Determination of methanogenic pathways through stable carbon isotope analysis in biogas production plants: Experimental and simulation studies for the two-stage fermentation of high-solids substrates

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Von der Fakultät für Bau- und Umweltingenieurwissenschaften der Ruhr-Universität Bochum zur Erlangung des akademischen Grades eines Doktors der Ingenieurwissenschaften (Dr.-Ing.) genehmigte Dissertation

vorgelegt von

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               Prof. Dr. Maria de Lourdes Florencio dos Santos

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Abstract

Anthropogenic activities severely affect the atmospheric levels of methane (CH$_4$), which increased by 150% since 1850. This is a very concerning issue as CH$_4$ is the most important atmospheric greenhouse gas (GHG) after carbon dioxide (CO$_2$). Approximately 20% of the CH$_4$ anthropogenic emissions derive from uncontrolled anaerobic degradation of organic waste in landfills. Nevertheless, the recovery of this CH$_4$ as fuel gas is possible through the fermentation of organic waste in biomethanation plants, thus, combining the mitigation of GHG emissions with the production of renewable energy. A very broad range of substrates are suitable for the conversion of CH$_4$ in such biomethanation plants, including organic waste (municipal, industrial and agricultural) and several types of plant biomass (terrestrial and aquatic) which can be added for enhanced energetic output. In addition, the digestate from these fermentation plants retains important nutrients, such as phosphorous (P) and nitrogen (N), which present an important alternative for industrialized fertilizers. The production of N$_2$ fixation for fertilizers, for instance, consumes about 2% of the global energy, while reserves of phosphate rocks for P extraction are becoming scarce. Consequently, the potential for anaerobic digestion-based technologies is enormous. However, the further expansion of these technologies depends on an efficient, predictable and stable process. This requires a comprehensive understanding of the anaerobic digestion processes.

In order to accurately describe the carbon flow in anaerobic digestion processes, this thesis investigates the methanogenic and acetate degradation pathways in a broad way. Primarily, it aims to identify and describe the contributions of the acetoclastic and hydrogenotrophic methanogenesis in anaerobic bioreactors. In order to differentiate the processes of both pathways, stable carbon isotope ratio analysis ($\delta^{13}$C) were performed, which, despite being used to identify the CH$_4$ precursors from several atmospheric CH$_4$ sources, presents a novel methodology for the investigation of anaerobic bioreactors. Additionally, batch assays using labeled $^{13}$C acetate were employed to distinguish the acetate consumption through methanogenic Archaea and acetate-oxidizing Bacteria.

A model plant was designed for these investigations, which was composed of three thermophilic (51 – 56 °C) leach bed reactors (LBRs) followed by two mesophilic (36.5 °C) acetogenic/methanogenic reactors: an upflow anaerobic filter (UAF) and a continuously stirred tank reactor (CSTR), operated for the control of the $\delta^{13}$C analysis. In sum, the total liquid volume of all reactors of the two-stage plant comprised 54 L: 27 L in the three LBRs, 9 L in the control CSTR, 11 in the UAF and 7 L in the UAF storage tank (STR$_{UAF}$). The UAF was designed to allow for a representative sampling of sessile biomass through a removable biofilm carrier system (50 strip, each 395 x 18 x 3 mm). Thus, batch assays
with biofilm and suspended biomass could be carried out separately. The specific methanogenic activity (SMA) and the acetate degradation pathway quantifications for each biomass group were determined in 300 mL batch assays vessels. In an additional effort to support the analysis, as well as the experimental design of the batch assays, a three-process model for aceticlastic methanogenesis and syntrophic acetate oxidation (SAO) was developed. The model distinguishes carbon atoms in light and heavy isotopes, $^{12}$C and $^{13}$C, respectively, which permitted the simulation of the isotope ratios variation in addition to gas production, gas composition and acetate concentrations. This model allows for a precise quantification of the syntrophically oxidized acetate fraction.

The influence of organic loading rates (OLR) on the different methanogenesis pathways was examined for the two-stage experimental plant. The LBRs were operated at increasing OLRs, from 1.0 to 3.9 g$_{COD}$ L$^{-1}$ d$^{-1}$ in batch-fed modus, with weekly feeding. This resulted in highly dynamic OLRs for the UAF, which varied from 1.7 to 16.8 g$_{COD}$ L$^{-1}$ d$^{-1}$, while the control CSTR was operated continually under 1.0 g$_{COD}$ L$^{-1}$ d$^{-1}$. At higher OLR stages, up to 23 L$_{CH_4}$ d$^{-1}$ were produced in the two-stage plant, whereby 50 – 55 % produced by the UAF. This corresponds to a maximal specific methane production of 1.0 L$_{CH_4}$ L$_{reactor}$ d$^{-1}$. Nonetheless, an enhanced performance is expected with the use of higher biofilm specific superficial areas. The designed removable biofilm carrier system resulted in a low superficial area of only 75 m$^2$ m$^{-3}$. Data from the batch assays with UAF biomass indicated towards a higher biofilm SMA rate when compared to the sludge biomass (0.7 and 0.5 g$_{CH_4,COD}$ g$_{VSS}$ $^{-1}$ d$^{-1}$, respectively).

Despite the continuous dominance of the aceticlastic Methanosaeta in the UAF, the CH$_4$ fraction derived from CO$_2$ reduction, f$_{mc}$, varied significantly over an investigation period of 200 days. At OLRs below 6.0 g$_{COD}$ L$^{-1}$d$^{-1}$, the average f$_{mc}$ value was 33 %, whereas at higher OLRs, the average f$_{mc}$ values was of 42 % with peaks up to 47 %. The experiments allowed for a clear differentiation of the isotope fractionation related to the formation and consumption of acetate in both stages of the plant. The data indicate constant carbon isotope fractionation for acetate formation at different OLRs within the thermophilic LBRs as well as a negligible contribution of homoacetogenesis. Nevertheless, calculations of the maximal H$_2$ production derived from the volatile fatty acid (VFA) oxidation points to an overestimation of the f$_{mc}$ values in the order of 10 – 15 %.

The oxidized fraction of acetate, f$_{ac,ox}$, varied from 7 to 18 %, which is aligned with the f$_{mc}$ and H$_2$ production from VFA oxidation calculations for the UAF. Hence, despite the low free ammonia (NH$_3$) inhibition potential for the aceticlastic methanogens within the UAF, the slow growing acetate oxidizers were not outcompeted. These findings highlight the importance of the biomass retention times (biofilm samples with 445 to 575 days retention time were utilized for these f$_{ac,ox}$ determinations) for the SAO pathway. Indeed, the UAF
ammonium nitrogen concentrations (NH₄-N) are strikingly low, below 0.6 g N L⁻¹, in comparison to literature values reported for the SAO occurrence in mesophilic reactors (over 2.9 g N L⁻¹). On average, the biofilm biomass had only slightly higher $f_{ac,ox}$ values than the assays with sludge inoculum. The isotope-based kinetic model developed here also describes the $\delta^{13}C$ variations in unlabeled assays accurately and has the potential to determine biological $^{13}C$ fractionation factors.

These results present the first quantification of methanogenic pathway ($f_{mc}$ values) dynamics for a continually operated bioreactor. Additionally, this thesis innovates by using isotopic analysis to determine the acetate oxidation fractions based on the mechanistic description of the isotope kinetic and equilibrium effects integrated within microbial growth processes. The use of both methodologies together allows for the determination of methanogenic pathway variations and quantification of the amounts of acetate degraded through the SAO pathway. Hence, the calculation of anaerobic degradation pathways through stable isotope measurements is a remarkable innovation that complements the current monitoring parameters for anaerobic digestion processes in biomethanation plants.
ACKNOWLEDGEMENTS

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<tr>
<td>$C_2H_4O_2$</td>
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<td>$C_2H_3O_2^-$</td>
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<td>Heavy carbon carboxyl acetate / labeled $[1^{-13}C]acetate$</td>
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<td>$^{13}CH_3^{13}COOH$</td>
<td>Heavy carbon acetate / labeled $[1,2^{-13}C]acetate$</td>
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<td>$^{13}CH_3^{12}COOH$</td>
<td>Heavy carbon methyl acetate / labeled $[2^{-13}C]acetate$</td>
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### STANDARD UNITS

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<td>H</td>
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<td>µm</td>
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<td>Liter</td>
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<tr>
<td>Ppm</td>
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</tr>
<tr>
<td>Ppb</td>
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</tr>
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<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>Bar</td>
<td>Bar (unit of pressure)</td>
</tr>
<tr>
<td>kJ</td>
<td>Kilojoule</td>
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<tr>
<td>TWh</td>
<td>Terawatt hour</td>
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### Abbreviations and Indexes

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<th>Description</th>
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<tbody>
<tr>
<td>Ac</td>
<td>Index relative to total acetate (sum of undissociated acetic acid and acetate ion)</td>
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<tr>
<td>AF</td>
<td>Anaerobic filter</td>
</tr>
<tr>
<td>An</td>
<td>Anions</td>
</tr>
<tr>
<td>ADM1</td>
<td>Anaerobic Digestion Model No.1</td>
</tr>
<tr>
<td>ATB</td>
<td>Leibniz Institute for Agricultural Engineering Potsdam-Bornim</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BC</td>
<td>Biofilm carrier</td>
</tr>
<tr>
<td>BM</td>
<td>Index relative to total biomass concentrations</td>
</tr>
<tr>
<td>Ca</td>
<td>Parameter index relative to the anaerobic acetate oxidation</td>
</tr>
<tr>
<td>Cat⁺</td>
<td>Cation</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CSTR</td>
<td>Continuously stirred tank reactors</td>
</tr>
<tr>
<td>DAE</td>
<td>Differential algebraic equation system</td>
</tr>
<tr>
<td>DIET</td>
<td>Direct interspecies electron transfer</td>
</tr>
<tr>
<td>DIN</td>
<td>German Institute for Standardization</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGSB</td>
<td>Expanded granular sludge bed</td>
</tr>
<tr>
<td>EPS</td>
<td>Extra polymeric substance</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GC-C-IRMC</td>
<td>Gas chromatography combustion isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse gas</td>
</tr>
<tr>
<td>IRMS</td>
<td>Isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>LBR</td>
<td>Leach bed reactor</td>
</tr>
<tr>
<td>Ma</td>
<td>Parameter index relative to the acetoclastic methanogenesis</td>
</tr>
<tr>
<td>Mc</td>
<td>Parameter index relative to the hydrogenotrophic methanogenesis</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Maize silage</td>
</tr>
<tr>
<td>MSW</td>
<td>Municipal solid waste</td>
</tr>
<tr>
<td>NfE</td>
<td>Nitrogen free extracts</td>
</tr>
<tr>
<td>PDB</td>
<td>PeeDee Belemnite</td>
</tr>
<tr>
<td>PR</td>
<td>Index relative to measured protein content</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>Symbol</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative 5'-nuclease polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RUB</td>
<td>Ruhr-Universität Bochum</td>
</tr>
<tr>
<td>SAO</td>
<td>Syntrophic acetate oxidation</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>STR</td>
<td>Storage tank reactor</td>
</tr>
<tr>
<td>TRFLP</td>
<td>Terminal restriction fragment length polymorphism</td>
</tr>
<tr>
<td>UAF</td>
<td>Upflow anaerobic filter</td>
</tr>
<tr>
<td>UASB</td>
<td>Upflow anaerobic sludge blanket</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
</tr>
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</table>
# List of symbols and abbreviations

