2 injected into ADs, considering both biological and chemical utilisation routes and proposing a hypothesis for CO_2 fate when additional H_2 is not supplemented. Tests were carried out in sewage sludge and food waste units, both of which were selected because of their anticipated differences in CH_4 formation pathways due to their different free ammonia nitrogen (FAN) concentrations. The FAN levels in food waste units allowed testing the impact of CO_2 injection when obligate acetoclastic activity was considered inhibited. ~~It is important to clarify that this investigation aimed to provide initial insights into the mechanism of CO_2 utilisation, and that the experimental set-up was not designed to further quantify benefits in CH_4 production as a consequence of CO_2 enrichment (as headspace was periodically diluted with CO_2). Benefits in biogas production associated with CO_2 enrichment were previously investigated by several research groups for different anaerobic processes and a review of previous work is available [2].~~ The impact of CO_2 enrichment on analytical parameters of the final digestate, its potential to form carbonated precipitates and its impact in methanogenic *Archaea* were investigated. Firstly, digestates of food waste and sewage sludge ADs with and without CO_2 enrichment were compared in terms of pH, alkalinity and FAN concentration, among other parameters. Secondly, fluorescence in situ hybridisation (FISH) was used to detect differences in methanogenic microbial populations (*Methanosaetaceae*, *Methanobacteriaceae* and *Methanosarcinaceae*) when CO_2 was added into the system. Thirdly, environmental scanning electron microscope (ESEM) and X-ray diffraction (XRD) were applied to detect

the potential of CO_2 enrichment to form or dissolve carbonated precipitates in food waste ADs. This investigation aimed to provide initial insights into the mechanism of CO_2 utilisation. The experimental set-up was not designed to further quantify benefits in CH_4 production as a consequence of CO_2 enrichment (as headspace was periodically diluted with CO_2). Benefits in biogas production associated with CO_2 enrichment have been previously investigated by several research groups for different anaerobic processes and a review of previous work is available [2].

2. Materials and methods

2.1 *Description and operation of anaerobic digesters*

Nine batch AD units were operated for 13 days for each of the two substrates treated: food waste and sewage sludge. Three operating conditions were tested in triplicate: control units, units enriched with CO_2 at the start of the digestion process only and units enriched with CO_2 periodically. Each AD unit consisted of a 1 L bottle with a four port cap (Fisher Scientific, Loughborough, UK). Two ports were used for gas injection (CO_2 or nitrogen (N_2)) through Pyrex diffusers with a porosity of 3 and 15 mm diameter (Fisher Scientific, Loughborough, UK). One port was coupled to a 'Y' shaped tubing connector from which one exit was connected to a MilliGascounter (Litre Meter Ltd., Buckinghamshire, UK) for biogas volume recording and the other was fitted with a septa for gas sampling. The last port was used for digestate sampling. The ADs were continuously stirred and maintained at a mesophilic temperature (38 ± 0.5°C).

A working volume of 700 mL and an inoculum to substrate volatile solids (VS) ratio of 2:1 were used in the batch ADs. The materials to operate food waste units (macerated food waste as feedstock and digested food waste as inoculum) were collected from a UK AD site treating 30,000 tonnes of organic waste per year. The materials for sewage sludge ADs (mixture of 60/40 % v/v primary/thickened waste activated sludge as

feedstock and digested sewage sludge as inoculum) were from a UK wastewater treatment plant serving a 2.5 million population equivalent.

Gas with a CO_2 molar fraction (y_{CO2}) of 0.9 (partial pressure (p_{CO2}) of 1.3 ± 0.2 bar) was bubbled through the test ADs (both enriched once or periodically with CO_2) for 20 minutes at the start of the digestion process. The desired CO_2 molar fraction was obtained by mixing CO_2 and N_2. The control ADs were bubbled with N_2 for 5 minutes. These gas injection times were based on previously reported results [12]. The ADs operated with the periodic CO_2 injection regime were enriched once every 48 hours for 5 minutes with gas at p_{CO2} 1.3 ± 0.2 bar. Both gases were supplied from gas cylinders (BOC, Manchester, UK) and their flowrate (1.0 $L \cdot min^{-1}$ combined flowrate) was controlled by mass flow controllers (Premier Control Technologies, Norfolk, UK).

2.2 *Fluorescence in situ hybridisation (FISH)*

Food waste and sewage sludge samples were fixed for FISH analysis immediately after collection from the batch ADs at commencement and after 13 days of operation. Sample aliquots (2 mL) were centrifuged at 4000 *g* for 10 minutes and the pellet re-suspended in 750 µL of phosphate buffer solution (PBS), treated with 250 µL of 4% paraformaldehyde solution (PFA) and incubated overnight at 4°C. The incubated sample was centrifuged under the same conditions and re-suspended in PBS twice to remove any residual PFA. Absolute ethanol was added to the final sample, which was then kept at -20°C until further analysis. On defrosting, 5 µL of fixed sample were placed on gelatin coated well slides, incubated at 46°C for 10 minutes and dehydrated for 3 minutes in each 50, 80 and 100% ethanol solutions. Each sample was loaded in 5 different wells.

Hybridization was carried out at 46°C for 2 hours with 5 µL of previously preheated hybridization buffer and 1 µL of each oligonucleotide probe. Hybridized samples were then rinsed and incubated in preheated (48°C) washing buffer for 20

minutes. Slides were finally rinsed with distilled water and air-dried in darkness. Details of the 16S rRNA-targeted oligonucleotide probes (Sigma-Aldrich, Madrid, Spain) used are summarised in Table 1. The following microorganisms were targeted: *Bacteria* with Eub338-Mix probe (Eub338, Eub338-II and Eub338-III in equimolar proportion), *Archaea* with Arch915 probe, *Methanosaetaceae* with Mx825 probe and *Methanobacteriaceae* with Mbac1174 probe. In food waste samples *Methanosarcinaceae* was targeted with MS1414 probe. The helper oligonucleotides hMS1395 and hMS1480 were used to improve the accessibility of the *Methanosarcinaceae* targeting probe to its binding site, as previously suggested by Crocetti et. al. [13]. These helpers were added during hybridization in equimolar proportion to the MS1414 probe. Non Eub338 probe was used as negative control. A poor cell wall permeability of the oligonucleotide probes was observed in food waste samples, which was addressed by adding freeze-thaw cycles consisting of 5 minute steps at -80°C and 60°C to the experimental protocol [14,15].

