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# Biowaste as feedstock for 2nd generation

**VALORGAS**

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## D3.3: Biokinetic and biosecurity data and operating protocols from optimisation of autoclave and cell disruption pre-treatment

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Revision [0]

**D3.3 Biokinetic and biosecurity data and operating protocols from optimisation of autoclave and cell disruption pre-treatment**

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**D3.3 Biokinetic and biosecurity data and operating protocols from optimisation of autoclave and cell disruption pre-treatment**

# 1 Introduction

The work described in this Deliverable Report presents results from laboratory-scale trials on pretreatment methods intended to improve methane yields and/or methane production rates from food waste (FW) and to reduce pathogen indicator numbers. The studied pre-treatments were novel technologies developed by VALORGAS partner SMEs, namely cell disruptor technology (ESI) and a double auger autoclave process (Aerothermal). The cell disruption technology was also trialled as a post treatment for food waste digestate to determine its potential to reduce pathogen indicator organisms and improve digestate dewaterability. The experimental work was carried out by MTT and Soton.

# 2 Materials and methods

## 2.1 Origin of materials

The source segregated domestic food waste used in this study was from Ludlow, UK. A sample of ~400 kg of food waste collected from the South Shropshire Biowaste digestion plant in Ludlow was removed from biodegradable bags and divided into two equal portions. One portion was pre-treated with a novel double-auger autoclave (160 °C, 6.2 bars) that allows improved mixing and steam penetration, which was developed and operated by Aerothermal. Both portions were then passed through a macerating grinder (S52/010 Waste Disposer, IMCLimited, UK). The samples were frozen and shipped to MTT, Finland.

The samples were divided into portions of ~700 g equivalent and stored at -20 °C. Each week sufficient material for one week's feed in the semi-continuous digestion trial was thawed and stored at 4 °C. The pH of the feed was measured once a month and total solids (TS), volatile solids (VS), ammonium nitrogen ($NH_4$-N), total Kjeldahl nitrogen (TKN), soluble chemical oxygen demand (SCOD) and volatile fatty acids (VFAs) were analysed each time a new box of either autoclaved FW or control FW was opened. Samples for hygiene indicator analysis were thawed and stored in a fridge for 2, 4 or 7 days before the analysis.

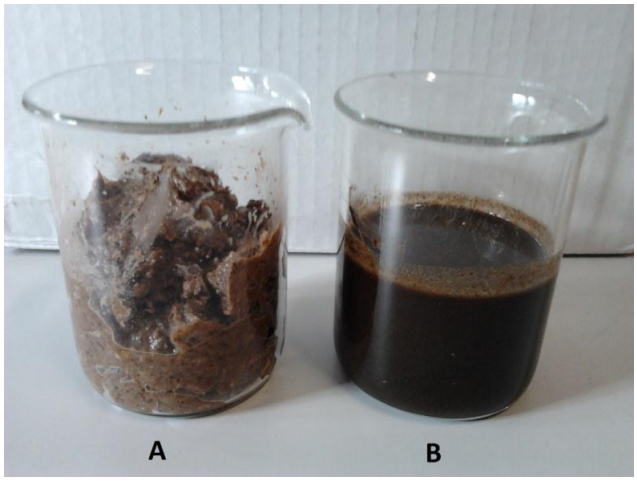



**Figure 1.** Control (A) and autoclaved (B) food waste samples.

For the cell disruption experiments source segregated household biowaste from Forssa region, Finland, was used. This material consists primarily of food waste but includes some other domestic biowastes such as pet litter (see VALORGAS Deliverable D2.1 for detailed compositional analysis). The waste was first hand-sorted and mixed again, after which it was mechanically crushed and screened for plastics at the waste management plant. The waste was then frozen in portions of 4-5 kg. For the cell disruption ($CO_2$ pressurisation) experiments, one 4-5 kg portion of biowaste was thawed as required. Subsequently, the biowaste was macerated with a laboratory knife mill (Retsch GM 300) at 2000 rpm for a mixing time of 2 min. The grinder was operated sequentially, changing the direction of rotation every 30 seconds. The $CO_2$ pressurisation experiments were carried out on biowaste and biowaste diluted 1:2 with tap water. For the experiments with 1:2 diluted biowaste, another 4-5 kg portion of biowaste was thawed, mixed and macerated with water. After maceration, the biowaste and diluted biowaste were divided into 400 g samples and stored at 2 °C until used (maximum 4 days).

## 2.2 Experimental set-up

### 2.2.1 CSTR digestion trials

Anaerobic digestion of the autoclaved and untreated (control) food waste was studied in four 11-litre continuously stirred tank reactors (CSTR) maintained at 37 °C. Digestate overflows from the reactor by gravity through a water lock which prevents gas escape (Figure 2). Hourly gas production and methane content were measured using an automatic measuring system (Metener Ltd, Finland), in which the produced biogas is collected into a small (~220 ml) gas storage reservoir on the top of the reactors and its methane content is measured automatically with infrared equipment during emptying (days 1-198). After day 198 gas production was measured with a volume-calibrated cylindrical gas collector based on water displacement after which the gas was collected in gas bags. The methane concentration of the gas from the bags was analysed with a handheld Combimass GA-m gas analyser (Binder Engineering, Germany). The reactors were fed manually five times a week using the feed inlet on the top.

**Figure 2.** CSTR reactors used for treating untreated and autoclaved food waste.

The reactors were inoculated with digestate from the Biovakka Suomi Oy Turku plant, a mesophilic CSTR digesting sewage sludge (Table 1). Reactors were started at an OLR of 2 kg VS $m^{-3}$ $day^{-1}$ and a hydraulic retention time (HRT) of 117 and 94 days for R1 and R3, respectively. On day 151 OLR was raised to 3 kg VS $m^{-3}$ $day^{-1}$and on day 256 to 4 kg VS $m^{-3}$ $day^{-1}$ (HRTs 78 d and 58 d for control, 63 d and 47 d for autoclaved food waste, respectively).

On day 179 all reactors were supplemented with 11 ml of trace element solutions containing selenium (0.2 g $l^{-1}$) and cobalt (1.0 g $l^{-1}$). From day 199 onwards all reactors were supplemented regularly with trace elements. The trace elements were added in two solutions containing respectively cations (g $l^{-1}$): Aluminium (Al) 0.1, Boron (B) 0.1, Cobalt (Co) 1.0, Copper (Cu) 0.1, Iron (Fe) 5.0, Manganese (Mn) 1.0, Nickel (Ni) 1.0, Zinc (Zn) 0.20; and oxyanions (g $l^{-1}$): Molybdenum (Mo) 0.2, Selenium (Se) 0.2, Tungsten (W) 0.2 (Banks et al., 2011). 1 ml of each solution was added weekly for every kilogram of digestate removed.

Parallel reactors treating control (R2) and autoclaved FW (R4) were started at OLR 3 kg VS $m^{-3}$ $day^{-1}$ on day 327 using digestate from R1 and R3 as inoculum. On day 418 the OLR in all four reactors was further increased to 6 kg VS $m^{-3}$ $day^{-1}$ which decreased the HRTs to 39 d and 31 days in the control and autoclaved reactors respectively.

Digestate samples for laboratory analyses (TS, VS, SCOD, $NH_4$-N, TKN) were collected routinely every two weeks and samples for VFA analysis once a week. Digestate pH was measured weekly. Samples for hygiene indicator analysis were collected from R1 and R3 on days 342, 349 and 356 (OLR 4 kg VS $m^{-3}$ $day^{-1}$) and analyses were started on the same day.

### 2.2.2 $CO_2$ pressurisation trials

Two identical $CO_2$ pressurisation units were set up at MTT and Soton to study the effects of cell disruption techniques. The pressurisation equipment was made of a filter housing, which is connected to a $CO_2$ bottle (Figure 2). The system can be operated at pressures up to 28 bar, and was first tested on water.

**Figure 3.** $CO_2$ pressurisation unit with $CO_2$ bottle.

At MTT, both solubilisation of biowaste and microbial inactivation were studied. Solubilisation of biowaste was tested by three sets of experiments that were performed at 25 bar pressure using treatment times of 10 min, 2h, 12 h, and 24 h. Experiments were performed with biowaste, 1:2 diluted biowaste and 1:6 diluted biowaste. All tests were performed in duplicate (referred to as A and B). Before each test, the sample (400 g) was taken from 2 °C to room temperature, and pH and temperature were measured. Samples were loaded into the pressurisation chamber manually and the lid was sealed. The pressure was increased to 25 bars with pressurised $CO_2$ and the gas valves were closed. Operating pressure was monitored by the gauge on the pressurisation equipment. The pressure chamber was then manually shaken for 1 minute in a vertical position. After shaking, the chamber was left in its holder for the remainder of the test period. The shaking time was included in the total treatment time. The pressures at set-up, after one minute of shaking and just before depressurisation were recorded. At the end of the treatment time the pressure was released. Rapid depressurisation was carried out, by fully opening the gas valve in the shortest time possible. After the treatment, the treated sample was removed from the chamber and temperature and pH were measured, after which the samples were stored at 4 °C until analysed (maximum storage period 2 weeks). For biochemical methane potential (BMP) tests, samples from duplicate tests (A and B) were combined, while other chemical analyses were performed for both samples.

As one set of experiments with different treatment times took about a week, the biowaste used was stored at 2 °C for maximum of 4 days. Thus two control samples were used to differentiate the effect of storage from the effects of the pressurisation treatment. One control sample was the waste immediately after maceration, while the other was macerated waste stored for 4 days at 2 °C.

The effect of $CO_2$ pressurisation treatment on the hygienic quality of food waste was also studied in the cell disruption experiments. Part of each pressure-treated sample was separated for hygiene analysis, which started on the same day as or on the next morning after the pressurisation experiment finished. In the experiments with biowaste and 1:2 diluted biowaste, food waste samples were used after freezing and subsequent thawing, as these conditions commonly occur in Finland in winter. It is possible that these conditions will favour the survival of organisms that are also relatively resistant to $CO_2$ pressurisation, and a further series of experiments was done with 1:6 diluted biowaste where the material was not frozen in any storage stage.

The effect of pressurisation on hygienic quality was also studied in separate experiments with *Salmonella enterica* serovar *typhimurium* culture. Inactivation was studied in pure culture and spiked into diluted biowaste. Experiments were performed in duplicate at 25 bar with treatment times of 2, 6, 16 and 24 hours and at temperatures of 20 and 35 °C. In the *Salmonella* inactivation experiments the pressure regulator and gas valve remained open throughout to maintain a pressure of 25 bar during the experiment.

The stock culture of *S. enterica* was grown in THG medium (containing Tryptone, yeast and glucose) and incubated at 37 °C for a one day. After incubation the stock culture was stored at 4 °C. The number of *S. enterica* in the stock culture was determined after incubation, and varied from $1.47 \times 10^7$ to $3.36 \times 10^7$ cfu $ml^{-1}$. Cell density was determined by total plate count with Rambach plates.

Pure cultures used in pressurisation experiments were suspended in 0.9 % NaCl solution in 500 ml glass bottles which were autoclaved before the experiments to prevent contamination. The volume of NaCl solution was 400 ml per bottle. To obtain a cell density of approximately 1 x $10^6$ cfu $ml^{-1}$, 50 ml of stock culture was added to 400 ml 0.9% NaCl solution giving a total volume of *S. enterica* pure culture of 450 ml.

*Salmonella* inactivation in biowaste was studied on diluted samples of both autoclaved and unautoclaved material, to allow comparison of the impacts on the presence of various microbial groups versus Salmonella monoculture. The autoclaved biowaste sample had previously been frozen and was subsequently thawed, macerated and diluted. The unautoclaved biowaste had not been frozen, and was collected fresh from the Forssa region. From both materials a dilution of approximately TS 5 % was prepared and spiked with *Salmonella enterica*. Experiments with diluted biowaste were done using the same procedures as experiments with saline water. In addition the effects of pressurisation on other hygienic indicators were determined for the unautoclaved biowaste samples. Diluted biowaste was weighed into 500 ml bottles, each containing 400 g of sample. Pressure treatment on the diluted biowaste was carried out for treatment times of 15 and 24 hours.