## OPERATIONAL AND MODEL PARAMETERS

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<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AP</td>
<td>%</td>
<td>Heavy isotope abundance percentage</td>
</tr>
<tr>
<td>$b_{\text{ac,VFA}}$</td>
<td>gCOD d$^{-1}$</td>
<td>Acetate load from the anaerobic VFA oxidation</td>
</tr>
<tr>
<td>$b_{\text{H2,VFA}}$</td>
<td>gCOD d$^{-1}$</td>
<td>Hydrogen load from the anaerobic VFA oxidation</td>
</tr>
<tr>
<td>COD</td>
<td>g L$^{-1}$</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>$F_i$</td>
<td>mol mol$^{-1}$</td>
<td>Fractional isotope abundance of the sample $i$ (e.g., $^{13}\text{C}/^{12}\text{C}$)</td>
</tr>
<tr>
<td>$f_{\text{ac,ox}}$</td>
<td>-</td>
<td>Fraction of acetate anaerobically oxidized</td>
</tr>
<tr>
<td>$f_{\text{mc}}$</td>
<td>-</td>
<td>Fraction of the methane production derived from carbon reduction</td>
</tr>
<tr>
<td>$f_{\text{H2,VFA}}$</td>
<td>-</td>
<td>Fraction of VFA anaerobically oxidized to $\text{H}_2$</td>
</tr>
<tr>
<td>HRT</td>
<td>d$^{-1}$</td>
<td>Hydraulic retention time</td>
</tr>
<tr>
<td>$K_a$</td>
<td>mol L$^{-1}$</td>
<td>Acidity coefficient</td>
</tr>
<tr>
<td>$K_{\text{hyd}}$</td>
<td>d$^{-1}$</td>
<td>First order kinetic hydrolysis rate</td>
</tr>
<tr>
<td>KH</td>
<td>Mol L$^{-1}$ bar$^{-1}$</td>
<td>Henry coefficient</td>
</tr>
<tr>
<td>$K_m$</td>
<td>mmol mmol$^{-1}$ d$^{-1}$</td>
<td>Substrate specific uptake rate</td>
</tr>
<tr>
<td>$K_{m,max}$</td>
<td>mmol mmol$^{-1}$ d$^{-1}$</td>
<td>Monod maximum specific uptake rate</td>
</tr>
<tr>
<td>$K_S$</td>
<td>mmol L$^{-1}$</td>
<td>Monod half-saturation constant for biological growth</td>
</tr>
<tr>
<td>MCP</td>
<td>ml$\text{CH}_4$ ml$\text{liq.}$ d$^{-1}$</td>
<td>Maximal methane ($\text{CH}_4$) production</td>
</tr>
<tr>
<td>OLR</td>
<td>gCOD L$^{-1}$ d$^{-1}$</td>
<td>Organic loading rate</td>
</tr>
<tr>
<td>$\text{OLR}_A$</td>
<td>gCOD m$^{-2}$ d$^{-1}$</td>
<td>Surface specific organic loading rate</td>
</tr>
<tr>
<td>$R_i$</td>
<td>mol mol$^{-1}$</td>
<td>Ratio of heavy to light isotopes in the sample $i$ (e.g., $^{13}\text{C}/^{12}\text{C}$)</td>
</tr>
<tr>
<td>RPM</td>
<td>n min$^{-1}$</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RS</td>
<td>-</td>
<td>Relative sensitivity index</td>
</tr>
<tr>
<td>$S_i$</td>
<td>mmol L$^{-1}$</td>
<td>Soluble concentration of the compound $i$</td>
</tr>
<tr>
<td>SMA</td>
<td>g$\text{CH}_4$ g$\text{COD}$ g$\text{VSS}$ d$^{-1}$</td>
<td>Specific methanogenic activity</td>
</tr>
<tr>
<td>SRT</td>
<td>d$^{-1}$</td>
<td>Solid retention time</td>
</tr>
<tr>
<td>TIC</td>
<td>mmol L$^{-1}$</td>
<td>Total inorganic carbon</td>
</tr>
<tr>
<td>TN</td>
<td>mmol L$^{-1}$</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>TOC</td>
<td>mmol L$^{-1}$</td>
<td>Total organic carbon</td>
</tr>
<tr>
<td>TS</td>
<td>%</td>
<td>Total solids</td>
</tr>
<tr>
<td>VS</td>
<td>%TS</td>
<td>Volatile solids</td>
</tr>
<tr>
<td>VSS</td>
<td>g L$^{-1}$</td>
<td>Volatile suspended solids</td>
</tr>
<tr>
<td>$X_i$</td>
<td>mmol L$^{-1}$</td>
<td>Particulate (&gt;0.45 µm) concentration of $i$</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>( Y_{ac,i} )</td>
<td>Acetate production yield from the substrate ( i )</td>
<td></td>
</tr>
<tr>
<td>( Y_{CH4} )</td>
<td>Methane production yield</td>
<td></td>
</tr>
<tr>
<td>( Y_{H2,i} )</td>
<td>Hydrogen production yield from the substrate ( i )</td>
<td></td>
</tr>
<tr>
<td>( Y_i )</td>
<td>Growth yield of the organism ( i )</td>
<td></td>
</tr>
<tr>
<td>( Y_{VFA} )</td>
<td>Volatile fatty acids production yield</td>
<td></td>
</tr>
<tr>
<td>( A )</td>
<td>Fractionation factor (relative; kinetic ratio of light to heavy isotopes)</td>
<td></td>
</tr>
<tr>
<td>( \Delta )</td>
<td>Fractionation factor (absolute; difference from product and source)</td>
<td></td>
</tr>
<tr>
<td>( \Delta G^0 )</td>
<td>Standard Gibbs free energy</td>
<td></td>
</tr>
<tr>
<td>( \Delta G )</td>
<td>Gibbs free energy</td>
<td></td>
</tr>
<tr>
<td>( \delta^{13}C_{ma} )</td>
<td>(^{13})C isotope abundance from the acetate derived methane</td>
<td></td>
</tr>
<tr>
<td>( \delta^{13}C_{mc} )</td>
<td>(^{13})C isotope abundance from the carbon dioxide derived methane</td>
<td></td>
</tr>
</tbody>
</table>
PUBLICATIONS INCLUDED WITHIN THIS THESIS

Papers published in refereed publications


Papers in preparation for submission in refereed publications


PUBLICATIONS RELATED TO THIS THESIS

Papers in refereed publications


Other publications


1. **INTRODUCTION**

1.1 General introduction

Biological methane (CH$_4$) production, methanogenesis, is an essential part of the global carbon cycle. Methanogenesis is the final degradation step of organic matter in a wide range of habitats, such as marine and freshwater sediments, marshes and swamps, flooded soils, geothermal habitats, animal gastrointestinal tracts and others (Zinder, 1993). It is estimated that around 2 % of the total carbon fixed through photosynthesis is converted biologically into CH$_4$ yearly, though most of this CH$_4$ diffuses into aerobic environments and is oxidized by methanotrophic Bacteria (Thauer et al., 2008). Calculations of total natural CH$_4$ emissions to the atmosphere are in the order of 218 to 347 Tg a$^{-1}$ (including geological sources) and the current CH$_4$ anthropogenic-derived emissions are in the same range, from 331 to 335 Tg CH$_4$ a$^{-1}$ (Ciais et al., 2013). Thus, anthropogenic activities strongly affect the CH$_4$ atmospheric concentrations. Atmospheric CH$_4$ levels have increased by 150 %, from 722 to 1,805 ppb, since 1850 (Hartmann et al., 2013). This is a very concerning issue as CH$_4$ has a 100-year global warming potential (GWP$_{100}$), over 20 times greater than carbon dioxide (CO$_2$). Indeed, CH$_4$ is the main atmospheric greenhouse gas (GHG) after CO$_2$ (Myhre et al., 2013). In addition, CH$_4$ emissions increase the ozone surface concentration, which is not only a GHG, but is also responsible for deteriorating the air quality and is related to adverse health effects (West et al., 2006).

Anaerobic degradation of organic waste in landfills, livestock manure and wastewaters are responsible for a yearly flow of 65 to 90 Tg CH$_4$ into the atmosphere (Ciais et al., 2013), i.e. ~ 20 % of the total anthropogenic emissions. Under a controlled engineered system, the same microbiologic consortia from natural ecosystems can produce biogas from organic waste and allow for the use of this biogenic methane as a renewable energy source. The conversion of 1 Tg CH$_4$ in a combined heat and power unit results in up to 5.4 TWh electrical energy and 9.4 TWh as heat; alternatively, it has an equivalent to 1.5 TL of diesel if used directly as a fuel. Nonetheless, beside this enormous energetic potential, the major benefits of anaerobic digestion technologies combine, on the one hand, the decrease of GHG emissions with, on the other hand, the recycling of organic waste nutrients. The remaining products of organic waste fermentation are a potential source of important nutrients, such as nitrogen (N) and phosphor (P), which can be
utilized as natural fertilizers (Gijzen, 2002). Nowadays, 100 Tg a⁻¹ of elemental nitrogen are fixed with hydrogen (H₂) to ammonia nitrogen (NH₃) in order to serve the production of chemical fertilizers through the very energy-intensive Haber-Bosch process (Ciais et al., 2013). This process alone consumes approximately 2 % of the global energy and 5 % of the annual natural gas production (Ritter, 2008). Nonetheless, the increasing use of P as a fertilizer, which, due to its non-renewable nature, strongly depends on the mining of phosphate rocks, is even more concerning. Ashley et al. (2011) estimate a P shortage already by the mid-21st century, highlighting the importance of P recycling to ensure the food production chain.

Targets for the reduction of GHG emissions in Europe motivated a rapid expansion of anaerobic digestion plants in the last decade. This development was mostly supported by the cultivation of crops with high growth yields and high CH₄ potential, e.g. maize and grass silages, for direct use in anaerobic plants. In Germany, Europe’s largest biogas producer, 3.1 % of the energy consumed in 2011 was produced in 7,100 biogas plants fed with these energy crops and animal slurries (Herrmann, 2012). Nevertheless, the competition of cultivable area with food crops and other environmental impacts related to energy crops monocultures may limit the further expansion of this crop-to-CH₄ model. Hence, the development of anaerobic technologies for the utilization of a broader spectrum of organic waste, such as crop residues, waste from downstream processing food and residuals of liquid biofuel production, is expected (Shilton and Guieysse, 2010). However, the further spread out of anaerobic technologies is only possible once the economic viability of the process is assured. Thus, the reliability of CH₄-based technologies depends on an efficient, predictable and stable process. A more comprehensive understanding of the anaerobic digestion processes can contribute to a larger adoption of anaerobic technologies.

1.2 Anaerobic degradation pathways

1.2.1 Anaerobic degradation of complex substrates

The biomethanation of organic substrates includes a complex chain of processes where polymers are consecutively degraded into shorter molecules until methane and carbon dioxide are the last remaining products (Zinder, 1993). The anaerobic digestion of complex substrates is commonly described in four sequential steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. In the hydrolysis step, the substrate proteins, carbohydrates and lipids are solubilized into amino acids, monosaccharides and long chain fatty acids (LCFAs); during the acidogenesis, amino acids and monosaccharides are converted mainly into volatile fatty acids (VFAs), e.g. propionate and butyrate. In the acetogenesis, VFAs and LCFAs are subsequently oxidized into
acetate and H₂. Both these compounds are the main CH₄ precursors for the methanogenesis. These processes are performed by a very diverse and complex microbial consortia, which includes bacterial and archaeal members, whereby the methane production is an exclusive metabolic property of Archaea from the phyla Euryarchaeota (Thauer et al., 2008). Despite the enormous developments obtained through molecular methods during the last few decades, interactions and metabolic regulation within this microbial consortia remain to be investigated in detail (Orell et al., 2013; Talbot et al., 2008).

Describing the several processes involved in anaerobic digestion can be a very complex task, depending on the level of detail required. Therefore, mathematical models can be an important tool for both process comprehension and optimization (Wichern et al., 2011), allowing not only qualitative, but also quantitative description of the mass and energy flows. The Anaerobic Digestion Model No. 1 (ADM1), developed by an international task group (Batstone et al., 2002), is a complex model that has synthetized the developments in the mathematical models for methane-producing systems since the first acetate degradation model from Andrews (1969). In Lübken et al. (2010) a detailed review on the historical development of anaerobic digestion models is provided. Practical aspects for model selection, validation and identification of parameters in anaerobic digestion models are described in Donoso-Bravo et al. (2011).

The ADM1 publication founded a basis for the wide spread of the dynamic simulation utilization for anaerobic digestion systems and is currently the most accepted framework for the description of anaerobic degradation processes in sewage sludge digesters and biogas plants (Donoso-Bravo et al., 2011; Nopens et al., 2009). In the model, 24 substrates and 19 processes, including seven different functional biomass groups, are incorporated together with a detailed description of the physicochemical processes. The description of the biomass growth follows Monod kinetics, with specific growth, decay and inhibition factors for each biomass group, resulting in a high number of parameters to be defined. These biological parameters are usually temperature specific, assuming optimal mesophilic (35 °C) or thermophilic (55 °C) conditions. Pavlostathis and Giraldo-Gomez (1991) give the temperature ranges of 30 – 38 °C and 50 – 60 °C, respectively, as the more suitable for methanogenic processes.