Images for FISH were acquired with a confocal laser scanning microscope (Nikon CS-1). Six images per well were captured at random positions, leading to 30 images per sample. In food waste samples there was a vast spatial distribution of the targeted cells in the z-axis. In order to obtain representative images, z-stack images were generated by compiling fields collected at every 0.5μm of the z-axis. A magnification of X600 was used in every case. The fluorescence of the images was enhanced with EZ-C1 software (Nikon, Melville, New York) and signal quantification was completed with Daime software version 2.0 (Vienna, Austria). The relative abundance of methanogenic *Archaea* was calculated by setting *Methanosaetaceae* (Mx825), *Methanobacteriaceae* (Mbac1174) or *Methanosarcinaceae* (MS1414) as specific probes in relation to the general *Archaea* probe (Arch915). This is further referred to as Abundance (%) and was used to compare relative presence of microbial populations within the same sample. Changes in *Archaea* microbial

diversity (*Methanosaetaceae*, *Methanobacteriaceae* and *Methanosarcinaceae)* between CO_2 augmented batch tests and controls at the end of the batch process were compared by the R_M ratio. The R_M ratio was calculated by dividing the fluorescence signal (i.e., pixel count) for each specific *Archaea* detected in the digestate of batch tests augmented with CO_2 by the fluorescence signal (i.e., pixel count) for each specific *Archaea* detected in the digestate of control experiments. The R_M ratio was used because the batch nature of the ADs operated in this study resulted in a general reduction of cells' activity over time, making R_M more suitable than Abundance to understand microbial differences between reactors.

2.3 *Ammonium or calcium carbonated precipitates detection*

The potential formation of carbonated precipitates in ADs enriched with CO_2 was examined by utilising XRD and ESEM in food waste digestate samples. Samples for these tests were collected from a food waste pilot-scale AD of 106 L working volume operating with total ammonia concentration (TAN) of 1798 ± 124 $mg \cdot L^{-1}$ and FAN concentration of 147 $mg \cdot L^{-1}$. The performance of this system was separately discussed in [16].

Samples of unaltered digestate and of digestate saturated with CO_2 (p_{CO2} 1.3 ± 0.2 bar) were frozen at -80°C and freeze-dried in an Alpha 1-2 LD freeze dryer (Martin Christ, Osterode am Harz, Germany) in order to condition the samples for XRD tests. Freeze drying was preferred over a heating process because of the thermal instability of ammonium carbonated species [17]. In order to reduce the noise of the XRD spectra, part of the digestate was centrifuged at 4000 *g* for 20 minutes, the supernatant centrifuged again for 40 minutes and the new supernatant separated for analysis. Both unaltered supernatant and supernatant enriched with CO_2 (p_{CO2} 1.3 ± 0.2 bar) were freeze-dried for XRD tests as described above.

The diffraction spectra of the samples was obtained by XRD using a D5005 unit (Bruker, Coventry, UK) with a 2-theta range of 10-90°, a step size of 0.04° and step time of one second. The spectrum of samples with and without CO_2 enrichment were compared for potential differences in calcium carbonate ($CaCO_3$), ammonium carbonate ($(NH_4)_2CO_3$), ammonium bicarbonate (NH_4HCO_3) and ammonium carbamate (NH_2COONH_4). Three sites per dried sample were imaged with a XL30 ESEM (FEI, Oregon, USA) in order to obtain the weight percentage of present elements.

2.4 ***Analytical methods***

Ammonia, soluble chemical oxygen demand (sCOD), volatile fatty acids (VFA), alkalinity, total solids (TS) and VS [18] were analysed at the beginning and end of the digestion process. Ammonia, sCOD and VFA tests were completed in the solid free fraction of the samples. This was obtained by filtering through 0.45 µm pore size syringe-drive filters (Millipore, Billerica, United States). TAN and sCOD were quantified with Spectroquant test kits (VWR, Lutterworth, UK). Quantification of VFA was completed by high performance liquid chromatography (HPLC) performed in a Shimadzu VP Series unit (Milton Keynes, UK). The concentration of acetic acid, propionic acid, n-butyric acid, iso-butyric acid, n-valeric acid and iso-valeric acid were quantified and their sum reported as total VFA (TVFA) concentration. Alkalinity was measured in the supernatant obtained after a double centrifugation process: samples centrifuged at 4700 *g* for 20 minutes and the supernatant centrifuged again for 40 minutes under the same conditions. It was expressed as mg $CaCO_3 \cdot L^{-1}$ and evaluated as partial alkalinity (PA) by titration to pH 5.75 [19] and as total alkalinity (TA) by titration to pH 4.3 [18]. The volume of biogas produced in the ADs and its composition (CH_4 and CO_2) were monitored daily with MilliGascounters (Littre Meter Ltd., Buckinghamshire, UK) and a CSi 200 Series Gas Chromatograph (Cambridge Scientific Instruments Ltd., Witchford, UK), respectively.

3. Results and Discussion

3.1 *Biogas production and digestate characterisation*

A CH_4 yield of 92 ± 16 mL·g VS^{-1}, 94 ± 2 mL·g VS^{-1} and 101 ± 9 mL·g VS^{-1} was recorded for sewage sludge ADs acting as control, enriched once and periodically with CO_2, respectively. All the sewage sludge test ADs achieved a 2.1-2.4 fold increase in daily CH_4 production in the 24 hours following the first CO_2 enrichment when compared to control units, which replicates the 2.4 fold increase obtained in a previous study [6] when using a similar p_{CO2} for enrichment (*ca.* 1.3 bars). In ADs enriched periodically with CO_2, a second injection boosted the daily CH_4 production by 1.3-1.5. A lower increase was observed for subsequent CO_2 injections, which was attributed to the batch nature of the ADs leading to the bulk of the CH_4 yield being produced during the first days of operation [20]. A biogas yield of 399 ± 14 mL·g VS^{-1}, 369 ± 22 mL·g VS^{-1} and 427 ± 17 mL·g VS^{-1} was recorded for food waste ADs acting as control, enriched once and periodically with CO_2, respectively. This is in agreement with the data from [6], which reported a moderate increase in CH_4 yield (5-13%) in ADs treating food waste enriched with CO_2 when compared with control units. Biogas data are presented in this study to evidence well-functioning reactors ~~AD operation~~. However, the experimental set-up was focussed on liquid phase sampling to provide initial insights into the mechanisms of CO_2 utilisation; hence the ADs' headspace was regularly diluted for periodically enriched units. Benefits in biogas production due to CO_2 enrichment in ADs can be consulted in previous studies [3,5,7–10,12,16].

Digestate characterisation data and removal efficiencies obtained for the different ADs are outlined in Table 2. The alkalinity, TAN, VFA, TS and VS content of the digestates was not significantly different between ADs with and without CO_2 enrichment. Only sCOD removal in ADs treating sewage sludge was increased from 23.4 ± 2.3% for control ADs to

27.9 ± 2.1% and 35.9 ± 2.5% for ADs enriched with CO_2 once and periodically, respectively. Single CO_2 injection did not alter the pH of the AD final digestate, whereas for ADs enriched periodically with CO_2 a slight decrease was observed, with a pH drop of 0.4-0.5 units for food waste ADs and 0.7 pH units for sewage sludge ADs (Table 2). In every case the final pH was above the value of 6 typically stated as inhibitory for methanogenesis [21].