Pure cultures were prepared in the microbiology lab on the same day as the pressurisation experiment. Samples of pure culture were subjected to pressurisation tests within 30 minutes of adding the stock culture. Immediately before starting the pressurisation experiment 50 g of pure culture were removed as a control sample and the rest was added to the 400 g of sample used in pressurisation experiment. The control sample was left at room temperature and pressure for the duration of the pressure treatment. The *Salmonella* content of the control sample and pressurised sample were both analysed. Where the experiments were done at 35 °C, a 25 ml control sample was stored at room temperature during the treatment. The other 25 ml of control sample was kept in a water bath at 35 °C. Salmonella concentration of control sample and pressure treated sample were determined simultaneously on the same day as the experiment was carried out.

At Soton, *Salmonella enterica* (ATCC 14028) was obtained from the National Collection of Industrial Marine and Food Bacteria (NCIMB) Aberdeen, UK and was grown in Buffered Peptone Water. The strain of *Escherichia coli* used in studies to show cell lysis and inactivation was isolated from primary sewage sludge obtained from Millbrook Wastewater Treatment Works (Southern Water Plc) Southampton, UK. Cultures were grown in nutrient medium to $10^6$ cfu $ml^{-1}$ and pressure treated at 28 bar for 24 hours before comparison with the original untreated culture by electron microscopy.

Enzyme activities of treated and untreated cells were determined using API ZYM strips (BioMérieux, Basingstoke, UK) which allow detection of the activity of 19 enzymes. Each strip consists of twenty small cupules to each of which 65µl of untreated or treated culture of *S. enterica* was added. The strips were then incubated for 4 hours at 37 °C after which one drop of ZYM A and ZYM B was added and any change in colour was noted and compared to a reference chart.

To determine the presence of cell lysis products resulting from cell disruption a UV spectrophotometric method was used. This allows the detection of UV-absorbing substances such as proteins and nucleic acids using the method described by Gerhardt (1981). To carry this out it was first necessary to transfer cells from the culture medium to an isotonic buffer solution before treatment to avoid any cross-contamination with proteinaceous material in the

medium. This was achieved by centrifuging the medium at 10000 g, 4 °C for 30 min and re-suspending the cell pellet in 0.85% NaCl solution. The isotonic suspension was then divided into two portions one of which was treated at 28 bar for 24 hours and the other kept at ambient temperature and pressure. After treatment, both samples were centrifuged again as before and the absorption of the supernatants was measured at 260 and 280 nm using a quartz cuvette with 0.85% NaCl solution as a blank.

## 2.3 Analytical methods

### 2.3.1 Hygienic quality

The hygienic quality of the FWs and digestates was analysed using *E. coli*, other coliforms, total coliforms, enterococcus and sulphite-reducing clostridia as indicator organisms. Analyses of different coliforms were performed according to Baylis and Patrick (1999) using Harlequin *E. coli* / coliform (LabM) culture medium. Enterococcus and sulphite reducing clostridia were determined according to standard methods, SFS 3014 (Finnish Standard Association 1984) and SFS-EN 26462-2 (Finnish Standard Association 1999), respectively. Analyses of *S. enterica* were performed with Rambach agar which is selective for Salmonella.

### 2.3.2 Chemical analyses

TS and VS were determined according to SFS 3008 (Finnish Standard Association, 2002). TKN was analysed according to Kjeldahl method 984.13 using a Foss Kjeltec 2400 Analyser Unit (Foss Tecator AB, Höganäs, Sweden) and $NH_4$-N according to McCullough (1967). For soluble COD analysis and solid liquid separation samples were agitated for 1 hour (prior to this FW samples were 1:10 diluted with water), centrifuged (3500 rpm, 15 min) after which the supernatant was further centrifuged (1320 rpm, 10 min) and stored in a freezer, then thawed before analysis. VFA was analysed according to Huhtanen et al. (1998). pH was determined using a VWR pH100 pH-analyser (VWR International).

Crude protein and nitrogen by Duma's method were analysed using Leco FP 428 nitrogen analyser (Leco Corp., St. Joseph; MI 49085; USA). The protein content was calculated by multiplying the N% by a factor of 6.25. Sugars were analysed according to Somogyi 1945 and lipids after hydrolysis with 3 M HCl (Anon 1971). NDF (Neutral Detergent Fiber) was analysed with a filtering apparatus according to Van Soest et al. (1991) where sodium sulfite was used in NDF-detergent solution and α-amylase in case of samples containing starch. ADF (Acid Detergent Fiber) and lignin (permanganate-lignin) were determined according to Robertson & Van Soest (1981). Hemicellulose content was calculated from the difference between NDF and ADF, when cellulose content was calculated from the difference between ADF and lignin. Iron concentration was analysed according to Luh Huang & Schulte (1985) and the measurement was performed with ICP-OES (inductively coupled plasma emission spectrometry) (Thermo Jarrel Ash Iris Advantage, Franklin, USA).

### 2.3.3 Biological methane potential, ammonification and post digestion assays

Biochemical methane potential (BMP), post digestion and ammonification assays were performed with automated testing equipment (Bioprocess Control AB, Figure 4). Tests were conducted in duplicate or triplicate each with a total liquid volume of 400 ml (FW and ammonification trials) or 200 ml (post digestion trials), at 37 °C. The inoculum to substrate

ratio in FW assays was 1:1 on a VS basis when only the substrate was incubated in post digestion trials. The reactor contents were mixed mechanically for one minute per hour. Carbon dioxide was fixed by NaOH before gas volume measurement. The equipment has automated methane volume measurement based on liquid displacement. The results are given as average values of the triplicate of duplicate assays.

In ammonification assays both autoclaved and control FWs were incubated using digestates from the laboratory CSTR reactors as inoculum. TS, VS and $NH_4$-N of the FWs and inoculums were analysed before and after the experiment.

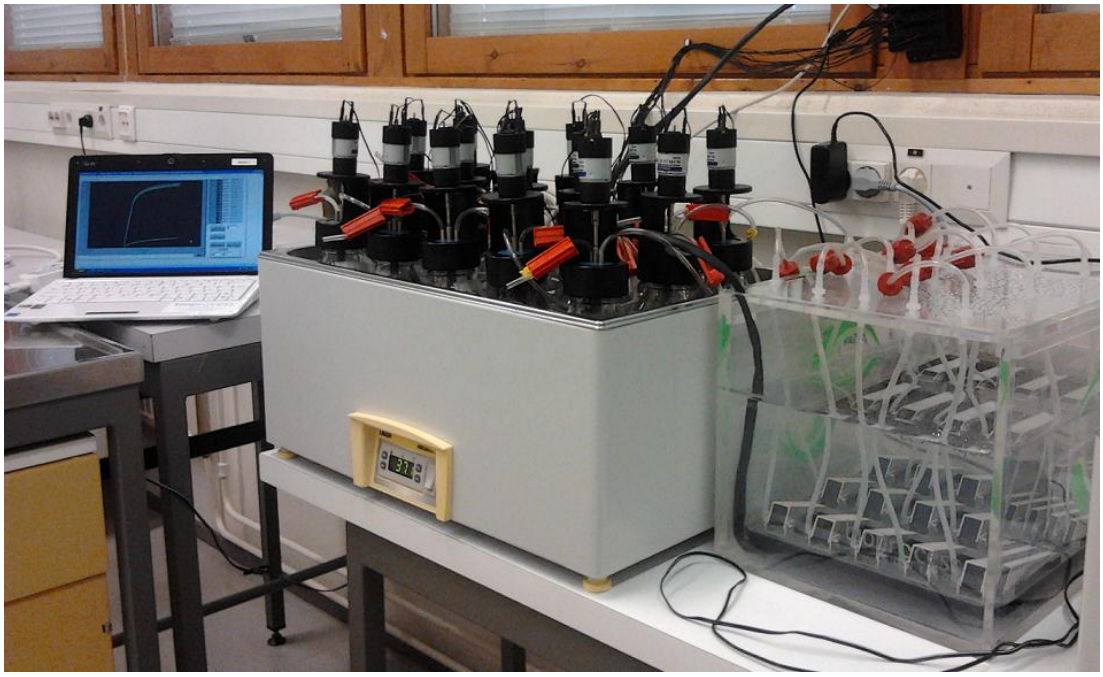


**Figure 4.** Biochemical methane potential testing equipment used in all assays.

## 2.4 Electron microscopy

*Transmission Electron Microscopy (TEM)*
Culture broths containing treated and untreated samples were centrifuged at 10000 g, 4 °C for 30 min (Sorvall legend XT/XTR, Thermo Fisher Scientific, UK). The supernatant was decanted and the cell pellets re-suspended in 1 ml primary fixative comprising 3% glutaraldehyde, 4% formaldehyde in 0.1 M PIPES buffer (pH 7.2). They were then embedded in alginate using method 4 of Page et al. (1994). The specimens were then rinsed in 0.1M PIPES buffer, post-fixed in 1% buffered osmium tetroxide (1 hour), rinsed in buffer, block stained in 2% aqueous Uranyl Acetate (20 mins), dehydrated in an ethanol series and embedded in TAAB resin (TAAB Laboratories, Aldermaston, UK). Gold sections were cut on an Ultra cut E ultra-microtome, stained with Reynolds lead stain and viewed on a FEI Tecnai 12 transmission electron microscope (Japan) with a SIS mega view III digital camera.

*Scanning electron microscopy (SEM)*
The preparation, processing, treatment and re-suspension in primary fixative were the same as for TEM. After primary fixation, the samples were again centrifuged and half of the primary fixative was removed followed by re-suspension of the cell pellet in the rest of the fixative. This small amount of sample was then rested for 30 min on the surface of a 13 mm

cover slip coated with 3-Aminopropyl triethoxysilane and kept in a glass vial. This was followed by buffer rinsing, post fixation in 1% buffered osmium tetroxide and dehydration in alcohol series. After this, the cover slip with the bacterial cells attached to it was dried in a critical point drier (Balzers - CPD 030) followed by mounting on aluminium stubs which were coated with gold palladium in a sputter coater (SEM coating unit E 5100 Polaron equipment limited). The samples were then viewed on a scanning electron microscope (FEI Quanta 200, Netherlands).

### 2.5 Calculations

The organic loading rate (OLR) in the CSTR experiments was calculated based on the substrate fed into the reactor volume in a given time: OLR = QS/V where OLR, organic loading rate (kg VS $m^{-3}$ $day^{-1}$); Q, substrate flow rate ($m^3$ $day^{-1}$; week feeding: 7 days feeding during 5 days); S, substrate VS concentration in the inflow (kg VS $m^{-3}$); V, reactor liquid volume ($m^3$).

HRT for CSTR was calculated with the ratio of reactor volume and the flow rate of the substrate: HRT = V/Q where HRT, hydraulic retention time (day); V, reactor liquid volume ($m^3$); Q, substrate flow rate ($m^3$ $day^{-1}$; week feeding: 7 days feeding during 5 days).

## 3 Results and discussion

### 3.1 Effect of autoclaving

Characteristics from the control and autoclaved feed and from the inoculum used in this study are presented in Table 1. In both control and autoclaved FW pH was around 5. This differs from the results of Bougrier et al. (2008) and Papadimitriou & Barton (2009) who found that the pH of autoclaved material was lower than the control, due to organic acid formation during autoclaving. The pH in the present study was measured after freezing and thawing of the material which could have affected the pH value. TS content in the autoclaved FW was lower, indicating steam condensation into the waste during the autoclave treatment. Autoclaving also solubilized FW components, resulting in higher SCOD and ammonium nitrogen contents in the autoclaved feed. TKN on fresh matter basis was observed to be lower in the autoclaved FW. In BMP assays the specific methane yields for food wastes were similar, at 392 and 415 $m^3$ $CH_4$ $t^{-1}$ $VS^{-1}$ for autoclaved FW and control FW, respectively.