The energy flow (given in terms of chemical oxygen demand: COD) obtained through the ADM1 is depicted in Figure 1-1 for the model solid substrate utilized in this work regarding maize silage (MS; see Section 2.2.1 for more details). The understanding of this detailed energy flow scheme is a very important aspect in the design and optimization of anaerobic digestion plants, allowing for the identification of the major process bottlenecks. However, the quality of the model output is ultimately limited by the precision
of the experimental observations. It is possible to validate most of the steps in this flow chart through current analytical techniques. Different approaches to characterization of the inflow substrates in terms of carbohydrates, proteins, lipids and inert material exist, e.g. elemental composition analysis, fodder analytics techniques (as depicted in Figure 1-1) and anaerobic respirometric tests (Lübken et al., 2015b), to adequately describe the disintegration and hydrolysis of particulate matter. In the following steps, typical anaerobic digestion operational parameters, such as COD, total and ammonia nitrogen, alkalinity and VFA concentrations, together with gas flow and composition data, allow for the estimation of diverse kinetic parameters (e.g., Donoso-Bravo et al., 2011; Koch et al., 2013; Lübken et al., 2015a; Wichern et al., 2009).

Nonetheless, there are important aspects that are very difficult to identify in mixed anaerobic communities. Specific biomass concentrations are usually unknown variables (Donoso-Bravo et al., 2011), although detailed identification of known microorganisms is possible through molecular biology methods. Application of methods for biomass quantification, such as quantitative 5’-nuclease polymerase chain reaction (qPCR) and fluorescence in situ hybridization (FISH), however, seldom support simulation studies (e.g. Hao et al., 2012; Lübken et al., 2007b). Another important challenge is the identification of the acetate degradation pathways (bold arrows, Figure 1-1); the unidentified mass flow for the acetate oxidation remains, which is not accounted for in the ADM1 implementation. Measurements of acetate, H₂ and CH₄ concentrations alone do not allow for the differentiation of both acetate degradation pathway, i.e. acetate-derived methanogenesis and acetate oxidation. The determination of the mass flow in the methanogenesis and acetate degradation is the focus of this work and is discussed in detail in the Sections 1.2.4, 1.4.3 and 1.4.4.
Figure 1-1: Substrate characterization and COD-degradation flow for MS (adapted from Batstone et al., 2002). Fractions of the solid substrate in %VS-values were determined through the extended Weender analysis (in gray; for more details see Lübken et al., 2015b). COD flow obtained through simulations with the ADM1 (Gehring et al., 2013) and are given in %COD-values (in black). NfE stands for nitrogen free extracts and numbered circles identify the different functional biomass groups: 1) sugar degraders, 2) amino acid degraders, 3) LCFA oxidizers, 4) propionate oxidizers, 5) butyrate/valerate oxidizers, 6) acetoclastic methanogens and 7) hydrogenotrophic methanogens. Mass flow differences in outflows between the substrate and their respective biomass group correspond to the biomass growth yield. The biological parameters utilized for these simulations can be found in Appendix I-A.

1.2.2 Hydrolysis and disintegration of particulate substrates

The hydrolysis rate in the fermentation of particulate substrates is one major limiting step together with the acetate degradation, hence, a key parameter for plant design. Hydrolysis is a complex multistep process, involving several microorganisms and enzymes (Lynd et al., 2002). There are various factors reported to affect the hydrolysis rate, such as pH, temperature, concentration of hydrolyzing biomass and type of
particulate organic matter (Pavlostathis and Giraldo-Gomez, 1991; Weiland, 2010). The use of first-order kinetics in the hydrolysis description is very common (i.e. the kinetics are not limited by the concentration of any biomass group), although, in certain circumstances, more detailed models are required (Vavilin et al., 2008a). The inclusion of specific inhibition parameters for the hydrolysis has been reported, e.g. total solids (TS) content (Koch et al., 2010), pH and VFA concentrations (Vavilin et al., 2006). Temperature influence on hydrolysis can be determined on the basis of the Arrhenius equation (Llabrés-Luengo and Mata-Alvarez, 1988; Veeken and Hamelers, 1999). However, the available substrate surface area, a fundamental aspect in the hydrolysis of particulate matter (Hobson, 1987; Rotter et al., 2008), is neglected in most of the models.

Substrates for CH₄-producing plants commonly derive directly from plant biomass (e.g. crops, food and yard waste) and have, therefore, a high content of lignocellulosic (sum of cellulose, hemicellulose and lignin) material (Eleazer et al., 1997; Vavilin et al., 2008a), the main product of plant photosynthesis. Table 1-1 gives an overview of first-order kinetic rates for several substrates with high lignocellulosic material. These substrates are generally slowly degradable: for comparison purposes, hydrolysis of wastewater treatment plant (WWTP) sludge is in the order of 0.1 – 0.5 d⁻¹ (Batstone and Jensen, 2011). Typical ranges for these biopolymer contents in plant dry weight are 35 – 50 % cellulose, 20 – 35 % hemicellulose and 5 – 30 % lignin (Lynd et al., 2002). Although lignin is not degradable under anaerobic conditions (Angelidaki and Sanders, 2004), a direct correlation of the lignin content alone to the substrate degradability is difficult (Eleazer et al., 1997; Tong et al., 1990). The nature of the lignin linkage in the three-dimensional polymeric matrix is the major aspect of the substrate microbial availability (Lynd et al., 2002). The higher the complexity level in the biopolymer structure, the higher the number of enzymes required for the hydrolysis, which implies a slower process kinetics (Lübken et al., 2010).
Table 1-1: First-order hydrolysis kinetic (k_{hydrolysis}) parameters for lignocellulosic-rich substrates (adapted from Lübken et al., 2010 and Vavilin et al., 2008a).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>k_{hydrolysis} (d^{-1})</th>
<th>T (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>0.10</td>
<td>35</td>
<td>Vavilin et al. (1996)</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.12</td>
<td>38</td>
<td>O’Sullivan et al. (2005)</td>
</tr>
<tr>
<td>Cellulose (MS)</td>
<td>0.04-0.14</td>
<td></td>
<td>Gujer and Zehnder (1983)</td>
</tr>
<tr>
<td>Grass</td>
<td>0.18</td>
<td>38</td>
<td>Lübken et al. (2015a)</td>
</tr>
<tr>
<td>Wood grass</td>
<td>0.266</td>
<td>40</td>
<td>Veeken and Hamelers (1999)</td>
</tr>
<tr>
<td>Napier grass</td>
<td>0.09</td>
<td>35</td>
<td>Tong et al. (1990)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>0.087</td>
<td>35</td>
<td>Tong et al. (1990)</td>
</tr>
<tr>
<td>Straw</td>
<td>0.14</td>
<td>40</td>
<td>Veeken and Hamelers (1999)</td>
</tr>
<tr>
<td>Leaves</td>
<td>0.386</td>
<td>40</td>
<td>Veeken and Hamelers (1999)</td>
</tr>
<tr>
<td>Paper and cardboard</td>
<td>0.012</td>
<td>35</td>
<td>Qu et al. (2009b)</td>
</tr>
<tr>
<td>Paper and cardboard</td>
<td>0.02</td>
<td>55</td>
<td>Qu et al. (2009b)</td>
</tr>
<tr>
<td>News print</td>
<td>0.057</td>
<td>35</td>
<td>Vavilin and Lokshina (1996)</td>
</tr>
<tr>
<td>Food waste</td>
<td>0.55</td>
<td>37</td>
<td>Vavilin et al. (2004)</td>
</tr>
<tr>
<td>Household solid waste</td>
<td>0.1</td>
<td>37</td>
<td>Vavilin and Angelidaki (2005)</td>
</tr>
</tbody>
</table>

1.2.3 Volatile fatty acids production and oxidation

The production of VFAs is considered as a fast degradation step in comparison to the hydrolytic and methanogenic processes (Pavlostathis and Giraldo-Gomez, 1991). The product formation from monosaccharides is highly dependent on the thermodynamic conditions in the environment. Hence, different acidogenic products are formed according to the pH, H₂ partial pressures and VFA concentrations. Regulation of the pH in the reactor can be used to control the production of ethanol, formic, acetic, lactic, propionic and butyric acids from carbohydrates and sewage sludge (e.g. Horiuchi et al., 2002; Zoetemeyer et al., 1982). Nonetheless, the COD flow in Figure 1-1 was defined on the basis of fixed stoichiometric products. Monosaccharide fermentation in the ADM1 is described through three different reactions pathways (all standard Gibbs free energy, ΔG⁰, given in the following were obtained or calculated with data from Thauer et al., 1977):

\[
C_6H_{12}O_6 + 2H_2O \xrightarrow{\Delta G^0 = -215.7 \text{ kJ}} 2C_2H_4O_2 + 2CO_2 + 4H_2
\]

(1-1)

\[
3C_6H_{12}O_6 \xrightarrow{\Delta G^0 = -311.3 \text{ kJ}} 4C_3H_6O_2 + 2C_2H_4O_2 + 2CO_2 + 2H_2O
\]

(1-2)
\[ C_6H_{12}O_6 \xrightarrow{\Delta G^0=-264.0} C_4H_8O_2 + 2CO_2 + 2H_2 \] (1-3).

The distribution between them is maintained constant (the following distribution was utilized for Figure 1-1: 49.5, 35.5 and 16 % for Equations 1-1, 1-2 and 1-3, respectively). This modeling approach is valid for constant pH values, as occurs in well-buffered anaerobic digestion reactors. A variable acidogenic stoichiometry approach is a more recommendable alternative (Rodríguez et al., 2006) for the description of fermentation processes with significant pH and/or H\(_2\) partial pressures variations. Amino acids fermentation can occur through paired Stickland oxidation-reduction reactions or by the oxidation of single amino acids with H\(_2\) or CO\(_2\) as electron acceptors, whereby the first pathway is more relevant (Batstone et al., 2002). A relation of stoichiometric products for diverse amino acid mixes calculated through Stickland reactions can be found in Lübken et al. (2015b).

The oxidation of the VFAs produced in the acidogenic processes and LCFAs is only feasible in a narrow thermodynamic range (Zinder, 1993). The \(\Delta G^0\) values for the oxidation of propionate (Equation 1-4), butyrate (Equation 1-5) and valerate (Equation 1-6) are unfavorable to the oxidizing Bacteria growth under standard conditions:

\[
\begin{align*}
C_3H_5O_2^- + 3H_2O & \xrightarrow{\Delta G^0=+71.7 \text{ kJ}} C_2H_3O_2^- + HCO_3^- + 3H_2 + H^+ \quad (1-4) \\
C_4H_7O_2^- + 2H_2O & \xrightarrow{\Delta G^0=+48.3 \text{ kJ}} 2C_2H_3O_2^- + 2H_2 + H^+ \quad (1-5) \\
C_5H_9O_2^- + 2H_2O & \xrightarrow{\Delta G^0=+48.3 \text{ kJ}} C_3H_5O_2^- + C_2H_3O_2^- + 2H_2 + H^+ \quad (1-6).
\end{align*}
\]

Thus, the anaerobic VFA oxidation requires a direct sink for the H\(_2\) produced and the other reaction products. Methanogens consuming these substrates are, therefore, essential for the VFA-oxidizing Bacteria and vice versa, characterizing a mutually dependent relationship, i.e. syntrophic growth. Exemplarily, the overall rate of the syntrophic butyrate fermentation to CH\(_4\) following the description by Schink (1997) is detailed below:

\[
2C_4H_7O_2^- + 2H^+ + 2H_2O \xrightarrow{\Delta G^0=-177 \text{ kJ}} 5CH_4 + 3CO_2 \quad (1-7).
\]

Firstly, 2 Mol butyrate are converted into 4 Mol acetate and 4 Mol H\(_2\) by VFA-oxidizing Bacteria. The 4 Mol acetate are converted into 4 Mol CH\(_4\) and CO\(_2\) by acetoclastic methanogens and the 4 Mol H\(_2\) are used to reduce one CO\(_2\) Mol into CH\(_4\) by a hydrogenotrophic methanogen. Hence, the \(\Delta G\) of -177 kJ – if standard conditions are assumed, Schink (1997) suggests that a \(\Delta G\) of -140 kJ is a better value for the conditions found in a sewage sludge digester – supports seven reaction steps: two conducted by
VFA oxidizers, and four by acetoclastic and one by hydrogenotrophic methanogens. Thus, the energy available for each step is in the range of the minimal energy quantum for ATP gain, about \(-20\) kJ Mol\(^{-1}\) (Schink, 1997). Hence, high rate methanogenesis is supported only by the syntrophic growth of VFA-oxidizing Bacteria with methanogenic Archaea, whereby the formation of microbial aggregates, as granules or biofilm structures, provides ideal conditions for this symbiosis (Stams et al., 2012). A schematic visualization of a syntrophic exchange of hydrogen in a microbial aggregate is shown in Figure 1-2. The H\(_2\) gradient results due to mass-transfer limitations of the H\(_2\) diffuse transport, limiting the distances between the cells to a maximal 10 µm (Giraldo-Gomez et al., 1992).