3.2 ***Microbial populations diversity by FISH analysis***

3.2.1 Microbial populations in batch ADs treating sewage sludge

A good signal was obtained for all the rRNA-targeted probes in samples from ADs treating sewage sludge (Fig. 1). The *ca.* 100% total *Archaea* Abundance obtained as sum of *Methanosaetaceae* and *Methanobacteriaceae* individual Abundances (Table 3) suggested good probe hybridization. *Methanosaetaceae* (~~responsible for the formation of CH_4 by conversion of acetate~~ obligate acetoclastic methanogen) was the dominant methanogen at the start of the batch digestion process, accounting for 86.4 ± 12.1% of the *Archaea* population (Table 3). Its Abundance is in agreement with previous studies stating that *Methanosaeta* sp. is the predominant methanogen in sewage sludge ADs [22] and reporting acetoclastic methanogenesis as the major contributor to total CH_4 formation (*ca.* 70%) in sewage sludge ADs [23]. The Abundance of *Methanobacteriaceae* at the start of the digestion trials was 11.0 ± 4.1% of the *Archaea* population (Table 3), which suggested a lower contribution of the hydrogenotrophic pathway towards total CH_4 formation.

Cell aggregates hybridised by the *Archaea* probe (Arch915) and not co-hybridised with the *Methanosaetaceae* (Mx825) or *Methanobacteriaceae* (Mbac1174) probes were clearly observed at the start of the digestion process (Fig. 2 (a) and (b)). This clustered cell structure has been consistently associated with agglomerations of *Methanosarcina sp*. [22,24,25]. Visual observation suggested a minor contribution of *Methanosarcinaceae* to

the total *Archaea* population in sewage sludge samples. However, an oligonucleotide probe targeting *Methanosarcinaceae* in sewage sludge samples was not used and is recommended for future studies.

At the end of the batch digestion, fluorescence from *Archaea* species was clearly reduced when compared to the start of the process; which was attributed to the batch nature of the ADs leading to a decrease in cells' activity with time and a reduction of the cellular rRNA content. Starving cells have previously been reported to derive faint fluorescent signals during FISH analysis [26]. However, the reduction of fluorescence signal from *Archaea* during the batch digestion was less pronounced in ADs enriched with CO_2 (Fig. 1). The R_M ratio (calculated by dividing the fluorescence signal of the specific *Archaea* in the digestate of batch tests augmented with CO_2 by the fluorescence signal in the digestate of control experiments) for ADs enriched once with CO_2 was 1.45 and 1.24 for $R_{Methanosaetaceae}$ and $R_{Methanobacteriaceae}$, respectively (p-value <0.05 and >0.05, respectively) (Table 3). For ADs enriched periodically with CO_2 the $R_{Methanosaetaceae}$ and $R_{Methanobacteriaceae}$ ratios increased to 1.79 and 1.32, respectively (p-value <0.05 and >0.05, respectively) (Table 3). These results demonstrated that ADs enriched periodically with CO_2 sustained higher *Archaea* related signal compared with ADs enriched once with CO_2 and with control units. The greater differences between digestates of control and test ADs was found for *Methanosaetaceae*, that emitted a statistically significant greater fluorescence signal in units enriched with CO_2 (Table 3). *Methanosarcina* spp. cell clusters were not visually noticeable in any of the digestate samples at the end of the digestion process (control, enriched with CO_2 once and periodically) (Figure 1).

3.2.2 Microbial populations in batch ADs treating food waste

A weak signal of the *Bacteria* probe (Eub338-Mix) was observed when performing FISH tests in food waste samples with the same protocol as for sewage sludge. The weak

signal from the Eub338-Mix probe was attributed to a poor penetration into the bacteria cells. In order to improve cell wall permeability, freeze-thaw cycles consisting of 5 minute steps at -80°C and 60°C were completed in the samples previously fixed with 4% PFA. A significantly enhanced hybridization was achieved when five cycles were completed, as previously reported by [14] and [15] and hence the method for FISH analysis was amended for all the food waste samples to include this additional step.

The sum of *Methanosaetaceae*, *Methanobacteriaceae* and *Methanosarcinaceae* individual Abundances was only 19-40% (Table 3). These results suggest still incomplete probe hybridization or a significant presence of *Archaea* not targeted by the specific probes used, and hence the quantitative FISH results for food waste on *Archaea* Abundance require cautious interpretation. Previous studies have reported a limited penetration of the oligonucleotide FISH probes in methanogenic cells and in particular for food waste ADs [27–29].

A vast spatial distribution of the targeted cells was observed when collecting images at random positions on the gelatin coated well slides, both in the xy-plane and the z-axis. *Methanosarcinaceae* clusters were found mainly in the centre of the xy-plane and higher positions in the z-axis. On the contrary, *Bacteria* and other *Archaea* cells (*Methanosaetaceae and Methanobacteriaceae*) were predominant in the edge of the wells and lower positions of the z-axis. The spatial distribution observed in the z-axis is evident in a video included as *supplementary data*. In order to account for this variability, images were collected at random positions of the xy-plane every 0.5µm of the z-axis and these fields were merged in a single z-stack image. A total of 30 z-stack images were used to complete the quantification analysis.

Methanosarcinaceae accounted for 19.4 ± 9.8% of the total *Archaea* population, being the most abundant methanogen within those targeted at the start of the digestion

process (Table 3). The Abundance of *Methanosaetaceae* and *Methanobacteriaceae* was recorded as 4.3 ± 1.7% and 1.8 ± 0.7%, respectively (Table 3). The significant presence of *Methanosarcinaceae* is in good agreement with the results of [22], who concluded that this *Archaea* was the prevailing methanogen in putrescible waste ADs.

The low Abundance of *Methanosaetaceae* was associated with the high ammonia concentration of food waste ADs. TAN and FAN concentrations of 3.4 $g \cdot L^{-1}$ and 139 $mg \cdot L^{-1}$, respectively, were recorded at the start of the digestion process and of 4.0 - 4.1 $g \cdot L^{-1}$ and 417 - 995 $mg \cdot L^{-1}$, respectively, at the end of the digestion process (Table 2). Previous studies have reported levels >3 $g \cdot L^{-1}$ of TAN as completely inhibitory for acetoclastic methanogens [30] and levels of FAN > 60 $mg \cdot L^{-1}$ as partially inhibitory for anaerobic digestion [31]. However, *Methanosarcina* presents a higher tolerance to free ammonia toxicity, due to the formation of big *Archaea* clusters [22,32]. These clusters reduce ammonia diffusion due to a high volume to surface area ratio [33] and protect the cells located inside the clusters from inhibitors present in the fluid matrix [34]. When comparing the size of *Methanosarcinaceae* clusters observed in food waste and sewage sludge samples (Fig. 2), the morphology of these *Archaea* appeared to be affected by the FAN concentration as larger clusters formed with higher free ammonia contents. *Methanosarcinaceae* were dispersed in smaller aggregates in sewage sludge, where lower TAN (0.8 - 1.2 $g \cdot L^{-1}$) and FAN concentrations (33 - 175 $mg \cdot L^{-1}$) (Table 2) were recorded.