**Table 1.** Characteristics of materials used in the CSTR study.

| | Control FW | Autoclaved FW | Inoculum |
|---|---|---|---|
| pH | 5.01 ± 0.13 | 5.02 ± 0.13 | N/A |
| TS (g $kg^{-1}$ WW) | 244.7 ± 5.0 | 205.2 ± 0.8 | 77.3 |
| VS (g $kg^{-1}$ WW) | 229.9 ± 4.8 | 189.1 ± 0.7 | 43.1 |
| VS/TS (%) | 92.3 | 92.1 | 55.8 |
| SCOD (g $kg^{-1}$ WW) | 99.5 ± 5.8 | 117.6 ± 11.1 | 11.9 |
| TVFA (g $l^{-1}$) | 3.23 ± 0.23 | 2.25 ± 0.14 | 24.0 |
| TKN (g $kg^{-1}$ WW) | 7.38 ± 0.32 | 6.80 ± 0.27 | 4.91 |
| NH4-N (g $kg^{-1}$ WW) | 0.34 ± 0.12 | 0.42 ± 0.11 | 2.4 |
| SMP ($m^3$ $CH_4$ $tonne^{-1}$ VS) | 392.2 | 415.5 ± 1.31 | N/A |
| SMP ($m^3$ $CH_4$ $tonne^{-1}$ TS) | 364.5 | 380.6 ± 1.2 | N/A |
| SMP ($m^3$ $CH_4$ $tonne^{-1}$ WW) | 92.5 | 78.0 ± 0.25 | N/A |

The organic composition (protein, fat, sugar, fibre) of the FWs was also analysed (Table 2). Proteins, fats and cellulose content were observed slightly to increase during the autoclaving while sugars and hemicelluloses content decreased. Hemicellulose starts to hydrolyse at temperatures above 150 °C (Garrote et al. 1999), which explains the reduced hemicellulose content after autoclaving, as also observed by Menardo et al. (2011). Lignin content was different between the materials: this was apparent when lignin was measured by two different methods. As lignin is a term referring to high molecular weight cross-linked material that is difficult to solubilise, it is possible that analogues are formed during the autoclaving process.

**Table 2.** Organic composition of control and autoclaved feed.

| Analysis (g $kg^{-1}$ TS) | Control feed | Autoclaved feed |
|---|---|---|
| Crude protein | 219.8 ± 24.7 | 223.5 ± 2.5 |
| Crude fat | 137.2 ± 7.6 | 146.9 ± 6.1 |
| Sugar | 112.5 ± 3.0 | 58.8 ± 7.0 |
| Cellulose | 52.7 ± 9.4 | 61.5 ± 13.4 |
| Hemicellulose | 57.8 ± 9.1 | 31.5 ± 3.8 |
| Lignin | 9.9 ± 8.5 | 86.4 ± 9.5 |

Increasing protein and decreasing sugar content was also observed by Bougrier et al. (2008) for sludge treated at 170 °C; protein content increased when digested slurries were autoclaved at 120 °C (Menardo et al. 2011) and when restaurant garbage was hydrothermally treated in temperatures from 100 to 180 °C (Ren et al. 2006). According to Ren et al. (2006) during hydrothermal treatment carbohydrate, especially starch, starts to deform and hydrolyses to reducing sugars. Subsequently, at high temperatures the reduced sugars form refractory compounds with proteins, more specifically with amino acids (Bougrier et al. 2008, Ren et al. 2006). These kind of compounds are formed, for example, through the Maillard reaction where the colour of the autoclaved substrate darkens and its odour changes, effects that are observed in this substrate (Figure 1) as well as in sludge and MSW when autoclaved (Bougrier et al. 2008, Takashima & Tanaka 2008). Maillard compounds start to form at temperatures around 100 °C and the formation is also highly dependent on the retention time as described by Nursten (2005) and Müller (2001). Formation of these compounds changes the biodegradability of the material making it harder or even impossible to degrade (Bougrier et al. 2007, 2008), which can also lead to a decrease in nutrients such as $NH_4$-N (Ren et al. 2006).

## 3.2 CSTR trials

### 3.2.1 Reactor operation

At the time of writing, the CSTR study with control and autoclaved food waste has been running for 470 days. During this period the OLR has been raised from 2 to 6 kg VS $m^{-3}$ $day^{-1}$, leading to a decrease of HRTs to about one third of the starting value. Table 3 presents a summary of the loading rates and average methane yields in the original reactors R1 (control) and R3 (autoclaved), and also for the parallel reactors R2 (control) and R4 (autoclaved) which started operation later. Most of the results presented in this report are from the longer-running reactors R1 and R3 because the data from these reactors is more comprehensive.

**Table 3.** Loading rates, retention times and methane yields in reactors R1, R2 (control) and R3, R4 (autoclaved).

| | OLR (kg VS $m^{-3}$ $day^{-1}$) | Days (d) | HRT (days) | Yield ($m^3$ $CH_4$ $tonne^{-1}$ VS) |
|---|---|---|---|---|
| R1 | 2 | 19-150 | 117 | 446 ± 54 |
| R3 | | | 94 | 394 ± 42 |
| R1 | 3 | 151-255 | 78 | 491 ± 33 |
| R2 | | 1-91 | | 527 ± 23 |
| R3 | | 151-255 | 63 | 434 ± 33 |
| R4 | | 1-91 | | 470 ± 15 |
| R1 | 4 | 256-417 | 58 | 500 ± 21 |
| R3 | | | 47 | 464 ± 25 |
| R1 | 6 | 418-439 | 39 | 468 ± 21 |
| R2 | | 92-439 | | 464 ± 4 |
| R3 | | 418-439 | 31 | 428 ± 14 |
| R4 | | 92-439 | | 416 ± 8 |

Weekly methane production during operation at an OLR of 2 kg VS $m^{-3}$ $day^{-1}$ was on average 446 and 395 $m^3$ $CH_4$ $tonne^{-1}$ VS for reactors R1 and R3 treating control and autoclaved waste, respectively (Table 3, Figure 5). At OLR 3 kg VS $m^{-3}$ $day^{-1}$ methane production for the control R1 was on average 491 $m^3$ $CH_4$ $tonne^{-1}$ VS and for the autoclaved waste R3 434 $m^3$ $CH_4$ $tonne^{-1}$ VS. In the parallel reactors methane yields were higher (527 $m^3$ $CH_4$ $tonne^{-1}$ VS in R2 and 470 $m^3$ $CH_4$ $tonne^{-1}$ VS in R4), but this difference can be explained by the different start-up strategies and inoculums, as the methane production in the parallel reactors showed a decreasing trend during this acclimation phase. As expected the increase of OLR from 3 to 4 kg VS $m^{-3}$ $day^{-1}$ in R1 and R3 did not greatly change the methane yield (500 $m^3$ $CH_4$ $tonne^{-1}$ VS for control, 464 $m^3$ $CH_4$ $tonne^{-1}$ VS for autoclaved FW). The methane concentration in both reactors varied between 55 and 65 % during the experiment. When the OLR was raised to 6 kg VS $m^{-3}$ $day^{-1}$ the methane yield and methane concentration started to decrease in all reactors (468 and 464 $m^3$ $CH_4$ $tonne^{-1}$ VS in control reactors, 428 and 416 $m^3$ $CH_4$ $tonne^{-1}$ VS in autoclaved), and the difference in $CH_4$ yields between original and parallel reactors was equalized. Whether the reactors will adapt to OLR 6 kg VS $m^{-3}$ $day^{-1}$ will be seen as the experiment continues; if so even higher OLRs may be applied. It should be remembered however that a loading of 6 kg VS $m^{-3}$ $day^{-1}$ is considerably higher than was possible with for this substrate at the start of the VALORGAS project, and is similar to the highest currently being achieved in the trials at Soton using the same trace element addition.

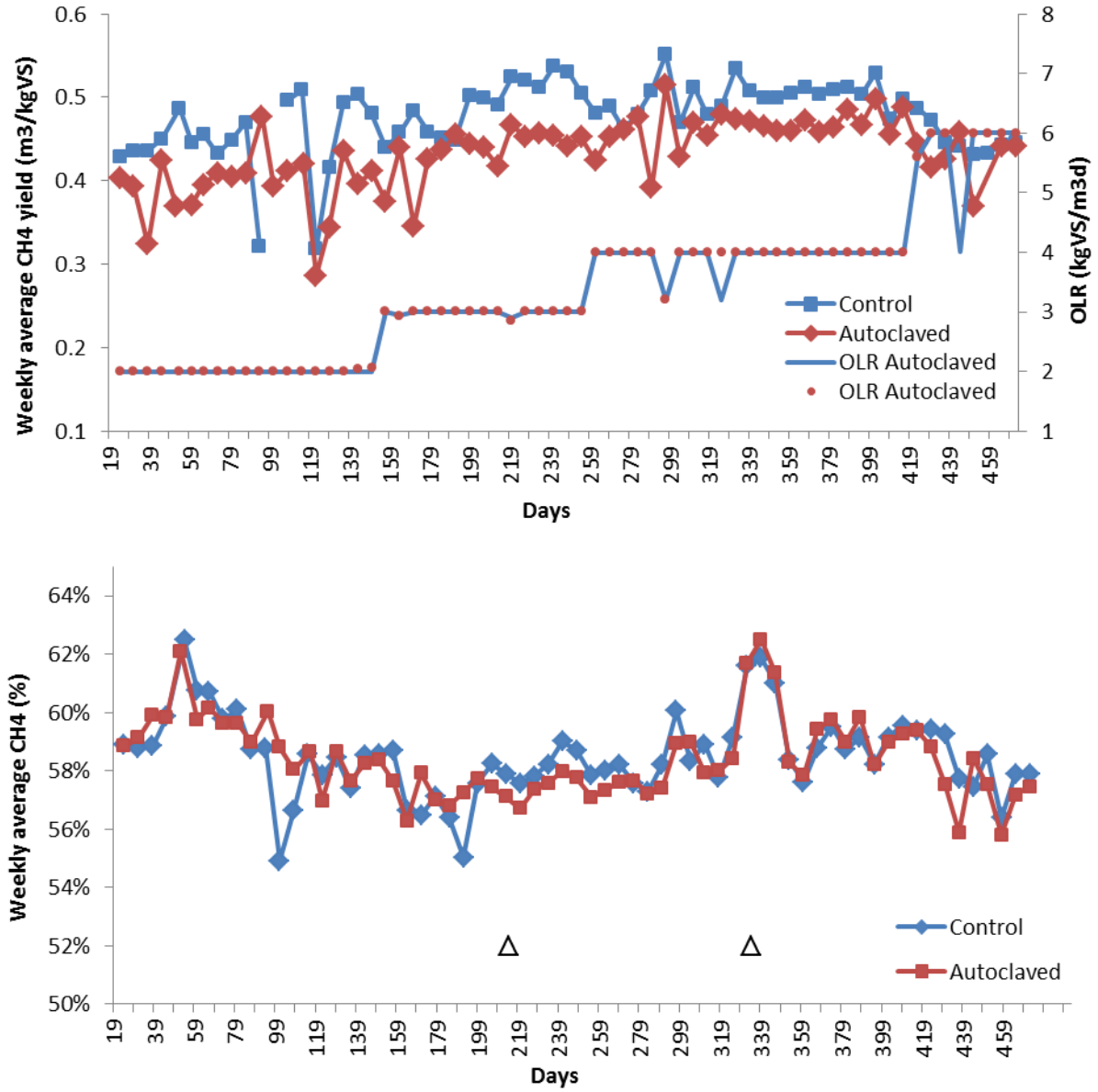



**Figure 5.** Weekly $CH_4$ yields and concentrations during days 19-388 in reactors R1 and R3 treating control and autoclaved food waste, respectively (Δ=change of $CH_4$ concentration measurements).