![Figure 1-2: Idealization of microbial syntrophic association in close spatial arrangements inside a bacterial aggregate. The graphs illustrate a typical H\(_2\) concentration profile from the producer to the consumers (adapted from Giraldo-Gomez et al., 1992), with \(r_{cp}\) as the distance between the cells of H\(_2\) consumers and producers.](image)

These thermodynamic constraints are most commonly implemented in models through an empirical noncompetitive inhibition (the equation is depicted in Section 2.6.2). Hoh and Cord-Ruwisch (1996) suggest that the inclusion of a limitation equation derived directly from thermodynamic calculations results in a more realistic basis to describe the processes and reduces the number of empirical parameters to be determined. Nevertheless, data measured from dissolved H\(_2\) would be required for this approach. Most simulation studies are based on headspace H\(_2\) partial pressure measurements, which define the H\(_2\) concentrations dissolved through gas exchange and equilibrium processes. However, this assumption is challenging, as these calculations underestimate the H\(_2\) dissolved (Pauss and Guiot, 1993). Moreover, the H\(_2\) in bulk liquid may not be the same that is experienced by the microorganisms within the biomass aggregates (Conrad et al., 1985; Giraldo-Gomez et al., 1992). By using an empirical noncompetitive equation, the H\(_2\) solubility and the mass-transport effects are integrated within the estimation of the H\(_2\) inhibition constant, allowing for consistent simulation results. Indeed, Batstone et al. (2006b), using a two-dimensional individual-based model, found that both simulation
1.2.4 Acetate degradation and methanogenic pathways

Acetate cleavage and CO\textsubscript{2} reduction with H\textsubscript{2} are the major methanogenic pathways. Acetoclastic methanogenesis accounts for about two-thirds of the global production of CH\textsubscript{4} and the remaining one-third can be almost completely related to hydrogenotrophic methanogenesis (Ferry, 2010). Other substrates metabolized by methanogenic Archaea are formate, methanol, methylated amines and sulfides (Zinder, 1993). The acetoclastic methanogenesis is undertaken exclusively by the Archaea genera *Methanosaeta* and *Methanosarcina* from the *Methanosarcinales* orders (Thauer et al., 2008). The acetate concentration in the environment is an indicator of which of them is the dominant genera: higher concentrations pointing to a dominance of the genus *Methanosarcina* and lower concentrations of the genus *Methanosaeta* (Jetten, 1990). Reported threshold concentrations for acetate consumption (concentration below microbial growth is not supported) are between 0.6 and 1.2 mmol for *Methanosarcina* and between 0.005 and 0.012 mmol for *Methanosaeta* (Zinder, 1993). The reduction of CO\textsubscript{2} with H\textsubscript{2} can be performed by most of the methanogenic Archaea, including the orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales* and some *Methanosarcina* species of the *Methanosarcinales* order (Thauer et al., 2008).

Besides growing on H\textsubscript{2} and CO\textsubscript{2}, some *Methanosarcina* species are also capable of oxidizing acetate (Krzycki et al., 1982; Lovley and Ferry, 1985). Thus, the presence of *Methanosarcina* is not necessarily an indicator of the acetoclastic methanogenic pathway. By contrast, *Methanosaeta* have, until now, been regarded as exclusively acetotrophic organisms (Ferry, 2010). Despite this inflexibility, they are probably the predominant global methane producer due their adaptation to very low acetate concentrations (Smith and Ingram-Smith, 2007). Recently, the capability of *Methanosaeta* to metabolize CO\textsubscript{2} in a direct interspecies electron transfer (DIET) with *Geobacter* was reported (Rotaru et al., 2014). This confirmed that the high electrical conductivities previously reported for these microbial aggregates were a DIET indicator (Morita et al., 2011). Because of this newly discovered metabolic flexibility, *Methanosaeta* may have an even larger contribution to global methane production. Nevertheless, further investigations on *Methanosaeta*-dominated environments are necessary to quantify the importance of this novel methanogenic pathway.

The pioneering studies of Jeris and McCarty (1965) and Smith and Mah (1966) indicated that the proportion of two-thirds of the CH\textsubscript{4} production through acetoclastic methanogenesis is also found in sewage sludge digesters. The derivation of the carbon flux in most anaerobic digestion models corresponds with these findings (Lübken et al.,
Introduction

2010; see Figure 1-1). However, there is an increasing amount of evidence indicating that, depending on the substrate characteristics, hydrogenotrophic rather than acetoclastic methanogenesis is the dominant pathway (Demirel, 2014; Karakashev et al., 2006; Nettmann et al., 2010). This pathway determination has important implications for the design and operation of anaerobic plants, as acetoclastic and hydrogenotrophic methanogens are known to differ significantly concerning growth rates, pH and inhibition factors (Batstone et al., 2002; Demirel and Scherer, 2008).

Nevertheless, the quantification of the methanogenic pathways in bioreactors remains a challenge. Molecular methods allow for the identification of the dominant methanogenic Archaea population (Klocke et al., 2008; Lübken et al., 2007a), but are neither sufficient for quantifying the specific metabolic activity from hydrogenotrophic and acetoclastic methanogens in a mixed community, nor capable of tracking variations on a short timescale. A discussion on the quantification of methanogenic pathway contributions through carbon isotope analysis follows in the Sections 1.4.3 and 1.4.4.

The acetate degradation pathway needs to be reconsidered in the environments where a dominance of hydrogenotrophic methanogens is identified. Acetate conversion to CH4 occurs through two known pathways (see Figure 1-1): either through the acetoclastic methanogenesis (Equations 1-8) or through a syntrophic process combining the anaerobic acetate oxidation with hydrogenotrophic methanogenesis (Equation 1-9):

$$C_2H_4O_2 \xrightarrow{\Delta G^0 = -31kJ} CH_4 + CO_2$$

$$C_2H_4O_2 + 2H_2O \xrightarrow{\Delta G^0 = +105kJ} 2CO_2 + 4H_2 \xrightarrow{\Delta G^0 = -135.6kJ} CH_4 + CO_2 + 2H_2O$$

Due to the high Gibbs free energy associated with the acetate oxidation step ($\Delta G^0 = 105$ kJ), the contribution of the syntrophic pathway is commonly disregarded in literature, especially for mesophilic conditions (Batstone et al., 2002). In thermophilic conditions the energetic constraints are more favorable for an anaerobic oxidation of acetate (a detailed calculation of the acetate oxidation dependence on the temperatures is given in Schink, 1997). Indeed, the importance of the SAO pathway has been recently confirmed predominantly for thermophilic conditions (Hao et al., 2011; Hao et al., 2012; Fotidis et al., 2013; Lü et al., 2013; Ho et al., 2014). Furthermore, Schnürer et al. (1996) only isolated the first mesophilic acetate oxidizers in association with hydrogenotrophic methanogens from a biogas plant in 1996; the first thermophilic coculture was isolated 12 years earlier by Zinder and Koch (1984). Hence, the mechanisms of syntrophic acetate oxidation (SAO) are still not fully understood (Angelidaki et al., 2011) and neither are all the trophic groups involved yet known (Hattori, 2008).
In comparison to acidogenic and methanogenic processes, information about the kinetics of acetate oxidation is scarce. Despite the discussions regarding its inclusion in the ADM1 (see Batstone et al., 2002), the acetate oxidation is neglected in most of the anaerobic digestion models with only a few exceptions (Shimada et al., 2011; Vavilin, 2012a, 2012b; Wett et al., 2012). Yet, it is clear that the overall methane production rates from acetate through the SAO pathway are significantly lower than from acetoclastic methanogenesis (Karakashev et al., 2006; Schnürer et al., 1999). Slow growth rates of only 0.04 d\(^{-1}\) were found for an isolated mesophilic acetate oxidizer, *C. Ultunense* (Hattori, 2008; Schnürer et al., 1996). For comparison purposes, the growth rates for the acetoclastic methanogens *Methanosaeta* and *Methanosarcina* reported are 0.2 and 0.6 d\(^{-1}\), respectively (De Vrieze et al., 2012). Consequently, while reduced hydraulic retention times (HRTs) diminish the SAO rates (Shigematsu et al., 2004; Westerholm et al., 2012), high solid retention times (SRTs) are likely to favor the slowly growing acetate oxidizers. In fact, one of the few mesophilic acetate oxidizers isolated so far, *Syntrophaceticus schinkii*, was found on an upflow anaerobic filter (UAF; Westerholm et al., 2010).

In spite of their slow growth rates, acetate oxidizers tolerate much higher free ammonia concentrations (NH\(_3\)) than acetoclastic methanogens (Schnürer et al., 1996; Westerholm et al., 2011; Wilson et al., 2012). This higher NH\(_3\) tolerance potentially explains the dominance of hydrogenotrophic methanogens in agricultural biogas plants and, consequently, the presence of acetate oxidizers over acetoclastic methanogens (Fotidis et al., 2013; Karakashev et al., 2005; Nettmann et al., 2010). Indeed, Polag et al. (2013) found that SAO can even completely dominate the acetate degradation on the mesophilic fermentation of maize silage. Nonetheless, most of these studies focusing on the SAO importance are applied to systems with high NH\(_3\) concentrations, and the retention time and/or biofilm formation importance of SAO are rarely mentioned (e.g., Westerholm et al., 2012) or investigated. Investigations regarding the SAO pathway also predominantly concern thermophilic rather than mesophilic conditions.

### 1.3 Methane production from high-solid substrates

#### 1.3.1 Potential high-solid substrates for methane production

There are plenty of organic waste and plants that can be efficiently converted to CH\(_4\) with reduced or no pretreatment (Gunaseelan, 1997). The consideration of the potential substrates for anaerobic CH\(_4\)-based technologies should be considered in a wide perspective. In the case of crop production for direct biomethanation purposes, the benefits of the renewable CH\(_4\) production need to be counterbalanced by other environmental impacts, e.g. substrate transport, digestate final destination, requirements of water, fertilizer and land, among others (De Meester et al., 2012). According to Shilton
and Guieysse (2010), the utilization of organic waste poses the only rational alternative to expand the biogas utilization. Nevertheless, energy crops may play an important role in supplementing the CH\(_4\) generation in waste-producing plants and contributing to the economic viability of such plants. It is possible through adequate cultivation techniques to enhance biomass yields and reduce environmental impacts related to crop production, e.g. the utilization of a regionally adapted crop rotation strategy, with different cultures in winter/autumn and summer/spring seasons, instead of monocultures, and the enhancement of the digestate utilization as natural fertilizer (Herrmann, 2012).

Municipal solid waste (MSW) has a great potential as a substrate for anaerobic digestion plants as it is continually available and potential liquid and gas emissions from landfill disposal are avoided through their controlled degradation in fermentation plants (Mata-Alvarez et al., 2000). In comparison to incineration, fermentation technologies are ranked as a more sustainable alternative; this more positive balance results mainly due to the remaining digestate that retains all nutrients present in the MSW, while the incineration ashes are a product with poor value (De Meester et al., 2012). The MSW is a very heterogeneous substrate and its composition reflects directly on its CH\(_4\) potential (Angelidaki and Sanders, 2004). An adequate sorting of the organic fraction of the MSW leads to methane yields as high as 390 to 430 L\(_{\text{CH}_4}\) kg\(_{\text{VS}-1}\) (see Table 1-2).