Additionally, high acetate concentrations have also been reported to cause inhibition of *Methanosaeta* spp., with *Methanosarcina* spp. shown to have a higher tolerance to acetate [35]. *Methanosarcina* spp. has been found as the dominant *Archaea* in environments with acetate concentrations >60 $mg \cdot L^{-1}$ as per Schmidt el al. [36] and >200 $mg \cdot L^{-1}$ as per Zheng and Raskin [37]. In food waste ADs, the initial acetic acid concentration was determined to be 7.7 times that of sewage sludge units (Table 2), which

may have led to a further inhibition of *Methanosaetaceae* populations in the food waste matrix.

At the end of the AD batch process, the Abundance of *Methanosarcinaceae* was quantified at 21.9 ± 9.0%, 32.4 ± 15.1% and 14.3 ± 6.4% for digestate samples of control ADs, ADs enriched once and periodically with CO_2, respectively. The low Abundance of *Methanosaetaceae* and *Methanobacteriaceae* observed at the start of the digestion process was maintained for all the digestates. *Methanosaetaceae* accounted for 5.4 ± 1.7%, 5.8 ± 2.1% and 3.5 ± 1.0% of the *Archaea* population in digestate samples of control ADs and test ADs with single and periodic CO_2 injections, respectively. The Abundance of *Methanobacteriaceae* was quantified at 3.1 ± 1.8%, 2.1 ± 0.8% and 1.1 ± 0.6% for control ADs and units with single and periodic CO_2 injections. Furthermore, in contrast with sewage sludge units, enrichment with CO_2 in food waste ADs did not lead to higher R_M ratios, as these were <1 for $R_{Methanosaetaceae}$, $R_{Methanobacteriaceae}$ and $R_{Methanosarcinaceae}$ (Table 3).

3.3 *Potential formation of carbonated precipitates and impact in ammonia levels when enriching ADs with CO_2*

Several tests were performed in the liquid and solid fractions of digestate samples in order to assess the potential of CO_2 enrichment to form or dissolve ammonium or calcium carbonated precipitates. A similar TAN concentration in the solid free fraction of test and control digestates was observed (*ca.* 4 $g \cdot L^{-1}$ for food waste and 1.2 $g \cdot L^{-1}$ for sewage sludge) (Table 2), implying that neither ammonia desorption nor precipitation of ammonium carbonated species were substantial when enriching ADs with CO_2. Within the ammonia carbonated precipitates which could be formed (e.g., NH_2COONH_4, $(NH_4)_2CO_3$ and NH_4HCO_3), the most likely to be present in a wet environment under mesophilic conditions, a pH of *ca.* 8 and excess CO_2 was NH_4HCO_3 [38,39]. However, this compound is highly soluble in water (366 $g \cdot L^{-1}$ at 40°C [17]) which could explain that it was not

precipitated in spite of the significant ammonia concentration in the food waste digestate samples and the excess of CO_2 available.

A similar TAN concentration was recorded in control and CO_2 enriched ADs, both in the case of sewage sludge and food waste units (Table 2). This indicated that ammonia stripping did not happen to a significant extent and this is in agreement with a study [16] where similar TAN concentrations were recorded in pilot scale food waste ADs operated conventionally and periodically injected with CO_2. Other studies have reported significant reductions in ammonia levels by biogas stripping, but they required high temperatures (*ca.* 70°C) and pH (*ca.* 10) in order to shift the ammonia equilibrium towards the free ammonia form [40,41].

It is of note that in spite of a constant TAN concentration between control and test units, FAN concentration was reduced because of the shift of the ammonia equilibrium towards the ionized form as a result of a moderate pH reduction (0.4 to 0.7 units, Table 2) after CO_2 dissolution. FAN levels of 885 ± 121 $mg \cdot L^{-1}$ and 417 ± 65 $mg \cdot L^{-1}$ were recorded for control ADs and ADs periodically enriched with CO_2, respectively, for food waste ADs and of 164 ± 30 $mg \cdot L^{-1}$ and 41 ± 6 $mg \cdot L^{-1}$, respectively, for sewage sludge ADs (Table 2).

The potential formation of carbonated precipitates in ADs enriched with CO_2 was further examined in digestate samples collected from a food waste pilot-scale AD of 106 L working volume and TAN concentration of 1,798 ± 124 $mg \cdot L^{-1}$. The elemental composition obtained by ESEM in freeze dried samples of food waste digestates showed that carbon and oxygen accounted for 49.1 ± 0.3% and 32.1 ± 0.8%, respectively, of the solid phase in digestates without CO_2 enrichment (Fig. 3). Similar values of 47.6 ± 0.2% and 32.0 ± 0.9% were obtained when the digestate was enriched with CO_2 prior to freeze drying. Consistent results were obtained when the tests were completed in the digestates' supernatant, where carbon and oxygen were found to account for 45.7 ± 0.9% and 32.9 ±

1.3%, respectively, when CO_2 was not injected and for 43.2 ± 0.4% and 33.2 ± 0.3% when enriching the supernatant with CO_2 prior to freeze drying. Furthermore, no significant differences between the XRD spectrum of samples with and without CO_2 enrichment were observed when compared for potential differences in calcium carbonate ($CaCO_3$), ammonium carbonate ($(NH_4)_2CO_3$), ammonium bicarbonate (NH_4HCO_3) and ammonium carbamate (NH_2COONH_4). These results indicate that there was no enrichment or depletion of carbon and oxygen in the solid fraction of digestates enriched with CO_2. Therefore it was concluded that precipitation or dissolution of carbonated precipitates did not occur to a significant extent in the tests performed.

3.4 *Discussion of mechanisms of CO_2 utilisation following AD CO_2 enrichment*

The complexity of the reactions involved in anaerobic digestion has led to conflicting hypothesis in the literature on the possible mechanisms for exogenous CO_2 utilisation when H_2 is not supplemented. Several studies have associated the benefits of CO_2 enrichment to a boost of the acetoclastic pathway of CH_4 formation [6,9]. This was hypothesised because a higher VFAs concentration (substrate availability) was observed, which was believed resulted from enhanced homoacetogenesis via the Wood-Ljungdhal pathway [5]. Other references support a reduction of exogenous CO_2 with H_2 by hydrogenotrophic *Archaea*, increasing the contribution of hydrogenotrophic methanogenesis to CH_4 formation [4]. A review of previous studies supporting these hypotheses can be found in [2].

Previous studies hypothesised a mechanism for CO_2 utilisation based on AD performance and the conditions under which CO_2 was injected. To illustrate, Salomoni et al. [5] observed a 46% uptake of the CO_2 injected in the first stage of a two phase anaerobic digester (TPAD), which was attributed to carbon assimilation by homoacetogeneis as no methanogenic activity was expected in the first stage reactor. Bajón

Fernández et al. [6] reported a substrate dependent response to CO_2 injection, with the higher increase in CH_4 formation observed in sewage sludge ADs being hypothesized due to a boost of acetoclastic methanogenesis after CO_2 utilization by homoacetogenesis. The benefits of CO_2 enrichment observed were less significant in food waste ADs, because acetoclastic methanogenesis was understood to be inhibited at the high TAN levels (4 $g \cdot L^{-1}$) recorded.