Key digestion parameters (pH, VFA, TS, VS, SCOD, $NH_4$-N, TKN) during the experiment are presented in Figure 6. The pH in the control reactor remained relatively stable (around 7.8) throughout the experiment. In the autoclaved reactor pH slowly decreased with increasing loading rate; from pH 7.6 at OLR 2 kg VS $m^{-3}$ $day^{-1}$ to 7.3 at OLR 6 kg VS $m^{-3}$ $day^{-1}$. The ammonium-N concentrations show a clear difference between control and autoclaved reactors. The $NH_4$-N concentration in control reactor increased during the first 100 days was close to 4 g N $l^{-1}$ and thereafter remained constant. In the autoclaved reactor, however, ammonium-N decreased from 2.2 g N $l^{-1}$ to 2 g N $l^{-1}$ when the OLR was increased to 3 kg VS $m^{-3}$ $day^{-1}$ and subsequently to 1.4 g N $l^{-1}$ on average when the loading was further increased to 4 kg VS $m^{-3}$ $day^{-1}$.

The decrease in ammonification, i.e. in formation of $NH_4$-N during anaerobic digestion, in the autoclaved reactor in compared to the control may be a result of the effect of autoclaving

on the hydrolysis of proteins in the autoclaved food waste. It is also known that ammonium-N concentration increases the buffer capacity of the material during digestion (Procházka et al. 2012) which explains the pH differences in the reactors: the control reactor with ammonium-N concentration of ~4 g N $l^{-1}$ had higher and more stable pH than the autoclaved reactor. High ammonium-N concentration can also inhibit the digestion process, but it is greatly dependent on the materials and acclimation times used (Chen et al. 2008, Procházka et al. 2012).

Total nitrogen content in both reactors increased during the experiment. The starting concentration was 4.9 g N $kg^{-1}$ (inoculum) and in both reactors the TKN content increased to ~7 g N $kg^{-1}$ in less than 150 days. In the control reactor R1 TKN content continued to increase slowly to a current concentration of 9 g $kg^{-1}$ while in R3 (autoclaved) the TKN content is slightly lower at about 8 g N $kg^{-1}$. As well as TKN, the TS and VS contents increased during the study. A probably explanation is an increase in the quantity of microbial biomass present in proportion to the increased food supply. It is also possible that some accumulation occurred because the digestate outflow was from top of the reactors so that the material may have stratified slightly despite continuous mixing.

The total VFA concentration remained relatively low (<250 mg $l^{-1}$) during the runs with OLR 2 kg VS $m^{-3}$ $day^{-1}$. When the loading was increased to 3 kg VS $m^{-3}$ $day^{-1}$, VFA increased to over 1500 mg $l^{-1}$ in the control reactor. In the autoclaved reactor the VFA concentration also rose but to a smaller extent and showed a more rapid recovery. At the time of the loading increase, the ammonia concentration in the control reactor had risen to around 4 g N $l^{-1}$, a level found to critical in the studies at Soton (Banks et al., 2011), while the concentration in the autoclaved reactor was only around 2.3 g N $l^{-1}$; this could explain the higher VFA seen in the control. In both control and autoclaved reactors acetic acid was the main constituent of the peak, with only a small accumulation of propionic acid. It was not until trace elements were added (selenium and cobalt on day 179 and full supplementation from day 199) that the control reactor stabilised. By day 233 VFA concentrations in both reactors were under 200 mg $l^{-1}$. SCOD concentrations in both reactors rose at similar rates and were not related to VFA concentrations. This phenomenon has been noted previously at long retention times and in digestion of solid substrates (e.g. Kuo et al., 1996; Rincon et al., 2011), and is being investigated in the VALORGAS project.

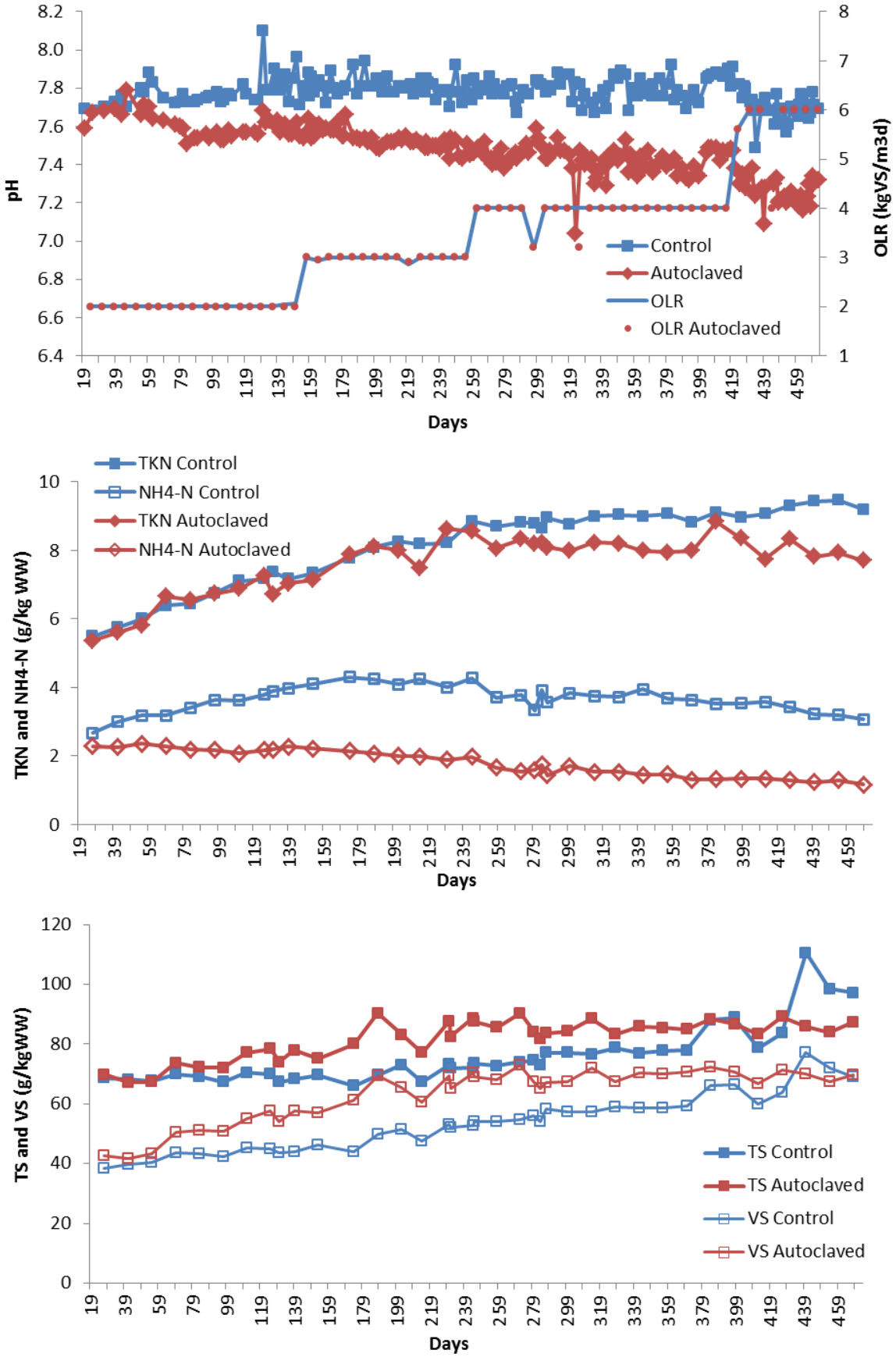



**Figure 6.** Reactor parameters from reactors R1 and R3 during days 19-470.

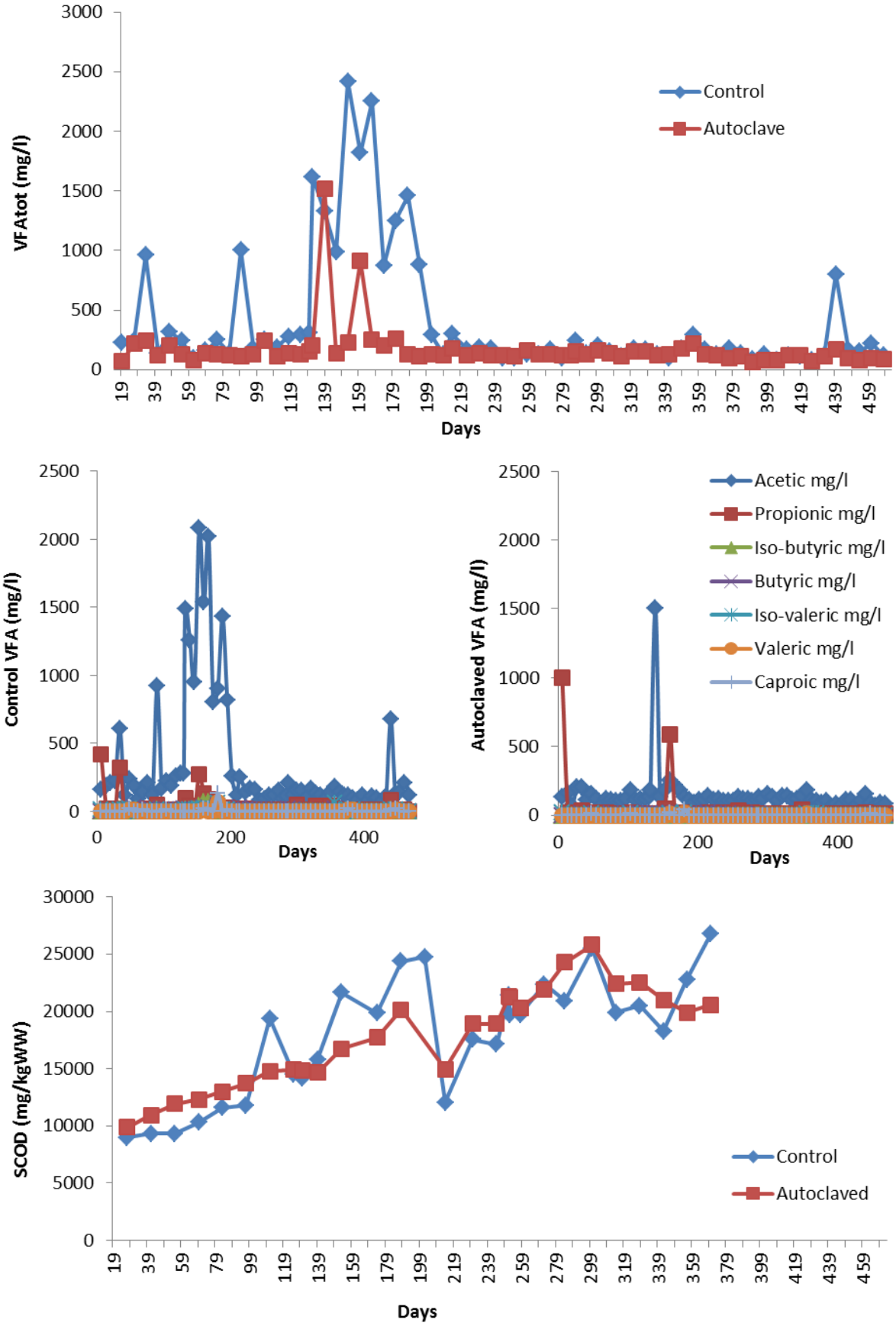



**Figure 6.** Reactor parameters from reactors R1 and R3 during days 19-470

### 3.2.2 Hydrogen sulphide

The hydrogen sulphide ($H_2S$) concentrations at OLR 3 and 4 kg VS $m^{-3}$ $day^{-1}$ are presented in Figure 7. Due to the lack of data results are shown only from day 166 to day 313. $H_2S$ concentrations at OLR 3 kg VS $m^{-3}$ $day^{-1}$ were relatively low in the control (>100 ppm) and autoclaved reactors (>75 ppm). After the OLR was increased to 4 kg VS $m^{-3}$ $day^{-1}$ $H_2S$ concentrations in the control reactors started to increase and reached 500 ppm, after which $H_2S$ data was no longer available. In the autoclaved reactor $H_2S$ concentrations remained low (>60 ppm).