Table 1-2: Methane yields (\(Y_{\text{CH}_4}\)) from selected high-solid substrates in anaerobic digesters reviewed in the literature.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>VS ((%_{\text{TS}}))</th>
<th>(Y_{\text{CH}<em>4}) ((\text{L}</em>{\text{CH}<em>4}\ \text{kg}</em>{\text{VS}-1}))</th>
<th>Pre-treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSW</td>
<td>&gt; 82</td>
<td>390 – 430</td>
<td>Sorting (organic fraction only)</td>
<td>Gunaseelan (1997)</td>
</tr>
<tr>
<td>Food waste</td>
<td>&gt; 95</td>
<td>409 – 529</td>
<td></td>
<td>Gunaseelan (1997)</td>
</tr>
<tr>
<td>Yard waste</td>
<td></td>
<td>118 – 412</td>
<td>Codigestion with sludge</td>
<td>Kosse et al. (2015)</td>
</tr>
<tr>
<td>Energy crops</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>85 – 98</td>
<td>340</td>
<td>Ensiling(^a)</td>
<td>FNR (2010)</td>
</tr>
<tr>
<td>Grass</td>
<td>70 – 95</td>
<td>310</td>
<td>Ensiling(^a)</td>
<td>FNR (2010)</td>
</tr>
<tr>
<td>Aquatic biomass</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine microalgae</td>
<td>90</td>
<td>570</td>
<td>Thermal pretreatment</td>
<td>Schwede et al. (2013)</td>
</tr>
<tr>
<td>Freshwater algae</td>
<td>90</td>
<td>410</td>
<td></td>
<td>Gunaseelan (1997)</td>
</tr>
</tbody>
</table>

\(^a\) Ensiling is a simple conservation technique which converts soluble carbohydrates, mainly lactic acid, resulting in low pH values and the inhibition of further microbiological activity (Weiland, 2010).
However, it is important to note that a substantial part of the energy produced in MSW plants may be required for the mechanical substrate sorting. De Meester et al. (2012) report an internal consumption of a MSW fermentation plant of 36% of the electrical energy produced against 6% from an agricultural biogas plant. The source sorting of food waste from processing industries or markets gives a highly degradable organic substrate (see Table 1-2) with CH$_4$ potentials ranging from 409 up to 529 L$_{CH4}$ kg$_{VS}^{-1}$. These values are higher than for a high energy crop such as maize. Urban biomass, such as leaves and other green waste, can also have high CH$_4$ yields which are still mostly unexplored, e.g. values of 392 and 412 L$_{CH4}$ kg$_{VS}^{-1}$ are reported for nettle and cow parsley, respectively (Kosse et al., 2015). Another alternative for the cultivation of energy crops growth are algae, as uncultivable land can be used and less water is required than for terrestrial plant cultures (Shilton and Guieysse, 2010).

### 1.3.2 Anaerobic digestion technologies

The total solids contents (TS) in the substrate is one key parameter, which defines the type of reactor to be utilized (Table 1-3). In general, wastewater, industrial or domestic, is ideally suited for fixed biomass digesters. The solids and the hydraulic retention times (SRT and HRT, respectively) in these reactors are decoupled, resulting in reduced reactor volumes. The most common mechanisms for solids (i.e. biomass) retention are through sludge bed formation (UASB: upflow anaerobic sludge blanket; EGSB: expanded granular sludge bed) or fixed through biofilms (AFs: anaerobic filters). On the one hand, high strength wastewater, such as that from breweries and other food processing industries, allows for very high organic loading rates (OLRs), up to 30 g$_{COD}$ L$^{-1}$ d$^{-1}$ (Bischofsberger et al., 2005), hence, these reactors are often denominated as high-rate digesters. On the other hand, the treatment of low strength substrates, COD concentrations below 1 g$_{COD}$ L$^{-1}$, such as domestic sewage, are also practicable in these systems. The inflow solids content is the major limitation as it can cause clogging of the system or accumulates in the sludge bed (in upflow systems) reducing the volume for biomass in the reactors.

Continuously stirred tank reactors (CSTR), or simply mixed tanks, are very well-established for sludge fermentation in WWTPs. Diverse configurations are possible, including mixed tanks in series with or without internal recirculation. There is an increased utilization of cosubstrates in WWTP sludge digesters (WWTP refers here to activated sludge plants). Through these additional substrates (which includes municipal, industrial and agricultural solid waste), much higher energetic outputs are possible. Moreover, the codigestion of sewage sludge may allow the degradation of substrates that are difficult to degrade alone, providing a better nutrient supply and/or diluting toxic compounds (Mata-Alvarez et al., 2014). Mixed tanks have also been the most common type of reactor of
biogas plants designed for energy crop fermentation during the last few decades in Germany (FNR, 2010). Despite the design and operational simplicity of these plants, they are limited to TS contents around 15 % due to the limitations of pumping and mixing systems (Bischofsberger et al., 2005). Moreover, the hydrolysis of solids substrates at solid contents over 10 % is difficult due to mass-transfer limitations (Abbassi-Guendouz et al., 2012; Koch et al., 2010). Another drawback of these systems are the long retention times required for substrates with slow hydrolysis kinetics (see Table 1-1), resulting in lower OLRs and larger reactor volumes. In the case of codigestions in WWTP digesters, it is important to note that a higher N-load for the nitrification processes occurs through the sludge dewatering, depending on the cosubstrate nitrogen content.

**Table 1-3:** Typical design parameters, TS content, HRT and OLR, for anaerobic reactors and for different types of substrate (adapted from Batstone and Jensen, 2011). The main design parameters are given in bold.

<table>
<thead>
<tr>
<th>Reactor type</th>
<th>Substrates</th>
<th>TS Content (%mass)</th>
<th>SRT (d)</th>
<th>OLR (gCOD L⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed tank</td>
<td>Sewage sludge and solids, animal manure, organic solids¹</td>
<td>2 – 7 (12)¹</td>
<td>15 – 30¹ (= HRT)</td>
<td>1.0 – 4.5ᵃ</td>
</tr>
<tr>
<td>(CSTR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed biomass / High-rate digesters (UASB/EGSB/AF)</td>
<td>Domestic and industrial wastewater</td>
<td>&lt; 0.2</td>
<td>&lt; 1</td>
<td>(1)ᵇ – 30</td>
</tr>
<tr>
<td>Solid phase reactor / Leach bed reactor</td>
<td>Organic solids</td>
<td>10 – 30</td>
<td>30 – 50 (batch)</td>
<td>6 – 10</td>
</tr>
</tbody>
</table>

¹ Maximal values suggested for codigestion in WWTP in Germany (DWA, 2009).
ᵇ Value for domestic wastewater UASB plants (e.g., Souza et al., 2011).

Solid phase reactors include batch and continuous systems. The leachate is recirculated through the solid substrates, thus avoiding mixing and pumping of the substrate, hence, the denomination of leach bed reactor (LBR). These degradation processes are comparable to the anaerobic stabilization in landfills, however, much more rapid decomposition is possible through the leachate recirculation (Chugh et al., 1999). Very high biomass and enzyme concentrations can be obtained in the percolate, resulting in high hydrolysis rates. Busch et al. (2008), for instance, report that the same degradation rates that are achieved in 18 days in a LBR require 60 days in a mixed tank. Other positive aspects of these systems versus mixed tanks is that no milling of the substrate is required.
and a simple process control through the leachate recirculation is possible. The utilization of plug-flow reactors is, in principle, more advantageous as a continual gas production is possible, though complicated and expensive mechanical systems are required (Batstone and Jensen, 2011). Similar stability in the gas production can be obtained by utilizing a series of sequentially fed batch solid phase reactors (Buschmann and Busch, 2009; Nizami and Murphy, 2011).

1.3.3 Combined solid phase and fixed biomass reactors

The use of LBRs is the most promising alternative to convert organic solid waste into \( \text{CH}_4 \) due to their capacity to degrade substrate with high-solid contents at high OLRs (Table 1-3). There is also the possibility of combining them with high-rate digesters composing a two-stage system. The hydrolysis of the solid substrates producing a leachate highly enriched in soluble organic substrates occurs at the first stage and, at the second stage, this leachate is circulated through a fixed biomass digester and high rates of \( \text{CH}_4 \) production are possible. The effluent from the second stage can be recirculated back to the LBRs, retaining nutrients and suspended biomass within the system. The benefits of this two-stage configuration versus single stage LBRs was demonstrated by Lehtomäki et al. (2008) in a comparative experimental study with grass silage. The two-stage system achieved an efficient substrate degradation under significantly lower SRTs. In the same study, a literature overview of data of two-stage systems, LBRs + AF or UASB, reports high specific methane yields for substrates such as fruit and vegetables waste (383 – 405 L\( _{\text{CH}_4} \) kg\( _{\text{VS}} \) d\(^{-1}\)), potato waste (258 – 390 L\( _{\text{CH}_4} \) kg\( _{\text{VS}} \) d\(^{-1}\)), sugar beet (440 L\( _{\text{CH}_4} \) kg\( _{\text{VS}} \) d\(^{-1}\)) and grass silage (270 – 390 L\( _{\text{CH}_4} \) kg\( _{\text{VS}} \) d\(^{-1}\)). Examples of full-scale plant designs utilizing this, or similar, combined reactor configurations for MSW degradation can be found in Bischofsberger et al. (2005). Another positive aspect from two-stage plants is that most of the \( \text{CH}_4 \) outflow comes from the high-rate methanogenic reactor reaching \( \text{CH}_4 \) partial pressures up to 85 %, which could be potentially directly used as fuel gas (Schönberg and Linke, 2012).

In this two-stage configuration, both major limiting steps in the solids fermentation, hydrolysis and acetate degradation (Lübken et al., 2010), are operated separately. The hydrolysis kinetics drive the design of the solid stage. It is noteworthy that the first-order hydrolysis kinetics overviewed in Table 1-1 were mostly found for mixed tank reactors, hence, higher rates can be expected in LBRs. Perculation rates and feeding intervals can be optimized to obtain increased degradation rates (Buschmann and Busch, 2009; Cysneiros et al., 2011; Nizami et al., 2010). In the second stage, the fixed biomass system is operated with the VFA-rich leachate from the solid-phase reactors, forming a highly specialized acetogenic-methanogenic consortium. A continual operation of the fixed biomass reactor in maximum loading conditions is possible if an adequate
operational strategy is chosen. The inflow rate can be stopped or reduced with a rapid response from the system at the first signs of the system overloading (Busch et al., 2008). Therefore, it is very important to understand the ongoing process and identify processes imbalance at an early stage. This demands a detailed understanding of the interaction between VFA-oxidizing Bacteria and methanogenic Archaea; whereby, acetate is quantitatively the most important VFA (Figure 1-1). In addition, Gijzen (2002) suggests that potential still exists for the development of systems with OLRs substantially higher than those depicted in Table 1-3. He compares the current anaerobic technologies with natural CH₄-producing environments, whereby, animals and insects’ gastrointestinal tracts can by far outcompete high-rate methanogenic reactors (considering the specific volumetric CH₄ production).

1.4 Methanogenic pathways determination through stable carbon isotope analysis

1.4.1 Carbon isotopes notation, analysis, mixing and fractionation

There are two stable carbon isotopes, ¹²C and ¹³C, and one radioactive, ¹⁴C, that are relevant for carbon cycle studies (Holmen, 1992). In the two stable forms, the number of protons in the nucleus is the same, six, and the number of neutrons varies, six or seven. In the radioactive form, ¹⁴C, there are eight neutrons in the nucleus and, through a beta decay, one of the neutrons decays into a proton yielding an ¹⁴N atom. With a half-life of 5,730 years, the ¹⁴C is widely used for radiocarbon dating in archaeological, anthropological, paleotemperature and zoological studies (Laeter et al., 2003). Nevertheless, the ¹⁴C abundance is very low and does not interfere in the estimation of the ¹²C and ¹³C isotope abundances (Holmen, 1992). Carbon, together with hydrogen, nitrogen, oxygen and sulfur, is found mostly in its light form. The distribution between light, ¹²C, and heavy, ¹³C, carbon lays approximately from 98.9 % to 1.1 % (Table 1-4).

Table 1-4: Isotopes for the light elements HCNOS (hydrogen, carbon, nitrogen, oxygen and sulfur; from Fry, 2006).