The analysis developed in the present study, provides experimental evidence on chemical species formation as well as shifts in methanogenic *Archaea* to provide initial experimental evidence on the mechanisms of exogenous CO_2 utilisation in AD. Formation of ammonium and calcium carbonated precipitates was not observed when enriching ADs with CO_2, suggesting a biological pathway for CO_2 utilisation. *Methanosaetaceae* was confirmed to be the dominant methanogen in sewage sludge ADs (Abundance of 83.8 – 98.8%) but was found to be scarce in food waste units (3.5 – 5.8%), where *Methanosarcinaceae* was more abundant (14.3 – 32.4%). Since *Methanosaetaceae* is an obligate acetoclastic methanogen, its Abundance among the *Archaea* population in sewage sludge was related to acetoclastic methanogenesis being the major contributing pathway for CH_4 formation. On the other side, *Methanosarcinaceae* (abundant in food waste ADs) is a versatile *Archaea* that can undertake methanogenesis by utilisation of acetate, CO_2 or methanol [42,43].

$R_{Methanosaetaceae}$ ratios of 1.45 and 1.79 were observed in sludge ADs enriched once and periodically with CO_2, respectively (p-value <0.05). This indicated a sustained higher *Methanosaetaceae* activity in sludge ADs enriched periodically with CO_2 and suggested that previously observed enhancements of CH_4 formation were due to a boost of acetoclastic methanogenesis. In the acetoclastic pathway of CH_4 formation (Fig. 4) CO_2 is a product rather than a substrate, which implies that the observed higher sustained activity

of *Methanosaetaceae* following CO_2 injection must be associated with an indirect pathway. Fluorescence microscopy with FISH allowed determination of a higher *Methanosaetaceae* activity ($R_{Methanosaetaceae}$) in test ADs, but cannot discern whether this was due to a boost of homoacetogenesis or to a lower toxicity to *Methanosaetaceae* as free ammonia concentration was reduced in ADs enriched with CO_2 (Table 2). Radiolabelling or stable carbon isotope studies are hence required for further investigations to clearly track the fate of the CO_2 added. However, significant differences in $R_{Methanosaetaceae}$ ratios were observed for both ADs enriched once and periodically with CO_2 (Table 3), while FAN concentration was only reduced in the later (Table 2). This suggests that the $R_{Methanosaetaceae}$ >1 observed was not only a result of a lower free ammonia inhibition and that additional CO_2 injected into ADs could have been biologically reduced by the Wood-Ljungdahl pathway, leading to formation of acetate that acted as substrate for acetoclastic methanogenesis. This is in line with results from Mohd Yasin et al. [10], who previously reported an increase in acetate formation in methanogens enriched from waste activated sludge and supplemented with CO_2, with a posterior consumption of acetate and CH_4 production.

The proposed hypothesis for utilisation of exogenous CO_2 injected into ADs is summarized in Fig. 4. This study provides a base that supports a boost of acetoclastic methanogenesis in ADs enriched with CO_2 when exogenous H_2 is not supplemented, while further investigation is required to confirm the proposed reduction of CO_2 via homoacetogenesis. Further studies are required to link results of microbial communities with CH_4 formation pathways and to investigate the source of reducing equivalents needed for the proposed mechanism.

4. Conclusions

Chemical and biological mechanisms for CO_2 utilisation in ADs were studied. Examination of XRD and ESEM outputs suggested that chemical CO_2 utilisation did not

occur to a significant extent and that CO_2 was utilised biologically. *Methanosaetaceae* was dominant in sewage sludge ADs (83.8 – 98.8%). *Methanosarcinaceae* was dominant in food waste units (14.3 – 32.4%), where free ammonia reached inhibitory levels for *Methanosaetaceae.* $R_{Methanosaetaceae}$ ratios of 1.45 and 1.79 were observed for sludge ADs enriched once and periodically with CO_2, respectively (p-value < 0.05), suggesting that previously reported CH_4 enhancements in CO_2 enriched units are due to a boost of acetoclastic methanogenesis. Radiolabelling or stable carbon isotope studies are proposed for further investigations to elucidate whether a boost of acetoclastic methanogenesis is triggered by CO_2 reduction in homoacetogeneis or by a lower free ammonia toxicity resulting from a mild pH drop.

5. References

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11.

6. Figure and table captions

- Table 1. Oligonucleotide probes used for FISH analysis, with details of sequence and target microbial group.
- Table 2. Material characterisation at start and end of digestion process and removal efficiencies for food waste and sewage sludge ADs. Format as average ± standard deviation.
- Table 3. Abundance (%) and R_M ratio of methanogenic microbial populations (*Methanosaetaceae, Methanobacteriaceae and Methanosarcinaceae*) in sewage sludge and food waste ADs. Reported as average ± standard deviation of 30 images obtained per sample.
- Fig. 1: FISH images obtained for sewage sludge ADs at the start of the batch digestion process (a) and at the end for control ADs (b), ADs enriched once with CO_2 (c) and ADs enriched periodically with CO_2 (d). Cells in purple are *Methanosaetaceae* co-hybridised by the *Archaea* (Arch915) and the *Methanosaetaceae* (Mx825) probes.
- Fig. 2: FISH images obtained for sewage sludge ((a) and (b)) and for food waste ((c) and (d)) at the start of the digestion process. In sewage sludge samples *Methanosarcinaceae* cells were those stained in red, only hybridized by the *Archaea* probe (Arch 915) and with a characteristic cluster structure. In food waste samples *Methanosarcinaceae* cells were stained in purple since they were co-hybridised by the *Archaea* (Arch 915) and the *Methanosarcinaceae* (MS1414) probes. Images (a), (b) and (c) correspond to a single field and image (d) to a z-stack image.
- Fig. 3: ESEM images and elemental analysis of food waste AD digestates with and without CO_2 enrichment. Samples collected from a food waste pilot-scale AD of 106 L working volume and total ammonia concentration of 1,798 ± 124 $mg \cdot L^{-1}$. Images correspond to: (a) supernatant of digestate without CO_2 enrichment, (b) supernatant of

digestate enriched with CO_2, (c) digestate without CO_2 enrichment, (d) digestate enriched with CO_2. Format of elemental composition as *average* ± *standard deviation* with results in weight percentage.

- Fig. 4: Hypothesised mechanism of exogenous CO_2 utilisation. Adapted from [16].