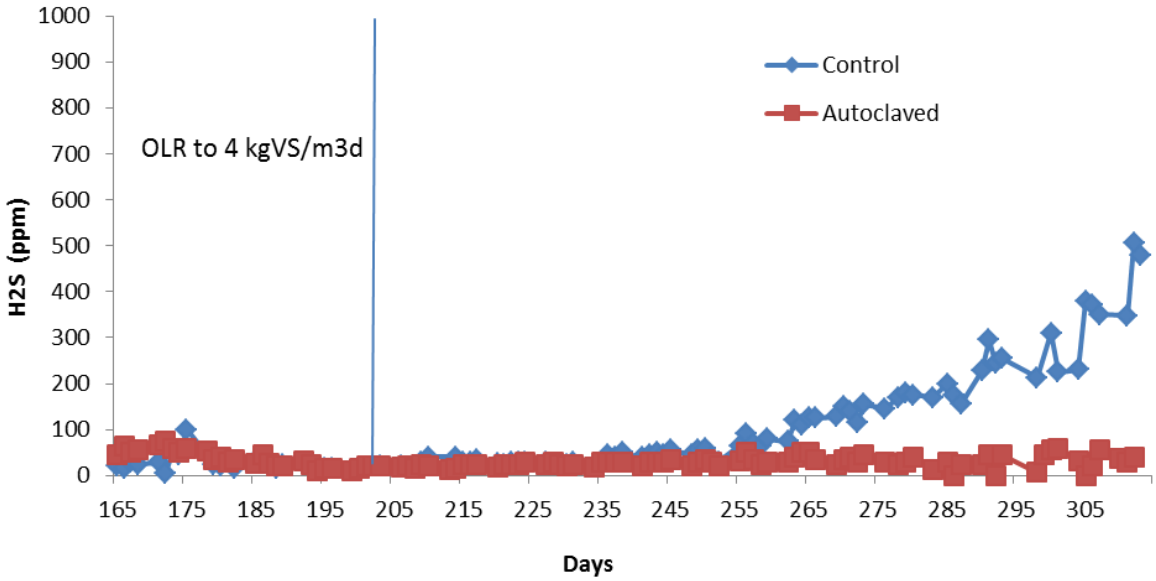



**Figure 7.** Hydrogen sulphide concentrations in control and autoclaved reactors during days 166-314.

A number of factors could have contributed to the increasing $H_2S$ concentrations in the control reactor. Firstly, the amount of sulphur entering the digester is higher due to the increased amount of daily feed; as sulphur-containing proteins are broken down soluble sulphides and $H_2S$ will be generated in increasing quantities. This is accompanied by a decrease in HRT, which may give sulphate-reducing bacteria (SRB) an additional competitive advantage. In the autoclaved reactor the lower $H_2S$ concentrations could be due to the autoclaving treatment affecting the proteins in the food waste and thus reducing the availability of sulphur. Other causes for low $H_2S$ concentrations are related to iron concentrations and to pH. It is known that iron forms iron sulphides and in this way decreases $H_2S$ formation. The iron content of the autoclaved FW was 22.7 ± 12.5 g $kg^{-1}$ TS which is 240 times higher than in the control FW (0.13 ± 0.014 g $kg^{-1}$ TS), possibly due to metal residues from the autoclave. SRBs are also affected by pH and according to O'Flaherty et al. (1998) have higher optimum pH levels than methane-producing bacteria. The lower pH in the autoclaved reactor may thus have inhibited the growth of SRBs causing lower $H_2S$ concentrations.

### 3.2.3 Biosecurity of the CSTR digestates

Biosecurity aspects of the FWs and the digestates were tested with hygiene indicators *E. coli*, other coliforms, total coliforms, enterococcus and sulphite-reducing clostridia. As expected,

autoclave treatment was found to be very effective and all hygiene indicator concentrations were under the detection limit (5 cfu $g^{-1}$). From the control FW only a few colonies of *E. coli* were discovered and no other coliforms. However, Enterococci (3.72 x $10^3$ cfu $g^{-1}$) and Clostridia (3.82 x $10^4$ cfu $g^{-1}$) were both discovered in the control FW samples (Figure 9).

Total coliforms in the CSTR reactor digestates were below the detection limit. Clostridia were detected in concentrations of 1.63 x $10^2$ cfu $g^{-1}$ in the reactor fed with autoclaved FW and 1.11 x $10^3$ cfu $g^{-1}$ in the control reactor. Concentrations of Enterococcus were discovered to be quite high, at 7.64 x $10^8$ and 2.64 x $10^8$ cfu $g^{-1}$ in reactors fed with control and autoclaved FW, respectively. Altogether, hygiene indicator concentrations between the digestates were quite similar, but very different when compared to the FW samples. This result suggests there may be some survival from the original inoculums or other minor sources of contamination, and the conditions in the digester are favourable for these organisms to multiply.

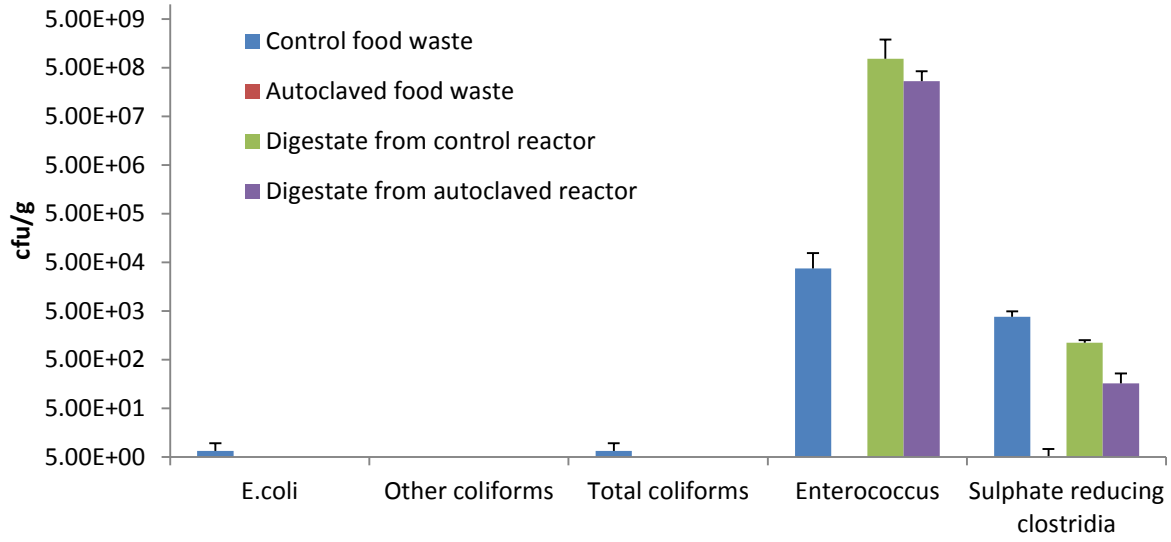



**Figure 9.** Hygiene indicator concentrations in reactor feeds and digestates.

The European Union's regulations concerning animal by-products (European Parliament and the Council 2009, European Commission 2011) and their digestion residues give acceptable values for *E. coli* or Enterococcaceae of 1000 cfu $g^{-1}$ when no Salmonella is detected in a 25 g sample. According to these regulations both the control and autoclaved digestates are suitable for land application in terms of their *E. coli* or Enterococcaceae content. It must be noted, however, that Salmonella was not analysed.

## 3.3 Effect of cell disruptor treatment

### 3.3.1 Microscopic examination, enzyme analysis and UV absorbance of treated and untreated cells in pure culture

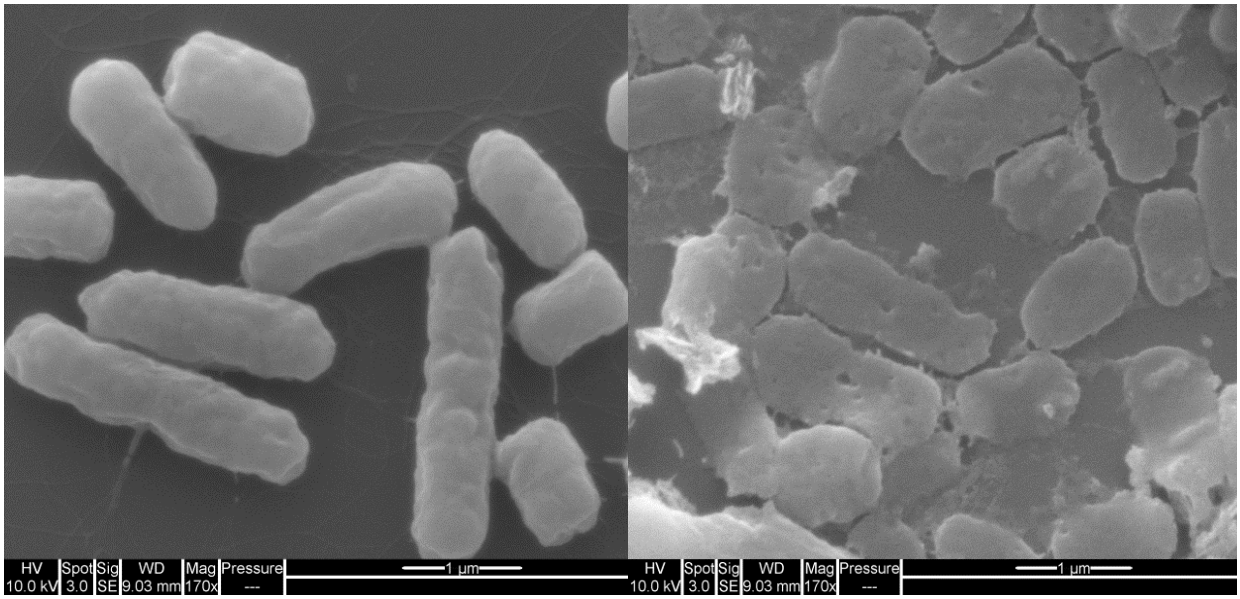
To investigate the effect of $CO_2$ pressurisation on *S. enterica* cells the process was tested on a pure culture grown to a cell density of $10^6$ cfu $ml^{-1}$ in Buffered Peptone Water and treated at 28 bar for 24 hours. Cell suspensions were then prepared for SEM and TEM as described above.

Figure 10 shows SEM images of untreated and treated *S. enterica* . The treated cells showed clear signs of rupture and damage compared to the relatively smooth appearance of untreated cells. The cells appeared flattened in comparison with the three dimensional appearance of normal cells, and perforations to the surface are clearly visible indicating the potential for loss of cell contents. In the TEM images (Figure 11) the cytoplasm in untreated cells is evenly distributed whereas the treated cells showed empty spaces in the cytoplasm, separation of the cell membrane and uneven distribution of cell components. Some cells were completely ruptured and their contents lost. On the basis of the electron micrographs, it is evident that the cells are mechanically damaged during the treatment process.

Although most work on $CO_2$ pressurisation has been carried out at much higher pressures (Garcia-Gonzalez et al., 2007), there is growing evidence that cell damage can also be induced within the pressure range used in the current work. This effect has also been shown using pure cultures of *E. coli* in which physical cells damage was also observed (Mushtaq et al, 2012).