<table>
<thead>
<tr>
<th>Element</th>
<th>Isotope abundance (%)</th>
<th>Mass difference (Relative)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low mass (L)</td>
<td>High mass (H)</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
<td>¹H 99.984</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
<td>¹²C 98.89</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
<td>¹⁴N 99.64</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
<td>¹⁶O 99.76</td>
</tr>
<tr>
<td>S</td>
<td>Sulfur</td>
<td>³²S 95.02</td>
</tr>
</tbody>
</table>

ᵃMass difference given as the mass ratio from the heavy to light isotopes, e.g. ¹³C/¹²C = 13/12 = 1.08.
In investigations concerning the natural abundances of the HCNOS stable isotopes, the δ-notation is the standard notation, but differences in the notations for isotopic abundance and especially for fractionation factors exist in the literature. A detailed overview of isotopic notations can be found in the works by Fry (2006) and Hayes (2002), among others. The notation adopted in this work is described in detail below. In the δ-notation, the difference of the abundance of the heavy isotope to a standard is given multiplied by a 1000-factor, resulting in per mil values (‰):

\[
\delta^{\text{HX}} = \left[ \frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right] \times 1000 \quad (\text{‰})
\]

(1-10),

where \(\delta^{\text{HX}}\) gives the abundance of the heavy isotopes \(\text{HX}\). The ratio of the heavy isotope of the sample \(R_{\text{probe}}\) is given as the ratio of heavy to light isotopes, as atom abundance percentage (AP) or molar fraction (F):

\[
R_{\text{Sample}} = \frac{\%^{\text{HX}}}{\%^{\text{LX}}} = \frac{\text{H}_F}{\text{L}_F}
\]

(1-11),

and the ratio of the standard \(R_{\text{standard}}\) is defined by international reference standards. The carbon standard is the PeeDee Belemnite (PDB; that can also be referred to as Vienna-PDB, or VPDB) that has a R-value of 0.011180, corresponding to an AP of 1.1056 \(^{13}\text{C}\) (Fry, 2006). Thus, negative δ indicates that the sample has a lower \(^{13}\text{C}\) abundance than the standard (and not a negative abundance). The main reasons for the δ-notation utilization are as follows: i) isotope mass spectrometers give relative and not absolute values; ii) reproducibility of measurements in different laboratories requires a standard; iii) absolute values and variation are very low; and iv) absolute variations are more important than absolute values (Scholz et al., 2010).

Studies using natural isotopic concentrations to identify environmental phenomena depend on very low variations of the isotope abundance, e.g. variations of the abundance of heavy carbons in the order of 0.01 % indicate the equilibrium difference between gaseous CO\(_2\) and dissolved bicarbonate (HCO\(_3^-\)). Figure 1-3 gives the natural ranges for the \(^{13}\text{C}\) isotope abundances as well as the \(^{13}\text{C}\) ranges in the biogenic CH\(_4\). Natural variations of the \(^{13}\text{C}\) abundance are in a very narrow range, below 0.2 % (\(\delta^{13}\text{C}\) from -130 to 38 ‰), and for the biogenic CH\(_4\) production the range is below 0.1 % (\(\delta^{13}\text{C}\) from -110 to -50 ‰). Hence, it is very important to consider the precision of the analytical methods.
Figure 1-3: Ranges of $^{13}$C isotopes from natural samples (gray line) and from biogenic CH$_4$ samples (black line) given as $\delta$-values and percentage abundances (AP). Atmospheric CO$_2$ and CH$_4$ $^{13}$C abundances are given by the circle and square points, respectively. Data from Laeter et al. (2003), Lassey et al. (2000) and Whiticar et al. (1986).

The isotope ratio mass spectrometry (IRMS) is the standard method for measurements of stable carbon isotopes (Fry, 2006). Carbon is analyzed in the form of CO$_2$, thus CH$_4$ and other organic carbonaceous compounds need to be previously oxidized to CO$_2$. The measurement principle consists of ionizing the CO$_2$ gas molecules that will be further accelerated through a magnetic field and separated by inertia (a heavier atom requires more force to be moved). The precision of IRMS measurements are typical around 0.1 ‰, thus, at three orders of magnitude higher than the $^{13}$C variation range found for biogenic CH$_4$ (Hayes, 2002). Recently, the possibility of utilizing near-infrared laser optical spectrometry for $^{13}$C in methane in a biogas plant was reported (Keppler et al., 2010). This method may be fundamental for further applications of stable isotope analysis in biogas plants, as the measuring devices are transportable, simpler to operate than IRMS and more suitable for online measurements. However, IRMS is still the more precise analytical method.

Studies involving stable isotopes depend on the description of fractionation and mixing processes. Fractionation and mixing processes are shown schematically in Figure 1-4 as two opposite processes, but, in reality, mixing and several fractionation processes occur simultaneously and can only be investigated separately in controlled laboratory experiments. The mixing of a compound from sources with different isotopic abundance, or signatures, allows the utilization of the isotopes as a natural tracer. Some examples are studies involving $\delta^{13}$C signatures to identify atmospheric CH$_4$ emissions sources (Lassey et al., 2000), quantification of estuaries’ contamination with domestic sewage (Gearing et al., 1991) and the identification of the carbon sources in sewage composition (Griffith et al., 2009). The $^{13}$C abundance that result from the mixing of two
or more sources can be calculated as the mass-weighted average of all sources (Hayes, 2002):

\[
\%^{H}X_{\text{mixed}} = \frac{M_{1} \cdot \%^{H}X_{1} + \ldots + M_{n} \cdot \%^{H}X_{n}}{M_{1} + M_{2} + \ldots + M_{n}} \quad (1-12),
\]

where \( M \) is the mass of \( X \) (Mol number of \( X \)) from each substrate source \( i \). Instead of the AP values, it is also possible to utilize the \( \delta \)-values directly. Although the utilization of the \( \delta \)-notation is more straightforward and avoids the conversion of the \( \delta \)-values to AP, it implies deviations of the values calculated. As the \( \delta \) gives the relative abundance of heavy atoms in relation to a standard and not an absolute value, the calculation errors increase with the distance of the \( \delta \)-value from zero (Fry, 2003). Calculations involving natural isotopic abundances led to reduced errors, normally below 0.02 \( \% \) (Hayes, 2002), hence, within the measurement’s uncertainties. However, the \( \delta \)-notation should be avoided for calculations involving highly enriched or depleted samples.

Figure 1-4: Schematic description of fractionation and mixing processes for a closed system (adapted from Fry, 2006). The colors indicate the heavy isotope abundances qualitatively (high = black, intermediate = gray and low = white). The calculations are only valid if the mass flows to both products (\( M_{1} \) and \( M_{2} \)) or both sources are the same. The utilization of \( \delta \)-notation allow for a more simple calculation, but should be avoided in cases where labeled substrates, i.e. if too high or too low \( \delta \)-values, are involved; see Fry (2003) and Hayes (2002) for more details.

Lighter isotopes have weaker chemical bonds and fast diffusion rates, which result in slightly higher reaction rates, defined as kinetic isotope effects (KIEs). These KIEs are not directly observable and are only identifiable through the isotopic abundance variation (Hayes, 2002). The investigation of KIEs allows for a differentiation of several physical, chemical and biological processes. The ratio from the kinetic rates involving light and heavy isotopes, \( ^{l}k \) and \( ^{H}k \), respectively, gives the fractionation factor, \( \alpha_{H} \) (Fry, 2006):

\[
\alpha_{H} = \frac{l_{k}}{H_{k}} \quad (-)
\]

(1-13),
where \(^{\text{H}}\text{k}\) and \(^{\text{L}}\text{k}\) stand for the kinetics for the light and heavy isotopes respectively.

The fractionation factor can also be described as the ratios of the isotope abundance ratios (Hayes, 1993):

\[
\alpha_H = \frac{R_{\text{Source}}}{R_{\text{Product}}} = \frac{\delta_{\text{Source}} + \delta_{\text{Product}}}{\delta_{\text{Product}} + \delta_{\text{Source}}} \quad (1-14)
\]

Positive \(\alpha\)-values indicate a depletion of heavy isotopes in the product and, consequently, negative values correspond to an enrichment. The arithmetical difference of the \(\delta\)-values from reagents and products leads to determination of the \(\Delta\)-value, also defined as a fractionation factor (Fry, 2003):

\[
\Delta = \delta_{\text{Source}} - \delta_{\text{Product}} \quad (1-15)
\]

and both fractionation factors can be converted:

\[
\Delta \cong (\alpha - 1) \times 1000 \quad (1-16)
\]

Due to the errors resulting from the \(\delta\)-notation approximations (see discussion about mixing calculations above) for processes with large isotope separations, \(\Delta\) over 25 \%, equations 1-13 and 1-14 are preferred (Whiticar, 1999). In biological processes, KIE effects are mainly relevant for autotrophic or for heterotrophic organisms absorbing and processing individual molecules with three or fewer carbon atoms (Hayes, 1993). The net sum of the effects of a chemical reaction where more compounds are continually in exchange are denominated isotopes, equilibrium isotopic effects (EIEs), whereby the heavy isotopes tend to concentrate where the bonds are stronger (Fry, 2006):

\[
\alpha_{\text{eq}} = \frac{R_{\text{heavy molecule}}}{R_{\text{light molecule}}} \quad (1-17)
\]

where \(\alpha_{\text{eq}}\) is the equilibrium factor between both species.

### 1.4.2 Carbon isotopes – global distribution

Some of the major processes and sources affecting the global \(^{12}\text{C}\) and \(^{13}\text{C}\) abundances are depicted in Figure 1-5. The carbon fixation through photosynthesis is one of the major fractionation processes in the global carbon cycle. The photosynthetic \(^{12}\text{C}\) fixation affects the isotopic distribution in the soil, fresh water and marine environments (Peterson and Fry, 1987). The photosynthetic pathway C3, present in most terrestrial and aquatic plants, and C4, mostly found in tropical grasses and desert plants, have different fractionation factors (Farquahr et al., 1989). In C3 plants, the fractionation factor is, on average, around 21 \%, while in the C4 pathway, the fractionation is much reduced, around 6 \% (see Figure 1-5). The C4 pathway was an adaption to obtain a maximum \(\text{CO}_2\) fixation with less loss of water (Hobbie and Werner, 2004) and is uncommon in vascular plants, whereby maize
and sugar cane are two exceptions. It is notable that both of these C4 plants are currently the two crops most explored for biofuel production. In addition to the differences in the overall isotopic composition of C3 and C4 plants, there is also a significant difference in the $\delta$-values within the C3 and C4 plant components. This leads to a significant difference in the fermentation products, such as glucose and ethanol, obtained from both plant types (Hobbie and Werner, 2004; Rossmann et al., 1991).

The EIEs between gaseous $CO_2$ and dissolved $HCO_3^-$ can be described as follows (Mook et al., 1974):

$$H^{12}CO_3^- + ^{13}CO_2 \leftrightarrow H^{13}CO_3^- + ^{12}CO_2$$

(1-18)

with the equilibrium constant defined as:

$$\alpha_{HCO_3,CO_2(g)} = \frac{R_{HCO_3}}{R_{CO_2}} (-)$$

(1-19)

![Diagram](image)

**Figure 1-5:** Selected $\delta^{13}C$ abundances and flows (adapted from Peterson and Fry, 1987). Carbon flows without fractionation (continuous lines) and involving fractionation (dashed lines) are depicted. Sewage sludge and treated wastewater $\delta^{13}C$-values obtained from Griffith et al. (2009). POM: particulate organic matter; DOM: dissolved organic matter.

The value of $\alpha_{HCO_3,CO_2(g)}$ depends mostly on the temperature, while the chemical equilibrium in the equation 1-17 is driven by the pH value. The $\delta^{13}C$ from the $CO_2$ ($\delta^{13}C_{CO_2}$) in the atmosphere is maintained mostly constant through the equilibrium with the $HCO_3^-$ in the oceans. However, the increasing emission of fossil fuels ($\delta^{13}C_{CO_2}$ of -27 ‰) has been responsible for a reduction in the atmospheric $\delta^{13}C_{CO_2}$ from -7 ‰ to the
current -8 ‰ over the last 100 years (Fry, 2006). Treated and untreated wastewater also cause important anthropogenic emissions that affect the δ\textsuperscript{13}C cycle (Gearing et al., 1991; Griffith et al., 2009).