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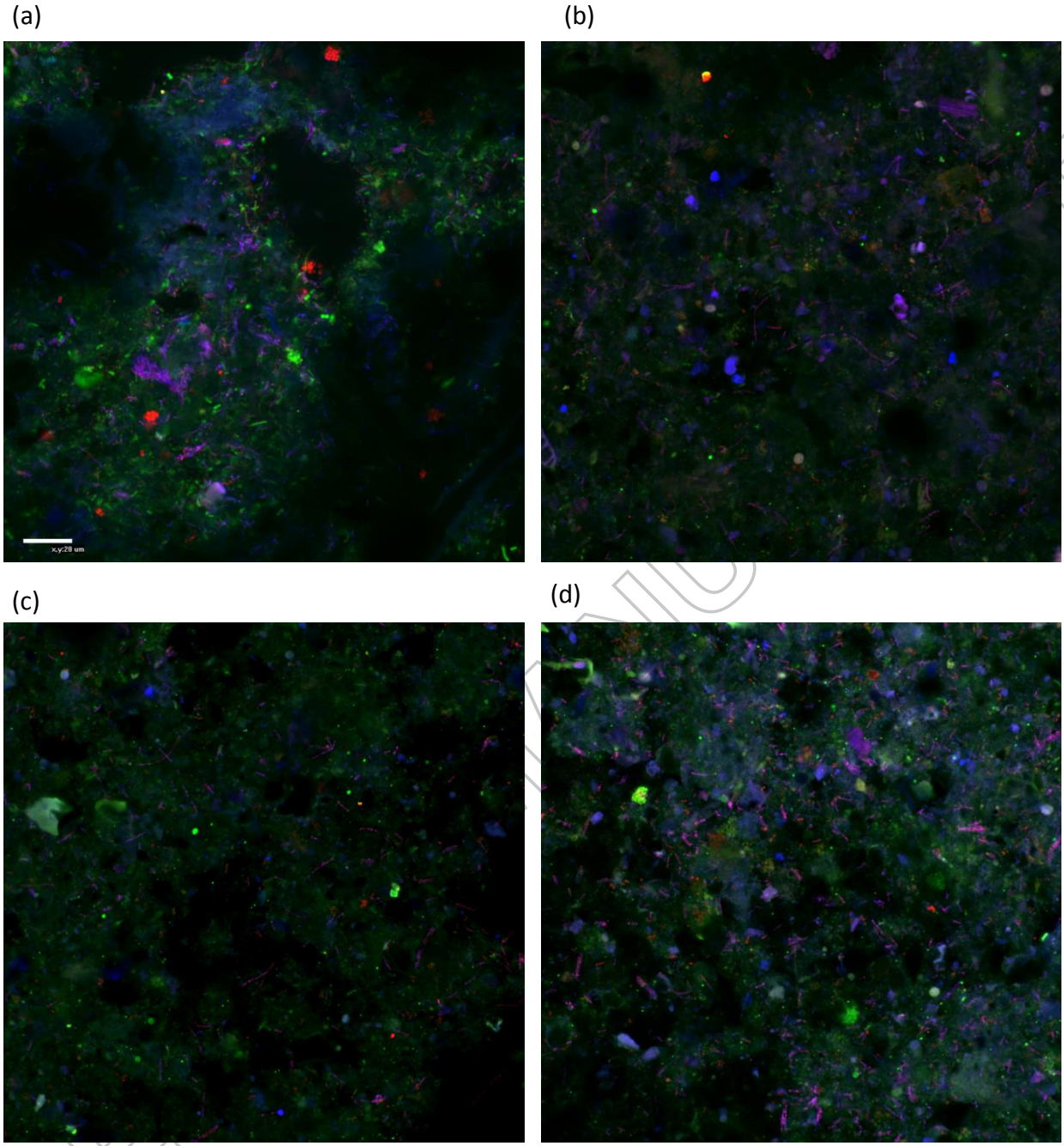

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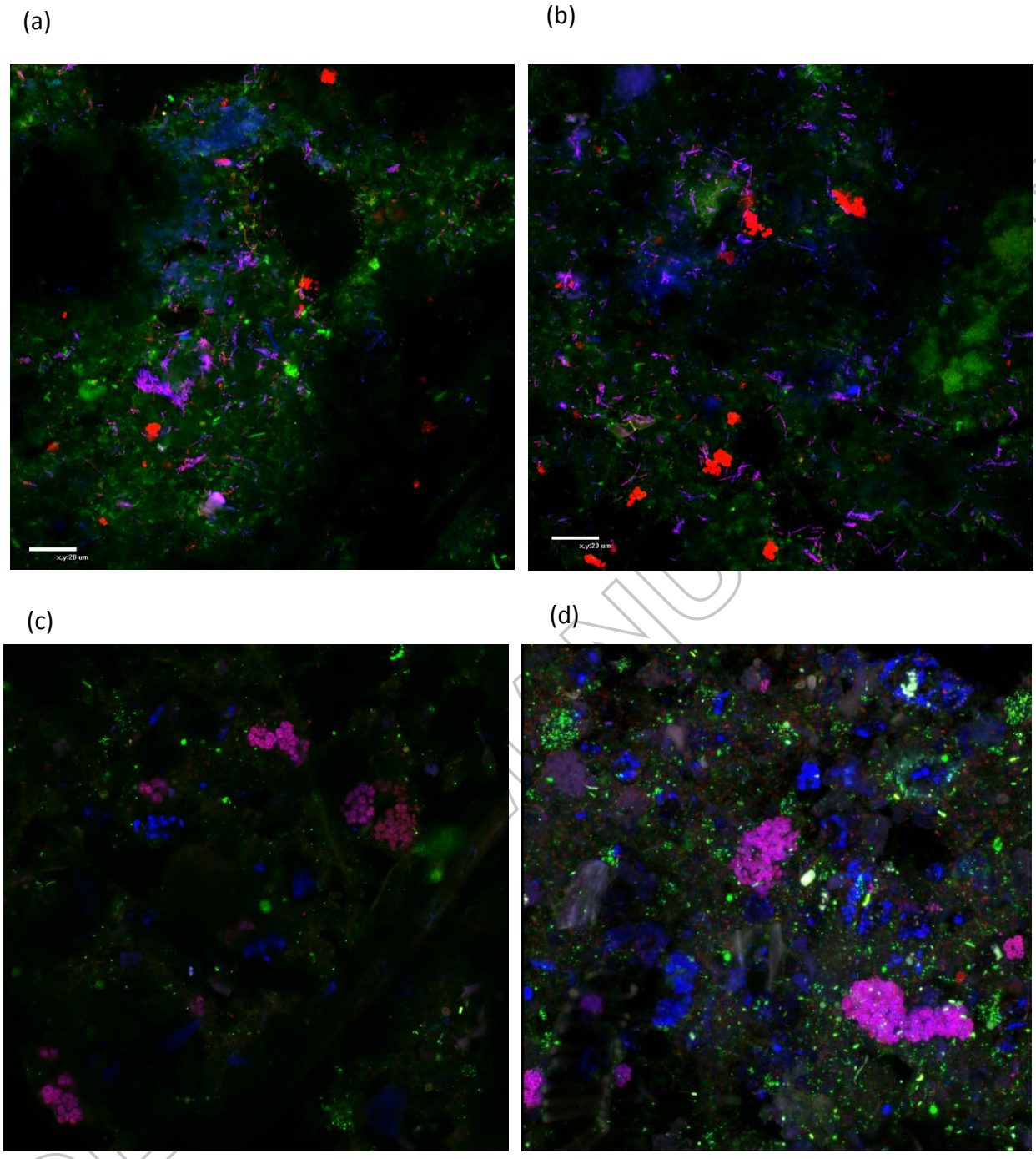
Note: To be reproduced in colour on the Web and in print.