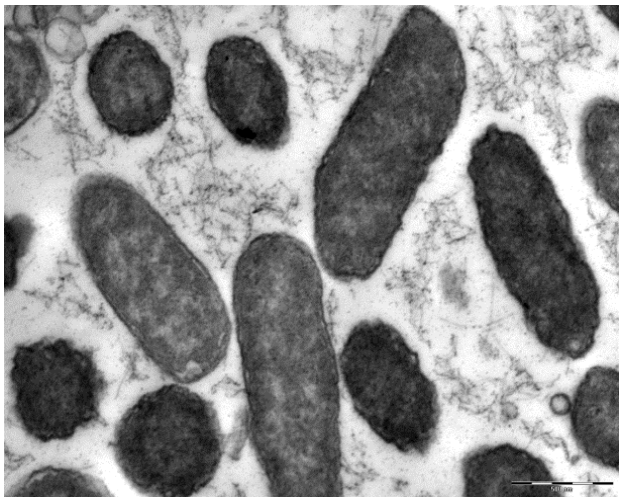
This view is further supported by the results for enzyme activity and spectrophotometric observation of culture medium to identify cell lysis products, which were carried out on both *S. enterica* and *E. coli*. The enzymes active in the untreated pure culture of *S. enterica* were alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, acid phosphatase, naphthol-AS-B1- phosphohydrolase and α glucosidase; and in untreated pure culture of *E. coli* were alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-B1-phosphohydrolase and β glucosidase. None of these enzymes were active in either of the treated cultures. UV absorbance in the centrifuged supernatant from treated cells was higher than for untreated cells for both *S. enterica* and *E. coli* (Table 4) indicating a loss of proteins and other UV-absorbing substances as a consequence of treatment.

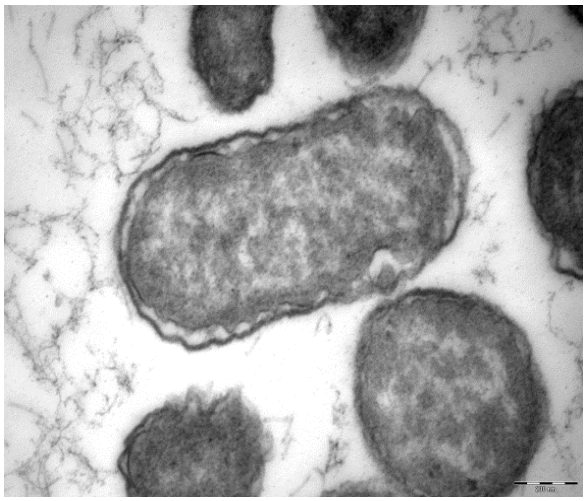
Although the current results do not rule out other effects of $CO_2$ pressurisation on the cell such as changes in intra-cellular pH it is clear that physical disruption of the cell wall and membrane plays a major role in inactivation.



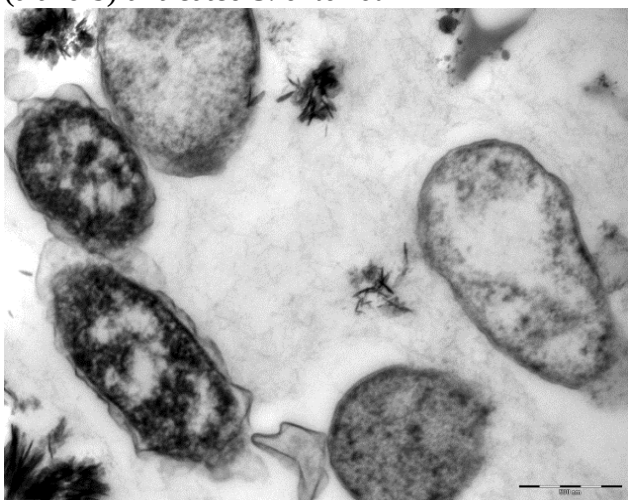

(a) untreated *S. enterica* (b) treated *S. enterica* (28 bar, 24 hours)

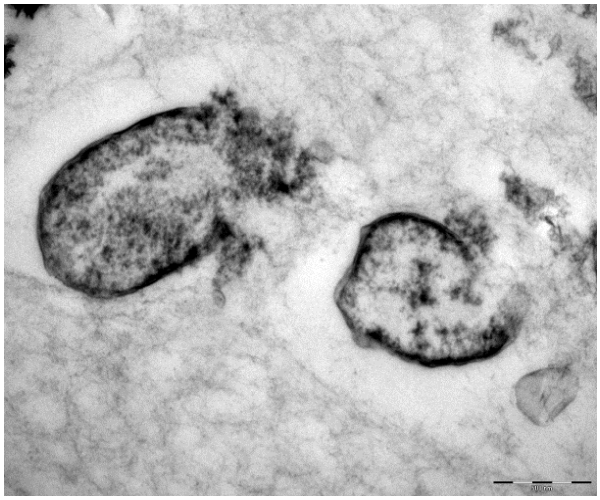
**Figure 10**. SEMs of untreated and treated *S. enterica*





(a and b) untreated *S. enterica*





(c and d) treated *S. enterica* (28 bar, 24 hours)

**Figure 11**. TEMs of untreated and treated *S. enterica*

**Table 4. UV absorbance by untreated and treated supernatant**

| | ***S. enterica*** | | ***E. coli*** | |
|---|---|---|---|---|
| Wavelength | Control | Treated | Control | Treated |
| 260 nm | 0.49 | 0.73 | 0.06 | 1.23 |
| 280 nm | 0.23 | 0.45 | 0.05 | 0.90 |

### 3.3.2 Inactivation of *Salmonella enterica* spiked into dilute biowastes

Inactivation of *Salmonella enterica* serovar *typhimurium* was studied in saline water and in diluted biowaste. The number of *S. enterica* at the beginning of the experiment was approximately 1 x $10^6$ cfu $ml^{-1}$. In saline water Salmonella inactivation was increased by extending the treatment time. A 2-hour treatment had no effect on concentration while 24-hour treatment decreased *S. enterica* concentration on average 6 logs. Salmonella pure culture in saline water was completely inactivated with a treatment time of 24 at 35 °C. At room temperature and with shorter treatment times total inactivation of *S. enterica* was not achieved. Salmonella concentration in the control sample at 35 °C was equivalent to the

concentration in the control sample at room temperature. Results from the pressure treatment of *S. enterica* pure culture in saline water are seen in Table 5 and Figure 12.

**Table 5**. Number of *Salmonella enterica* in pressure treated saline water samples (treatment temperature 20 °C or 35 °C) and in controls at 20 °C and 35 °C (treatment temperature 35 °C).

| Treatment time | Control cfu ml$^{-1}$ | Control, 35°C cfu ml$^{-1}$ | Pressure treated cfu ml$^{-1}$ |
|---|---|---|---|
| 2 hours, 20 °C | 4.70E+06 | | 8.09E+05 |
| 6 hours, 20 °C | 8.73E+06 | | 1.02E+04 |
| 15 hours, 20 °C | 6.73E+07 | | 4.21E+02 |
| 24 hours, 20 °C | 1.19E+08 | | 6.98E+01 |
| 24 hours, 35 °C | 1.37E+08 | 3.81E+08 | 0.00E+00 |

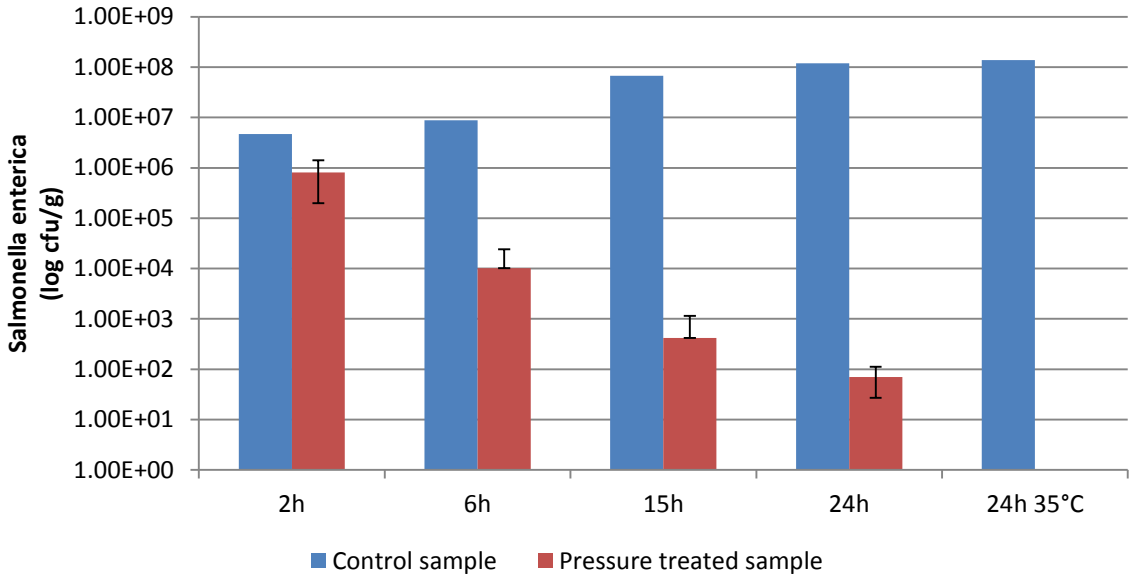



**Figure 12**. Amount of *Salmonella enterica* in control and pressure treated samples. Treatment pressure 25 bars, treatment temperature 20 °C and 35 °C ± 2 °C.

*S. enterica* was spiked into autoclaved and unautoclaved biowaste samples. The characteristics of the biowastes used are given in Table 6.

**Table 6.** Characteristics of materials used in *Salmonella enterica* inactivation experiments.

| | BW 5% autoclaved | BW 5% unautoclaved |
|---|---|---|
| pH | 5.11 ± 0.11 | 4.36 ± 0.47 |
| TS (g kg$^{-1}$ WW) | 48.7 | 47.4 |
| VS (g kg$^{-1}$ WW) | 44.7 | 44.8 |
| VS/TS (%) | 91.8 | 94.5 |

Inactivation of *S. enterica* in diluted biowaste was not as effective as in saline water pure culture, with a 1 log reduction after 24 hours at 20 $^{o}$C and 4 log reduction at 35 $^{o}$C for biowaste compared to 7 and 8 log reductions in the same conditions in saline water (Table 5 and 7). From autoclaved diluted biowaste *S. enterica* was not totally inactivated by any tested treatment. Raising the treatment temperature to 35 °C improved the inactivation efficiency significantly. Pressure treatments for unautoclaved biowastes were only carried out at 35 °C. In both the 15 and 24-hour treatments Salmonella was almost completely inactivated;

however, the controls at 35 °C also had a lower number of Salmonella than controls at room temperature, indicating that inactivation happened without the pressure-treatment. The differences in inoculum density and in the time and temperature conditions used prevented comparison between the autoclaved and unautoclaved biowastes.

**Table 7**. Number of *Salmonella enterica* in 5 % TS diluted biowaste samples.

| Material, treatment time and treatment temperature | Control, 20 °C cfu ml$^{-1}$ | Control, 35°C cfu ml$^{-1}$ | Pressure treated cfu ml$^{-1}$ |
|---|---|---|---|
| *Autoclaved* | | | |
| 24 hours, 20 °C | 2.83E+05 | | 4.05E+04 |
| 24 hours, 35°C | 1.11E+05 | 1.04E+05 | 2.50E+01 |
| *Unautoclaved* | | | |
| 15 hours, 35°C | 1.21E+05 | 2.53E+02 | < 5 |
| 24 hours, 35°C | 5.00E+02 | < 5 | < 5 |

The results for other hygiene indicating bacteria present in the diluted unautoclaved biowaste are given in Table 8 and only shown limited reductions.

**Table 8.** Hygiene indicating bacteria in treated diluted biowaste. (Salmonella results as in Table 7)

| | *S. enterica* cfu ml$^{-1}$ | *E. coli* cfu g$^{-1}$ | Other coliforms cfu g$^{-1}$ | Total coliforms cfu g$^{-1}$ | Faecal enterococci cfu g$^{-1}$ | Sulphite-reducing clostridia cfu g$^{-1}$ |
|---|---|---|---|---|---|---|
| 15 hours control | 1.21E+05 | 5.53E+03 | 1.43E+04 | 1.98E+04 | 2.42E+04 | 5.36E+02 |
| 15 hours control in 35°C | 2.53E+02 | 2.00E+01 | 9.72E+04 | 9.72E+04 | 1.99E+04 | 5.36E+02 |
| 15 hours, pressure treated | < 5 | 5.00E+00 | 1.16E+02 | 1.16E+02 | 2.91E+03 | 6.26E+02 |
| 24 hours control | 5.00E+02 | 2.55E+03 | 2.91E+03 | 5.45E+03 | 8.00E+03 | 5.95E+02 |
| 24 hours control in 35°C | < 5 | 5.00E+00 | 4.66E+04 | 4.66E+04 | 8.50E+02 | 7.30E+02 |
| 24 hours, pressure treated | < 5 | 5.00E+00 | 5.00E+01 | 5.00E+01 | 1.87E+02 | 7.14E+02 |

The results showed that Salmonella in saline water pure culture is destroyed by the pressurisation process at longer treatment times. However, with biowaste the impacts of pressurisation are low and or long treatment times are required. There is some indication that operation at higher temperature could improve the effects of pressurisation.