### 1.4.3 Methanogenic carbon isotope fractionation

Commonly, methane originating from hydrogenotrophic methanogenesis is lighter than that originating from acetoclastic methanogenesis (Whiticar, 1999). The difference in the fractionation factors in both pathways, together with information concerning the carbon isotopic distribution on acetate, CO\textsubscript{2} and CH\textsubscript{4} allows one to disentangle the contribution of each substrate to the total methane formation (Conrad, 2005). Although this method requires a high number of parameters and is usually less precise than labeled isotope experiments (Conrad, 2005), no interference with the system investigated is necessary. Therefore, this method is independent of system scale and is potentially applicable to both laboratory reactors and full-scale plants. Although still a novel approach to analyze engineered systems, methanogenic pathway determination through the analysis of stable isotopes has already been reported for a wide range of anaerobic ecosystems, including crop field soils, boreal peatlands and lake sediments (Conrad, 2005; Conrad et al., 2014). Notably, some findings from these studies in natural methanogenic environments present a convergence with current investigations on bioreactors (see Section 1.2.4): the SAO may be a relevant process in the carbon flow in some environments, such as rice field soils and lake sediments (Conrad and Klose, 2011; Conrad et al., 2010; Nuesslein et al., 2003), and hydrogenotrophic methanogenic contribution can be the main methanogenic pathway in soils depending on the content of organic matter (Penning and Conrad, 2007).

In the conversion from complex organic matter to methane (see Figure 1-1), a differentiation of the carbon isotopic abundance within anaerobic digestion subproducts also occurs. Nevertheless, most of the present literature data concentrate on the fractionation driven by methanogenic Archaea from CO\textsubscript{2} reduction, acetate and other methylate compounds (Conrad, 2005). A review of the fractionation parameters involved is given in Table 1-5. Data on carbon fractionation previous to the acetate formation is still rare (e.g., Conrad et al., 2014; Penning and Conrad, 2006). The flow scheme of the isotopic fractionation in the methanogenesis according to Conrad (2005) is shown in Figure 1-6, and the related equations are detailed in Section 2.5.1. A rough estimate of the distribution between the acetoclastic and hydrogenotrophic pathways can be obtained through the difference between the δ-values from CO\textsubscript{2} and CH\textsubscript{4}. Differences over 65 ‰ for both δ-values are characteristic of a dominance of the CO\textsubscript{2}-derived methanogenesis, while differences below 55 ‰ indicate a predominance of acetoclastic methanogenesis (Conrad, 2005).
An integration of the isotopic variations in a model for complex substrate degradation was developed for the degradation of MSW and cellulosic wastes (Vavilin, 2012a, 2012b). In this model, the $^{13}$C abundance from products (SP), $f_{SP}$, is dynamically calculated from the isotopic abundance in the source substrate (SS), $f_{SS}$, and the fractionation factor, $\alpha$:

$$\frac{df_{SP}}{dt} = \frac{dS_P}{dt} \cdot \left( \frac{d^{13}S_P}{dt} - f_{SP} \right) \approx \frac{dS_P}{dt} \cdot \left( \frac{1}{\alpha} \cdot f_{SS} - f_{SP} \right)$$

(1-20).

Hence, it is possible to obtain the contributions of the hydrogenotrophic and acetoclastic methanogenesis directly from the model by fitting the isotopic ratios in the methanogenic products, CO$_2$ and CH$_4$. Only KIEs related to the hydrogenotrophic methanogenesis were considered in these simulations. Acetoclastic driven KIEs and the EIEs between the CO$_2$ and dissolved HCO$_3^-$ are not included in the model. The simulations indicated that CO$_2$ was the main precursor for CH$_4$ production in the batch fermentation of MSW and that the contribution of the acetoclastic methanogenesis varied significantly (Vavilin, 2012b).

**Figure 1-6:** Scheme C flow and $^{13}$C-isotopic change in a methanogenic environment. The scheme does not explicitly show the C flows via acetate carboxyl, which is included in the reaction of $\delta_{\text{org}}$ to $\delta_{\text{CO}_2}$ (from Conrad, 2005). TMA: Trimethylamine.

Besides the biological fractionation, the assessment of methanogenic pathways may also involve physical and chemical fractionation processes. The presence of $^{12}$C or $^{13}$C in the CH$_4$ and CO$_2$ interferes with the molecule solubility. In the CH$_4$, the heavier molecule is more soluble, as happens in most of the gases, while in CO$_2$, the heavier molecule is less soluble, due to the specific structural properties of the CO$_2$ molecule (Bacsik et al., 2002). Nevertheless, the solubility effect is considerably lower ($\Delta$ values around ± 1) than the biological fractionation (see Table 1-5), and diffusive transport effects are more relevant in environments where the liquid gas exchange is more difficult, e.g. in wetlands.
(Chanton, 2005). Though the dissolved HCO₃⁻ to gaseous CO₂ equilibrium factor is stronger (ΔHCO₃⁻CO₂g ~ 10), its direct influence in the methanogenesis fractionation is reduced, as CO₂ is the species utilized by the methanogens (Conrad, 2005). An exemplary calculation for acetate degraded through the acetoclastic and SAO pathways is given in absolute ¹²C and ¹³C concentrations and in AP and δ notations in Appendix I-B.

Table 1-5: Review of relevant fractionation parameters given as Δ-values and α-values from methanogenic environments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Process description</th>
<th>Δ-value</th>
<th>α-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δₐ₀</td>
<td>Acidogenesis/acetogenesis</td>
<td>-3 – 0</td>
<td>0.997 – 1.000</td>
<td>Penning and Conrad (2006)</td>
</tr>
<tr>
<td>Δₖₐ</td>
<td>Homoacetogenesis</td>
<td>40 – 68</td>
<td>1.040 – 1.068</td>
<td>Blaser et al. (2013)</td>
</tr>
<tr>
<td>Δₖₑ</td>
<td>Acetate oxidation</td>
<td>0 – 10</td>
<td>1.000 – 1.010</td>
<td>Conrad and Klose (2011)</td>
</tr>
<tr>
<td>Δₘ₄ᵃ</td>
<td>Methanogenesis from acetate</td>
<td>21 – 27</td>
<td>1.021 – 1.027</td>
<td>Conrad (2005)</td>
</tr>
<tr>
<td>Δₘ₅ᵇ</td>
<td>Methanogenesis from acetate</td>
<td>7 – 18</td>
<td>1.007 – 1.018</td>
<td>Conrad (2005)</td>
</tr>
<tr>
<td>Δₘₖᶜ</td>
<td>Methanogenesis from CO₂</td>
<td>31 – 77</td>
<td>1.031 – 1.077</td>
<td>Conrad (2005)</td>
</tr>
<tr>
<td>Δₘₖᵗ</td>
<td>Methanogenesis from TMA</td>
<td>50 – 71</td>
<td>1.050 – 1.071</td>
<td>Conrad (2005)</td>
</tr>
<tr>
<td>Δₘₙᵐᵐ</td>
<td>Methanogenesis f. methanol</td>
<td>74 – 94</td>
<td>1.074 – 1.094</td>
<td>Conrad (2005)</td>
</tr>
</tbody>
</table>

Equilibrium isotopic effects

- Δₜ₉⁻¹³C includes the solubility factor for ¹²CO₂/¹³CO₂.
- Δₜ₉⁻¹³C ranges for 0 ºC and 30 ºC.

There are a few applications using the analysis of the stable isotopes natural abundances in engineered systems up to the present time. Most of these investigations concentrate on batch systems (Hao et al., 2011; Hao et al., 2012; Ho et al., 2014; Laukenmann et al., 2010; Lü et al., 2013; Qu et al., 2009a). There have been no studies so far, to the best of my knowledge, which present the determination of methanogenic pathways through stable carbon isotope analysis for continually operated bioreactors. Nikolausz et al. (2013) utilized δ¹³C and δ²H analysis for gas samples of five laboratory reactors and
could confirm that the predominant methanogenic pathway agreed with the dominant methanogenic communities identified. Polag et al. (2013) utilized δ¹³C batch incubations from the same biogas plant degrading MS and manure over two years. In this period, a high variation of the isotope signature in biogas was observed. Although it characterized a clear dynamic between the acetoclastic and hydrogenotrophic methanogenic pathways during this period, the exact quantification of each pathway was not calculated. Lv et al. (2014) also investigated the fermentation of MS in a comparative study with chicken manure. The long-term investigations included stable isotope analysis, however, the study focus was on the δ¹³C as an indicator of process failure and metabolic pathways were not quantified. A high variation of δ¹³C was observed prior to the acidification of the experimental reactors, indicating the potential of the isotopic measurements as an operational monitoring parameter for biogas plants.

1.4.4 Tracer experiments with labeled [¹³C]acetate

The use of labeled isotopes is based on the fact that methane originates only from the methyl carbon in acetate in acetoclastic methanogenesis (ma) (Ferry, 1992; Zehnder et al., 1980), while, after the acetate oxidation (ca), both carbon atoms, methyl and carboxyl, are available for hydrogenotrophic methanogenesis (mc) in the form of CO₂:

\[
2\text{CH}_3\text{COOH}^{ma} \rightarrow 2\text{CH}_4 + 2\text{CO}_2 \quad (1-21)
\]

\[
2\text{CH}_3\text{COOH} + 4\text{H}_2\text{O}^{ca} \rightarrow 2\text{CO}_2 + 2\text{CO}_2 + 8\text{H}_2 \rightarrow 7\text{CH}_4 + 2\text{CO}_2 + 2\text{CO}_2 + 4\text{H}_2\text{O} \quad (1-22)
\]

Equations 1-21 and 1-22 have the same net reaction, with two Mol of labeled [2-¹³C]acetate or [2-¹⁴C]acetate (identified by the underlined carbon atom) being converted into two Mol of CH₄ and two Mol of CO₂. Only labeled methane is present in acetoclastic methanogenesis (Equation 1-21), while the SAO (Equation 1-22) yields a uniform distribution from the labeled carbons in methane and carbon dioxide. The addition of the radioactive carbon isotope, ¹⁴C, allows for a straightforward data interpretation, since there is no presence of ¹⁴C other than the added tracer. By contrast, by adding labeled substrates with heavy stable carbon, ¹³C, the stoichiometry of Equation 2-22 becomes more complex. The CO₂ originating from the labeled acetate is immediately mixed into the inorganic carbon pool from the reactor, making its direct distinction from this background carbon source virtually impossible (Hori et al., 2011). Thus, a more elaborate experimental design and a more detailed description of the phenomena involved is required in order to provide a precise pathway distinction in experiments with labeled [¹³C]acetate.

Different approaches exist to quantify the acetate degradation pathways in investigations with ¹³C labeled substrates. A methodology developed by Shigematsu et al. (2004) and further improved by Hori et al. (2011) and Sasaki et al. (2011) is based on selected ion
monitoring to quantify the SAO contribution for acetate degradation. This method utilizes assays with methyl, carboxyl and completely labeled acetate. The [1,2-\textsuperscript{13}C]acetate is applied to determine the background influence, which is subtracted from the [1-\textsuperscript{13}C]acetate and [2-\textsuperscript{13}C]acetate assays. The conversion of the methyl and carboxyl groups to CH\textsubscript{4} (or CO\textsubscript{2}) in independent assays is used to calculate the SAO minimal and maximal fractions, f_{\text{ac,ox}} (fraction of acetate degraded through the SAO pathway). In the study by Shigematsu et al. (2004), the f_{\text{ac,ox}} for two mesophilic laboratory reactors was determined in the ranges of 60 – 92 % and 1 – 5 % for high and low OLRs, respectively. Sasaki et al. (2011) found f_{\text{ac,ox}} values for thermophilic systems ranging from 74 – 88 % in the fermentation of solid waste, while Hori et al. (2011) determined considerably lower f_{\text{ac,ox}} values, 13 – 21 %, in a reactor fed with synthetic wastewater. It is noteworthy that the ranges given for these three studies refer to one series of assays (with the three different labeled acetates).

Werner et al. (2014) utilized a preliminary treatment with pure light acetate, [1,2-\textsuperscript{12}C]acetate, to determine the \textsuperscript{13}C background, prior to assays with high concentrations of labeled [2-\textsuperscript{13}C]acetate. This approach allows for the calculations of SAO rates directly from the amounts of \textsuperscript{13}CO\textsubscript{2} formed, which were determined through a IRMS. The standard deviations from triplicate serum bottle assays were < 1 %. Werner et al. (2014) investigated mesophilic reactors fed with swine manure operated at high ammonia (4.4 – 4.8 gN L\textsuperscript{-1}) concentrations, and found f_{\text{ac,ox}} values of 16 – 25 % (range given for seven measurements from two reactors over 70 days) against 5 – 10 % from the two low ammonia reactors (1.8 gN L\textsuperscript{-1}). In the investigations of Polag et al. (2013), the relative \textsuperscript{13}C ratios from assays with 7 \% [1-\textsuperscript{13}C]acetate and [2-\textsuperscript{13}C]acetate against unlabeled assays are utilized to identify the SAO contributions. In a series of experiments, the same ratio increase (R_{\text{labeled}} to R_{\text{unlabeled}}) was found for CH\textsubscript{4} and CO\textsubscript{2}, inferring a 100 \% f_{\text{ac,ox}} value (see Equation 1-20).