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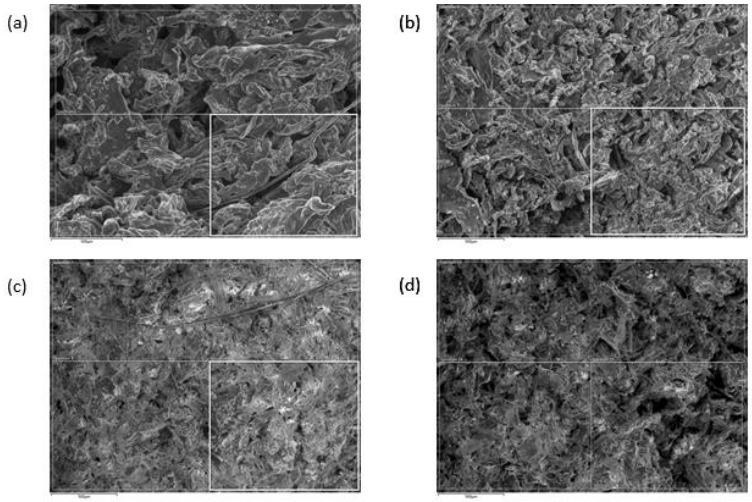

	C (%)	O (%)	Na (%)	Mg (%)	Al (%)	Si (%)	P (%)	S (%)	Cl (%)	K (%)	Ca (%)	Fe (%)
Supernatant of digestate without CO_2enrichment[a]	45.7 ± 0.9	32.9 ± 1.3	8.6± 0.1	0.4 ± 0.0	-	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	6.4 ± 0.6	3.2 ± 0.1	2.1 ± 0.1	-
Supernatant of digestate enriched with CO_2[a]	43.2 ± 0.4	33.2 ± 0.3	8.7 ± 0.1	0.4 ± 0.0	-	0.2 ± 0.1	0.5 ± 0.0	0.2 ± 0.0	7.5 ± 0.2	4.2 ± 0.1	2.0 ± 0.1	-
Digestate without CO_2 enrichment	49.1 ± 0.3	32.1 ± 0.8	4.5 ± 0.2	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.2	0.9 ± 0.0	0.7 ± 0.0	4.9 ± 0.3	3.3 ± 0.2	2.8 ± 0.3	0.8 ± 0.2
Digestate enriched with CO_2	47.6 ± 0.2	32.0 ± 0.9	4.9 ± 0.5	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	0.7 ± 0.0	5.6 ± 0.3	3.7 ± 0.2	3.0 ± 0.3	0.8 ± 0.0

[a] Supernatant obtained after a double centrifugation process as described in the *materials and methods* section.

Fig. 3: ESEM images and elemental analysis of food waste AD digestates with and without CO_2 enrichment. Samples collected from a food waste pilot-scale AD of 106 L working volume and total ammonia concentration of 1,798 ± 124 mg·L^{-1}. Images correspond to: (a) supernatant of digestate without CO_2 enrichment, (b) supernatant of digestate enriched with CO_2, (c) digestate without CO_2 enrichment, (d) digestate enriched with CO_2. Format of elemental composition as *average* ± *standard deviation* with results in weight percentage.

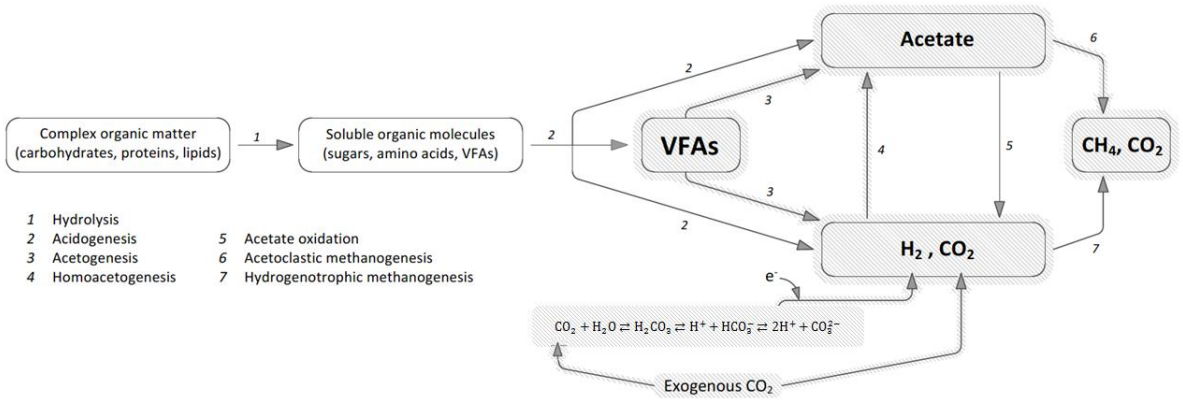

Fig. 4: Hypothesised mechanism of exogenous CO_2 utilisation. Adapted from [16].

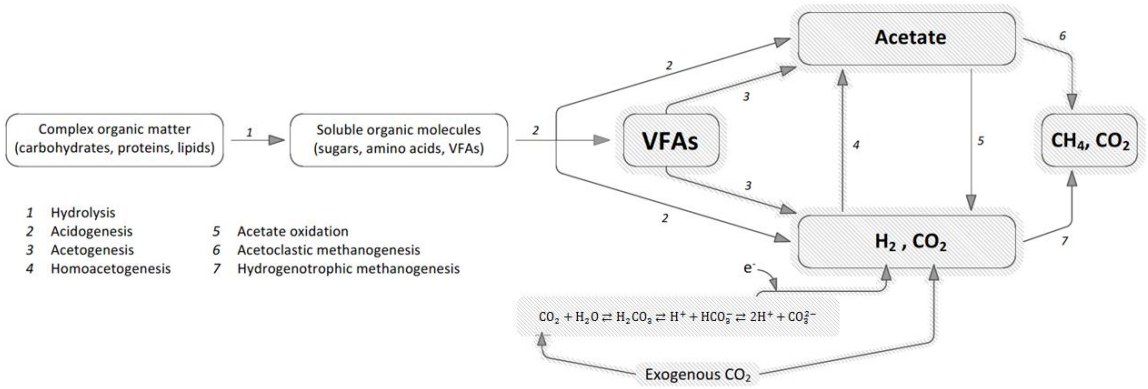

Table 1. Oligonucleotide probes used for FISH analysis, with details of sequence and target microbial group.