### 3.3.3 Forssa biowaste - pathogen indicators, solubilisation and BMP

The effects of cell disruption as a pre-treatment on the methane production, chemical characteristics and hygienic quality of biowaste from the Forssa region were tested at pressure of 25 bar and with treatment times of 10 min, 2 hours, 14 hours and 24 hours. Experiments were performed using biowaste with a TS of 30 %, biowaste diluted with tap water to a TS of 15 % and biowaste diluted with tap water to TS of 5 % (Table 9). Pressure, pH, temperature changes and visual effects (Table 10) were monitored during the experiments.

**Table 9.** Characteristics of materials used in $CO_2$ pressurisation experiments.

| | BW | BW 15% | BW 5% |
|---|---|---|---|
| pH | 5.10 ± 00.1 | 4.92 ± 0.04 | 4.34 ± 0.28 |
| TS (g $kg^{-1}$ WW) | 298.8 ± 10.89 | 147.9 ± 0.00 | 41.4 ± 1.27 |
| VS (g $kg^{-1}$ WW) | 262.0 ±10.54 | 130.9 ± 0.64 | 36.6 ± 1.56 |
| VS/TS (%) | 87.67 | 88.47 | 88.39 |
| SCOD (g $kg^{-1}$ WW) | 97.2 ±3.4 | 47.9 ± 0.24 | 13.5 ± 0.13 |
| TVFA (g $l^{-1}$) | 3.03 ±1.1 | 2.18 ±0.03 | 1.27 ± 0.2 |
| TKN (g N $kg^{-1}$ WW) | 6.92 ±0.08 | 3.58 ±0 | N/A |
| NH4-N (g N $kg^{-1}$ WW) | 0.14 ±0.16 | 0.14 ±0.02 | 0.07 ±0 |
| SMP ($m^3$ $CH_4$ $tonne^{-1}$ VS) | 425 ±10 | 422 ±3 | 396 ±0.8 |
| SMP ($m^3$ $CH_4$ $tonne^{-1}$ TS) | 371 ±9 | 372 ±3 | 353 ±0.8 |
| SMP ($m^3$ $CH_4$ $tonne^{-1}$ WW) | 108 ±3 | 55 ±0.4 | 15 ±0.03 |

The pressure at the start of every treatment was set to 25 bar, but dropped during one minute of mixing as well as during the treatment period. The pressure drop during mixing varied from 0.75 to 1.25 bar for biowaste, from 1.25 to 1.5 bar for 1:2 diluted biowaste, and from 1.0 to 1.5 for 1:6 diluted biowaste, indicating that the dissolution of $CO_2$ into the sample was more efficient in the diluted samples (Table 10). More significant pressure drops occurred during longer treatment times compared with shorter treatments, due to the longer time available for $CO_2$ absorption into the biomass. Temperature changes were dependent on the starting temperature, which varied from 6.8 °C to 24.4 °C in all samples. During testing of the equipment with pure water, the pressure drops were 2 bar after 1 min mixing and 2.5 bar after 10 min. After treatment the treated biowaste was effervescent, indicating dissolution of $CO_2$. In the diluted biowaste this visual effect was more evident as after pressurisation the waste samples bubbled intensively.

**Table 10.** Pressure, pH and temperature changes in biowaste, 1:2 diluted biowaste and 1:6 diluted biowaste during pressurisation experiments. The initial pressure was 25 bar.

| | **Pressure drop (bar)** | | **pH decrease**** | **Temperature (°C)** | |
|---|---|---|---|---|---|
| Biowaste | during mixing* | during treatment | | before | after |
| 10 min | 0.75 | 1.25 | N/A | 17.50 | 19.20 |
| 2 hours | 0.75 | 2.5 | N/A | 6.80 | 16.80 |
| 14 hours | 1 | 3 | N/A | 19.30 | 19.40 |
| 24 hours | 1.25 | 3 | N/A | 9.55 | 18.95 |
| 1:2 diluted biowaste | | | | | |
| 10 min | 1.25 | 1.5 | -0.03 | 14.40 | 16.65 |
| 2 hours | 1.5 | 2.25 | 0.15 | 15.50 | 19.55 |
| 14 hours | 1.25 | 2.25 | 0.67 | 19.60 | 21.25 |
| 24 hours | 1.5 | 3.5 | 0.66 | 11.85 | 20.85 |
| 1:6 diluted biowaste | | | | | |
| 10 min | 1.25 | 1.75 | -0.16 | 23.10 | 23.40 |
| 2 hours | 1.25 | 2.50 | 0.03 | 22.05 | 21.90 |
| 14 hours | 1.5 | 3.50 | 0.30 | 19.55 | 20.05 |
| 24 hours | 1 | 4.0 | 0.42 | 21.80 | 22.05 |

*Pressure drop during mixing is included in overall pressure drop
** pH-values measured immediately before and after treatments
N/A=not available

pH was measured immediately before and after pressure treatments with the 1:2 diluted biowaste samples, and with the biowaste samples immediately after the last pressure treatment. $CO_2$ pressurisation had a minor effect on biowaste pH, while in the experiments

with 1:2 diluted biowaste pH changes of up to 0.73 units were observed in individual samples. The changes indicated that $CO_2$ penetrated better into the diluted biowaste. Moreover, the pH fell more with longer treatment time which indicated carbonic acid formation. In 1:2 diluted biowaste samples the pH decreased by a maximum of 0.67 units (mean value of duplicates). The pH decrease with 1:6 diluted biowaste was lower than with 1:2 diluted biowaste, due to the lower initial pH of 1:6 diluted material.

**Pathogen indicators**

The effect of cell disruption on the hygienic quality of the biowaste was monitored using hygiene indicators (*E. coli*, total coliforms, other coliforms, enterococcus and sulphite-reducing clostridia) for untreated biowaste as well as for 10 min, 2 hour, 14 hour and 24 hour treatments. During the 14 and 24 hour treatments some reduction in *E. coli* and total coliforms was detected (Figure 13). 24-hour treatment achieved reductions of 1 log in *E. coli* and 2 log in total coliforms for both undiluted and diluted biowaste. Enterococcus and sulphite-reducing clostridia were not affected. The effects of storage time on the hygienic quality of control samples were not monitored for undiluted and 1:2 diluted biowaste.

**Chemical characterisation**

A series of chemical analyses indicating solubilisation, total VFA, soluble COD and $NH_4$-N were performed on the $CO_2$ pressure treated samples. As a control for the cell disruption experiments, chemical characterisation was carried out for freshly macerated biowaste as well as macerated biowaste stored for four days at 2 °C. TS, VS and TKN in the biowaste and 1:2 diluted biowaste remained unchanged during the four days of storage (data not shown). Storage did not affect the pH.

SCOD, VFA and $NH_4$-N concentrations showed some changes in the treated samples (Figure 14) but these were quite similar to those observed in the stored control samples during 4 days, and thus the effects of the studied pressurisation treatments were negligible. This was also supported by the very similar methane yields of the treated samples as compared to the control sample.

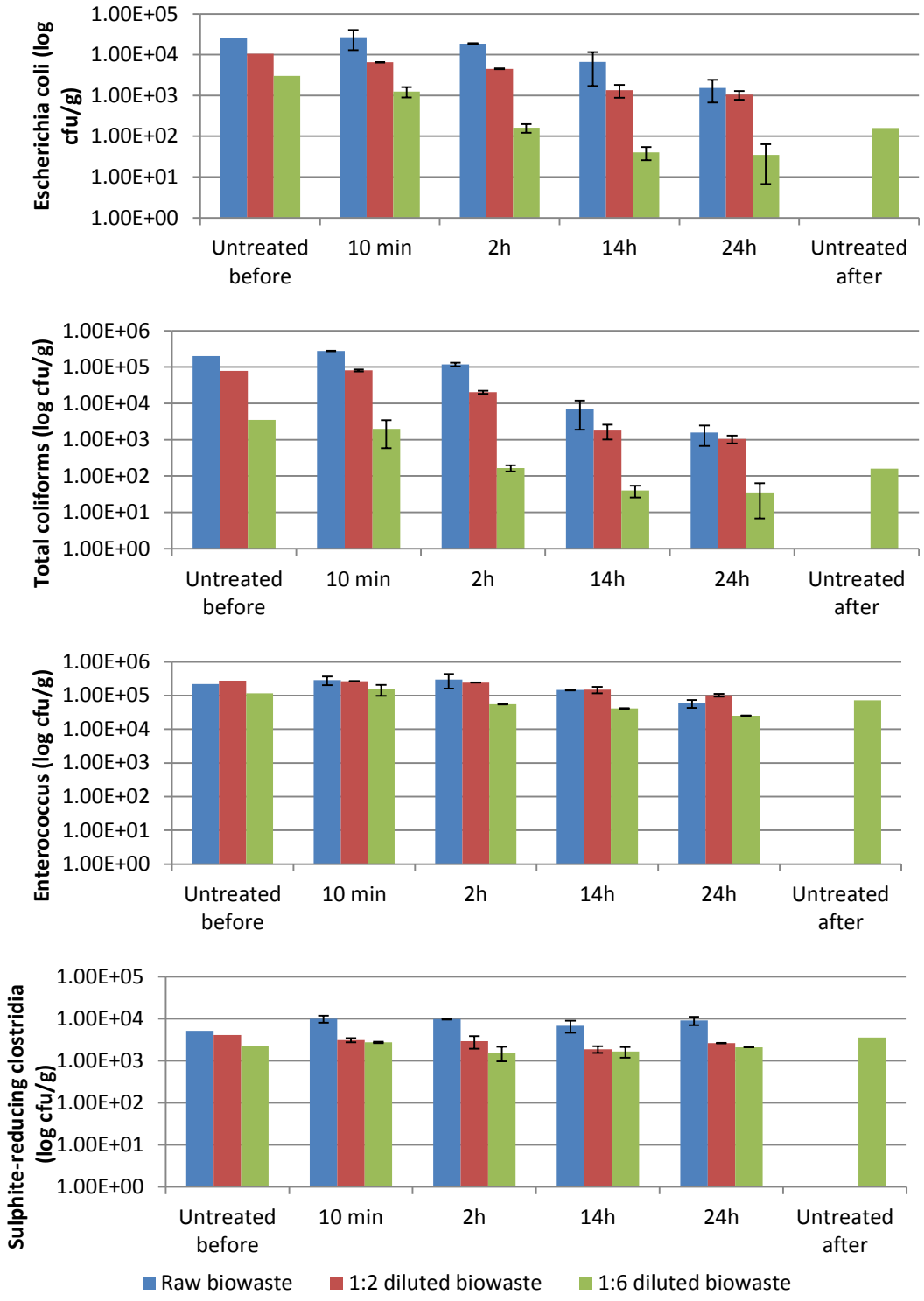



**Figure 13.** Amounts of *E .coli*, other coliforms, enterococcus and sulphite-reducing bacteria in biowastes treated at 25 bars for 10 min, 2, 14 and 24 h as well as in control samples in logarithmic scale including randomised standard deviations (log CFU $g^{-1}$ of wet weight).

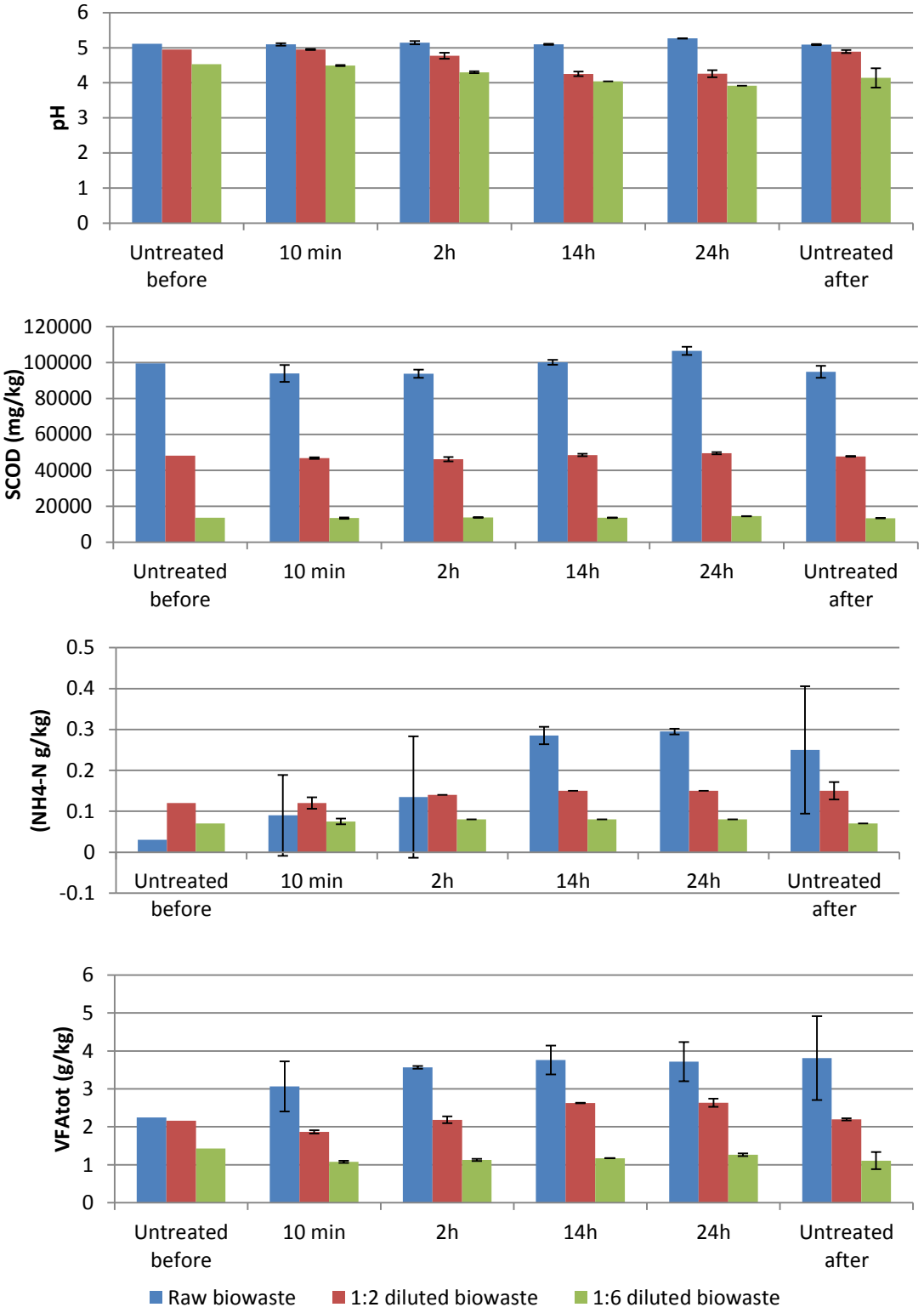



**Figure 14**. Effect of $CO_2$ pressurisation (10 min to 24 hours) on the pH, NH4-N, SCOD and VFAtot of biowaste as well as the effects of storage (4 °C, 4 days) on untreated samples. Averages with randomised standard deviations (RSD) are shown.

## Effects on methane potential

The methane potential of untreated biowaste and diluted biowastes was on average 414 $m^3$ $CH_4$ $tonne^{-1}$ VS. The effect of pressurisation treatment on methane potential as well as on the rate of methane production was insignificant (Figure 15). Dilution had no effect on methane potential, as seen in Table 11.

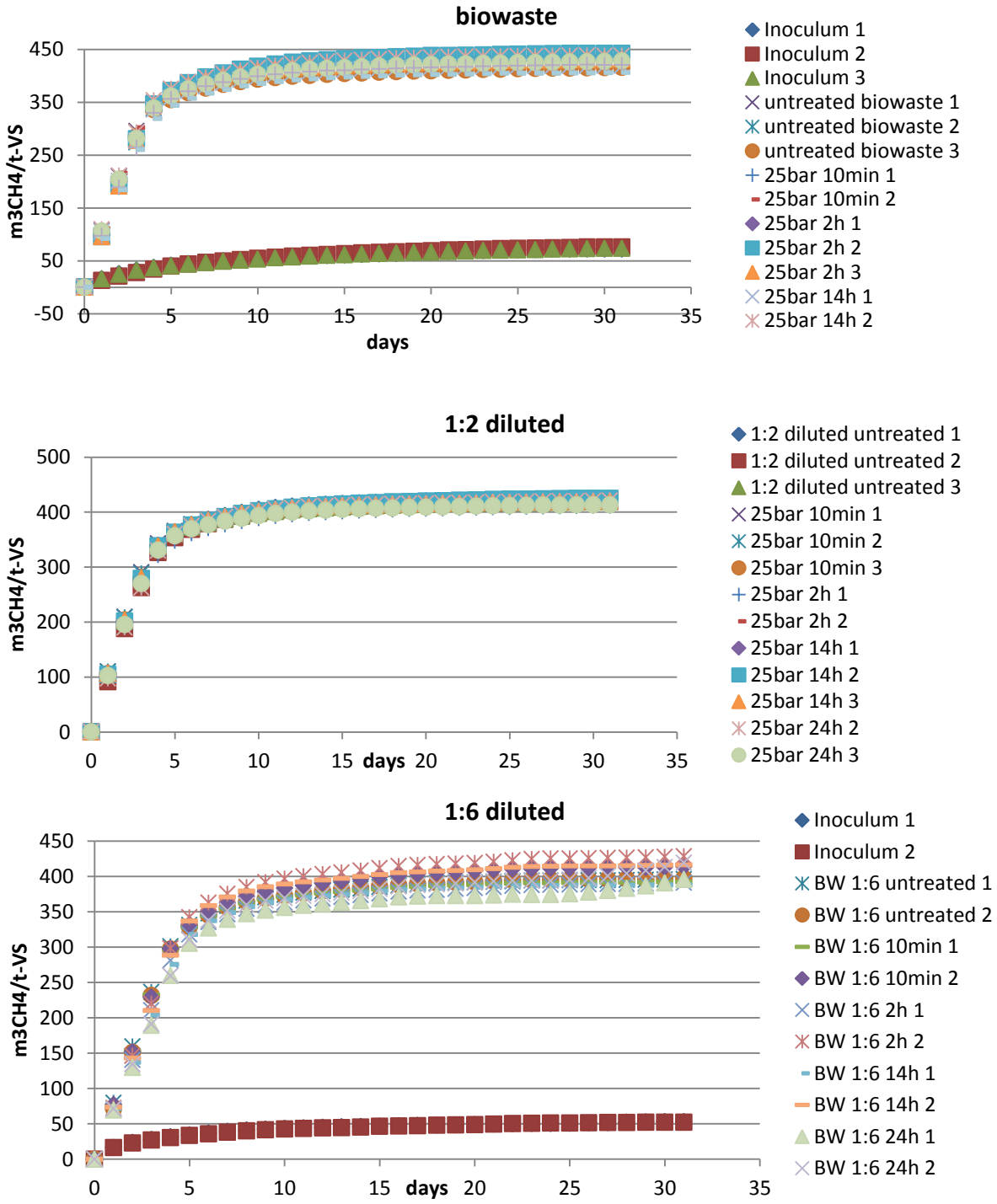



**Figure 15.** Specific methane productions of biowaste, 1:2 diluted biowaste and 1:6 diluted biowaste with different pressure treatment times

**Table 11.** Methane yields with standard deviations of the studied biowaste and diluted biowaste with different treatment times at 25 bar.

| | Untreated | 25 bar 10 min | 25 bar 2 h | 25 bar 14 h | 25 bar 24 h |
|---|---|---|---|---|---|
| *Raw biowaste* | | | | | |
| $CH_4$ (l) | 3.7 ±0.09 | 3.5 ±0.4 | 3.8 ±0.06 | 3.7 ±0.09 | 3.5 ±0.2 |
| $CH_4$ ($m^3$ $tonne^{-1}$ VS) | 425 ±10 | 406 ±47 | 435 ±7 | 428 ±10 | 401 ±23 |
| $CH_4$ ($m^3$ $tonne^{-1}$ TS) | 371 ±9 | 355 ±41 | 380 ±6 | 375 ±9 | 350 ±20 |
| $CH_4$ ($m^3$ $tonne^{-1}$ WW) | 108 ±3 | 103 ±12 | 111 ±2 | 109 ±3 | 102 ±6 |
| *1:2 diluted biowaste* | | | | | |
| $CH_4$ (l) | 3.7 ±0.03 | 3.7 ±0.006 | 3.4 ±0.3 | 3.7 ±0.03 | 3.5 ±0.2 |
| $CH_4$ ($m^3$ $tonne^{-1}$ VS) | 422 ±3 | 423 ±1 | 394 ±29 | 421 ±4 | 404 ±23 |
| $CH_4$ ($m^3$ $tonne^{-1}$ TS) | 372 ±3 | 373 ±1 | 347 ±25 | 371 ±3 | 356 ±20 |
| $CH_4$ ($m^3$ $tonne^{-1}$ WW) | 55 ±0.4 | 55 ±0.09 | 51 ±4 | 55 ±0.5 | 53 ±3 |
| *1:6 diluted biowaste* | | | | | |
| $CH_4$ (l) | 2.6 ±0.006 | 2.6 ±0.1 | 2.7 ±0.2 | 2.6 ±0.1 | 2.7 ±0.1 |
| $CH_4$ ($m^3$ $tonne^{-1}$ VS) | 396 ±0.8 | 403 ±15 | 410 ±26 | 402 ±20 | 407 ±17 |
| $CH_4$ ($m^3$ $tonne^{-1}$ TS) | 353 ±0.8 | 359 ±14 | 365 ±23 | 358 ±18 | 363 ±15 |
| $CH_4$ ($m^3$ $tonne^{-1}$ WW) | 15 ±0.03 | 15 ±0.6 | 15 ±1 | 15 ±0.8 | 15 ±0.6 |

## 4 Conclusions

Autoclaving of food waste appeared to result in a slightly lower specific methane yield in semi-continuous digester operation at loadings of up to 6 kg VS m-3 day-1. Autoclaving also resulted in lower concentrations of ammonia in the digestate and of hydrogen sulphide in the biogas. This may be due to the formation of recalcitrant compounds from proteinaceous material which immobilises some of organic nitrogen present in the feedstock. Without trace element supplementation, digestion of untreated waste was more sensitive to a shock loading increase, as evidenced by a peak in volatile fatty acids; this is probably linked to the higher ammonia concentration. Digesters fed on both autoclaved and untreated feedstock showed accumulation of soluble COD in the digestate which was not in the form of volatile fatty acids.

The effects of $CO_2$ pressurisation were evident in pure culture where treatment at 25 bar for periods of only a few hours resulted in substantial die-off of selected hygiene indicator organisms. Pure culture work using Salmonella enterica and Escherichia coli also showed cell damage as a result of the treatment confirmed by microscopic examination, loss of enzyme activity and release of cellular proteins into the surrounding medium. In food waste and digestates, however, the effects were greatly reduced. There was no visible effect on specific methane yield from the pressurisation process.

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