Probe	Probe sequence (5' – 3')	Target microbial group	Fluorochrome	Colour	Reference
Eub338[a]	GCTGCCTCCCGTAGGAGT	*Bacteria*	FITC	Green	(Amann et al., 1990)
Eub338-II[a]	GCAGCCACCCGTAGGTGT	*Bacteria*	FITC	Green	(Daims et al., 1999)
Eub338-III[a]	GCTGCCACCCGTAGGTGT	*Bacteria*	FITC	Green	(Daims et al., 1999)
Arch915	GTGCTCCCCCGCCAATTCCT	*Archaea*	Cy3	Red	(Stahl and Amann, 1991)
Mx825	TCGCACCGTGGCCGACACCTAGC	*Methanosaetaceae*	Cy5	Blue[c]	(Raskin et al., 1994)
Mbac1174	TACCGTCGTCCACTCCTTCCTC	*Methanobacteriaceae*	Cy5	Blue[c]	(Raskin et al., 1994)
MS1414[b]	CTCACCCATACCTCACTCGGG	*Methanosarcinaceae*	Cy5	Blue[c]	(Raskin et al., 1994)
hMS1395[b]	GGTTTGACGGGCGGTGTG	*Methanosarcinaceae helper*	-	-	(Crocetti et al., 2006)
hMS1480[b]	CGACTTAACCCCCCTTGC	*Methanosarcinaceae helper*	-	-	(Crocetti et al., 2006)
Non Eub338	ACTCCTACGGGAGGCAGC	Non bacteria	FITC	-	(Wallner et al., 1993)

[a] Eub338, Eub338-II and Eub338-III used in equimolar proportion as Eub338-Mix.

[b] Probe only used in food waste samples.

[c] Purple fluorescence when co-hybridized with Arch915.

Table 2. Material characterisation at start and end of digestion process and removal efficiencies for food waste and sewage sludge ADs. Format as *average* ± *standard deviation*.

	Food waste				Sewage sludge			
	AD start up	DC[a]	Dsingle[b]	Dperiodic[c]	AD start up	DC[a]	Dsingle[b]	Dperiodic[c]
Liquid phase								
pH	7.5 ± 0	8.3 ± 0.1	8.4 ± 0.0	7.9 ± 0.1	7.5 ± 0	8.1 ± 0.1	8.1 ± 0.1	7.4 ± 0.1
TAN (NH_4-N $mg \cdot L^{-1}$)	3,350 ± 0	3,963 ± 103	3,998 ± 297	4,138 ± 138	798 ± 0	1,194 ± 49	1,149 ± 34	1,185 ± 27
FAN ($mg \cdot L^{-1}$)[e]	139 ± 0	885 ± 121	995 ± 102	417 ± 65	33 ± 0	164 ± 30	175 ± 17	41 ± 6
TA (mg $CaCO_3 \cdot L^{-1}$)	-	15,854 ± 349	16,083 ± 581	16,444 ± 668	3,367 ± 0	4,637 ± 32	4,663 ± 5	4,674 ± 93
PA (mg $CaCO_3 \cdot L^{-1}$)	-	13,569 ± 969	14,394 ± 418	14,287 ± 694	2,665 ± 0	3,796 ± 140	3,923 ± 42	3,641 ± 64
TVFA ($mg \cdot L^{-1}$)[d]	2,424 ± 0	797 ± 54	825 ± 55	861 ± 124	423 ± 0	201 ± 13	310 ± 43	288 ± 28
Acetic acid (as % of TVFA)	52 ± 0	0 ± 0	0 ± 0	0 ± 0	39 ± 0	0 ± 0	0 ± 0	0 ± 0
Removal efficiencies								
TS (%)	n/a	27.4 ± 1.8	25.5 ± 0.3	29.4 ± 2.6	n/a	20.8 ± 0.8	18.9 ± 0.9	20.9 ± 1.6
VS (%)	n/a	36.3 ± 1.7	36.8 ± 1.0	39.6 ± 1.3	n/a	29.5 ± 0.6	27.9 ± 1.2	28.0 ± 2.5
sCOD (%)	n/a	51.8 ± 4.0	52.0 ± 2.1	48.1 ± 9.6	n/a	23.4 ± 2.3	27.9 ± 2.1	35.9 ± 2.5

[a] DC: Control digester.
[b] Dsingle: Digesters enriched with y_{CO2}=0.9 once at the start of the batch process.
[c] Dperiodic: Digesters enriched periodically with y_{CO2}=0.9.
[d] Calculated as sum of acetic acid, propionic acid, n-butyric acid, iso-butyric acid, n-valeric acid and iso-valeric acid.
[e] Calculated with total ammonia nitrogen, pH and temperature as reported by Hansen et al. [24].

Table 3. Abundance (%) and R_M ratio of methanogenic microbial populations (*Methanosaetaceae, Methanobacteriaceae* and *Methanosarcinaceae*) in sewage sludge and food waste ADs. Reported as *average* ± *standard deviation* of 30 images obtained per sample.

	Sewage sludge ADs[a]				Food waste ADs					
	Methanosaetaceae		*Methanobacteriaceae*		*Methanosaetaceae*		*Methanobacteriaceae*		*Methanosarcinaceae*	
	Abundance (%)[b]	R_M[c]	*Abundance (%)*	R_M	*Abundance (%)*	R_M	*Abundance (%)*	R_M	*Abundance (%)*	R_M
Start of ADs	86.4 ± 12.1	n/a	11.0 ± 4.1	n/a	4.3 ± 1.7	n/a	1.8 ± 0.7	n/a	19.4 ± 9.8	n/a
Digestate of control ADs	98.8 ± 21.6	n/a	7.2 ± 1.8	n/a	5.4 ± 1.7	n/a	3.1 ± 1.8	n/a	21.9 ± 9.0	n/a
Digestate of ADs with single CO_2 injection	83.8 ± 12.4	1.45*	8.0 ± 2.6	1.24	5.8 ± 2.1	0.67**	2.1 ± 0.8	0.38**	32.4 ± 15.1	0.72**
Digestate of ADs with periodic CO_2 injections	94.9 ± 17.5	1.79*	6.0 ± 1.8	1.32	3.5 ± 1.0	0.69**	1.1 ± 0.6	0.38**	14.3 ± 6.4	0.77**

[a]An oligonucleotide probe targeting Methanosarcinaceae was not used in sewage sludge samples.

[b]*Abundance (%)* was calculated by setting *Methanosaetaceae* (Mx825), *Methanobacteriaceae* (Mbac1174) or *Methanosarcinaceae* (MS1414) as specific probes in relation to the general *Archaea* probe (Arch915) for a given sample.

[c]R_M ratio was calculated by dividing the fluorescence signal (i.e., pixel count) for each specific *Archaea* detected in the digestate of batch tests augmented with CO_2 by the fluorescence signal (i.e., pixel count) for each specific *Archaea* detected in the digestate of control experiments.

*Statistically significant difference in pixel count for specific *Archaea* between test and control ($P<0.05$)

** Statistically significant difference in pixel count for specific *Archaea* between test and control ($P<0.05$). However, food waste FISH results require cautious interpretation as evidenced by the low sum of individual *Archaea* abundances, which suggests incomplete probe hybridization or a significant presence of *Archaea* not targeted by the probes used.

Microbial spacial distribution food waste.avi-muxed.mp4