Although it is clear that using \textsuperscript{13}C-labeled substrates is more complicated than \textsuperscript{14}C-labeled substrates, it avoids radioactive material utilization. Moreover, an adequate description of the fate of the \textsuperscript{12}C and \textsuperscript{13}C isotopes in labeled experiments may lead to important insights for the comprehension of the natural fractionation processes within methanogenic environments. The variations of \textsuperscript{13}C in the dissolved HCO\textsubscript{3}\textsuperscript{-} pool (as result of the labeled substrate addition and pH variations), for instance, is not accounted for in these SAO quantification methodologies reviewed, neither are the biological fractionation from methanogens or acetate oxidizing Bacteria. A mechanistic description of the \textsuperscript{12}C and \textsuperscript{13}C variations may, therefore, be a valuable tool to support the conduction of these kinds of assays.
1.5 Objectives of this study

The previous sections highlight the relevance and potential of CH₄ generation derived from organic waste and biomasses in fermentation plants. These plants present an alternative that allows for the integrated recovery of significant amounts of energy in the form of CH₄ and valuable nutrients as fertilizers, such as N and P. Moreover, anaerobic technologies can play an important role in the mitigation of GHG anthropogenic emissions. The utilization of combined solid phase and high-rate methanogenic reactors poses a system configuration that allows for high OLRs of high-solids substrates with a high potential for process control and, consequently, operational stability. The design and operation of these anaerobic plants depends on a comprehensive understanding of the ongoing processes, which demands a rigorous investigation of the degradation pathways from acetate, the main intermediate in the fermentation to CH₄, which is still not fully understood.

In summary, recent investigations of acetate degradation and methanogenic processes in anaerobic digesters indicate that: i) the once assumed fixed distribution of the methanogenesis pathway’s contribution of two-thirds acetoclastic and one-third hydrogenotrophic should be experimentally reevaluated, or further validated; ii) the importance of SAO is underestimated and the influence of the operational factor to these syntrophic consortia (as SRT and NH₃-N concentrations) requires further investigation. Consequently, analytical methods to determinate the methanogenic pathways and the amounts of SAO are an essential element towards a further understanding of the CH₄ formation in anaerobic plants.

In order to address these issues, the work at hand applies novel methods based on stable isotope analysis to investigate the fate of acetate in detail in an experimental two-stage anaerobic digestion plant. This study presents the methanogenic pathway determination of a mesophilic AF operated over a period of 500 days. The NH₄-N concentrations in the AFs were below the typical high free-ammonia concentration found in SAO studies. Hence, this investigation focuses on the contribution of the biomass' long retention times for the SAO pathway. The AF was designed to allow a separated sampling of biofilm and suspended biomasses, which enabled the determination of the metabolic routes for each of both biomass groups separately in batch experiments with labeled acetate. A mathematical model for anaerobic acetate degradation is developed with distinction of the ¹²C and ¹³C isotopes in order to support the analysis of the experimental data.

The main objectives of this study are to:
• Evaluate the utilization of δ^{13}C analysis to quantify methanogenic pathways in a continually operated methane-producing bioreactor without labeled substrates addition in a long-term investigation.

• Identify the effects of the OLR variation to the methanogenic pathways contribution.

• Develop a methodology to quantify the SAO through stable isotope measurements.

• Describe the main mechanisms of carbon isotope fractionation involved in anaerobic acetate degradation in a mathematical model.

• Determine the difference between the acetate oxidation rates for suspended and sessile biomass in an anaerobic filter.
2. MATERIALS AND METHODS

2.1 Analytical methods

2.1.1 Physicochemical parameters
Total and volatile solids (TS and VS) were determined according to the German standards (DEV, 1981). Total nitrogen (TN), total organic and inorganic carbon (TOC/TIC) were determined in a DimaTOC-2000 (Dimatec, Germany). Alternatively, TN was also measured after reduction with Devarda’s alloy and catalytic reaction (DIN 38409-28:1992-04). Measurements of total ammonium nitrogen (NH$_4$-N) and COD were spectrophotometrically executed in a DR-5000 device (Hach Lange, Germany). Protein concentrations were measured with folin phenol reagent (Lowry et al., 1951), with calibration against bovine serum albumin. Particulate proteins (X$_{PR}$) were determined after filtration of the samples in a 0.45 μm cellulose filter. Volatile fatty acids were measured using a Clarus 580 gas chromatograph (Perkin Elmer, Germany) fitted with an Elite-FFAP column (length: 30 m, internal diameter: 0.25 mm) with the following temperature program: 120 °C, 150 °C in 6 °C min$^{-1}$, holding for 5 min, analysis 10 min. Gas composition was determined in a second gas chromatograph, Elmer Clarus 580 (Perkin Elmer, Germany) at 120 °C. The elemental composition (C, H, O, N and S) was determined according to standard methods DIN 51732 and EN 14582. The digestate samples were previously dried at 105 °C and milled prior to the elemental analysis. If no other identification is given, all the physicochemical parameters were determined at the Institute of Urban Water Management and Environmental Engineering, Ruhr-Universität Bochum (RUB).

2.1.2 Stable carbon isotope analysis
Biogas isotopic ratios were determined through gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMC) analysis at the Institute of Geology, Mineralogy and Geophysics, RUB. The CO$_2$ and CH$_4$ from the gas samples (30 – 50 μL) were separated in a Trace GC Ultra (Thermo Scientific, Germany) fitted with a CP Pora Plot Q column (length: 27.5 m, internal diameter: 0.32 mm) at a constant temperature of 60 °C. Methane oxidation to CO$_2$ was carried out in a GC Combustion III (Thermo Scientific, Germany) at 960 °C (Hilkert, 1995). An IRMS Delta S (Thermo Finnigan, Germany) was used to determine the $^{13}$C/$^{12}$C ratios of the CO$_2$ gas. All measurements were conducted
at least three times. Acetate carbon isotope ratios in the leachate were determined at the Max Planck Institute (MPI) for Terrestrial Microbiology, Marburg, Germany, as described by Conrad et al. (2007). The samples were stored at -20 °C before analysis. Maize silage isotopic analyses were carried out at the Institute for Geology, Leibniz Universität Hannover, Germany. The MS samples were dried at 105 °C and milled prior to isotope analysis. The isotopic ratios were determined with an organic elemental analyzer (Thermo Scientific Flash 2000) connected online to a Thermo Scientific Delta V Advantage mass spectrometer. The isotope ratios are given as δ¹³C values per mil (‰) against the PeeDee Belemnite (PDB) standard. Calculations of statistical measures (standard errors, SE, and confidence intervals, CI) of highly enriched samples, i.e. by using labeled substrates, utilized the fractional abundance instead of δ¹³C values (Fry, 2003).

### 2.1.3 Microbiological analysis

Biomass samples were fixed with 3.7 % formaldehyde directly after sampling and stored at -20 °C. The microscopic analyses were carried out by confocal laser scanning microscopy (TCS SP8, Leica Microsystems, Germany) using LAS AF Leica software at the Institute of Urban Water Management and Environmental Engineering, RUB. The following system settings were used: scan mode xyz – pinhole 1.50 airy, Acusto-Optical Tunable Filter (AOTF) 488 nm (5 % laser intensity), AOTF 633 nm (2 % laser intensity); sequential scan settings for *Aleuria aurantia* Lectin labeled with AlexaFluor488 – 488 nm, photo multiplier tubes (PMT) 490 – 550 nm; sequential scan settings for SYTO60 – 633 nm, PMT (HyD) 637 – 690 nm. The settings for picture size, gain and offset were varied during the experiment to reach the best image resolution and fluorescence signal strength.

Analyses of qPCR and terminal restriction fragment length polymorphism (TRFLP) were conducted at the Leibniz Institute for Agricultural Engineering Potsdam-Bornim (ATB). The enumeration of copy numbers for the bacterial and archaeal 16S rRNA genes was carried out using a qPCR assay, as previously described by Yu et al. (2005) and Bergmann et al. (2010). Terminal restriction fragment length polymorphism analyses were conducted to characterize the archaeal community structure in the mesophilic reactors. The primer pairs Ar109f and Ar912r were used to amplify the archaeal 16S rRNA gene, as previously reported by Rademacher et al. (2012), and 16S rRNA gene sequence libraries were constructed to identify the TRFs detected. DNA amplification was performed using the same primer pairs (unlabeled) and PCR conditions as for the TRFLP. The PCR products were purified using Nucleospin® Gel and PCR Clean-up (Machery Nagel, Germany). Cloning was performed according to Rademacher et al. (2012) and sequenced by GATC Biotech AG (Germany). All operational taxonomic units
have been submitted to the EMBL database under accession numbers LN624342 – LN624363.

2.2 Operation of the two-stage experimental fermentation plant

2.2.1 Model substrate
Maize (zea mays) silage was chosen as the model solid substrate for these experiments and, after the plant inoculation, was the only feeding substrate. This guaranteed a constant stable carbon isotope ratio (δ¹³C) inflow to the plant. Maize silage (MS) was chosen as the model substrate for these experiments due to three main reasons:

i. as a very common example of a C4 plant, detailed δ¹³C literature data for zea mays and the products derived is available (Fernandez et al., 2003; Rossmann et al., 1991);

ii. due to the low lignin content of the MS (Gehring et al., 2013), no kinetic limitation in the hydrolytic step is to be expected. Furthermore, degradation of MS in combined LBR and AF systems, as utilized in this work, had already been successfully reported (Busch et al., 2008; Cysneiros et al., 2008; Ficara and Malpei, 2011);

iii. the mathematical modeling of the monofermentation of MS had already being investigated in previous studies (Gehring et al., 2013, 2009; Lübken et al., 2015a, 2013), offering a good basis for the experimental design.

The typical ranges in literature of TS, COD and δ¹³C distribution are depicted in Table 2-1. The variations on the COD distribution of MS are apparently not significant for the total CH₄ production (Gehring et al., 2013). Zea mays is composed mostly of cellulose, hemicellulose and starch material (carbohydrates). Hence, these components almost define the δ¹³C of the whole plant (δ¹³C_carbohydrates ~ δ¹³C_bulk material). Proteins, and especially lignin, are more depleted in ¹³C, but together compose just a little more than 10 % of the TS material.
Material and methods

Table 2-1: Ranges in literature for TS and COD distribution in carbohydrates, proteins, lipids and lignin (data from Gehring et al., 2013) and their respective δ¹³C in maize (*zea mays*) silage (data from Fernandez et al., 2003).

<table>
<thead>
<tr>
<th>Plant fractions</th>
<th>Composition (%)</th>
<th>δ¹³C-values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TS fractions</td>
<td>COD fractions</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>86.2</td>
<td>79.1 – 84.7</td>
</tr>
<tr>
<td>Proteins</td>
<td>8.0</td>
<td>8.5 – 10.9</td>
</tr>
<tr>
<td>Lipids</td>
<td>2.6</td>
<td>4.7 – 7.2</td>
</tr>
<tr>
<td>Lignin</td>
<td>3.2</td>
<td>2.4 – 3.7</td>
</tr>
<tr>
<td>Bulk material</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

⁰Values for cellulose and non-ADF (acid-detergent fiber) material, respectively.

2.2.2 Experimental plant design and operational parameters

The experimental plant consisted of three thermophilic leach bed reactors (LBR) followed by a mesophilic upflow anaerobic filter (UAF) and a mesophilic control continuously stirred tank reactor (CSTR; Figure 2-1). Each of the LBRs was connected directly to a storage tank reactor (STR), comprising three pairs of reactors: LBR₀₁-STR₀₁, LBR₀₂-STR₀₂ and LBR₀₃-STR₀₃. A fourth storage tank reactor, STR_UAF, was used for UAF internal recirculation and for the interface between the LBRs and UAF systems. Glass reactors with an inner diameter of approximately 200 mm and a total volume of 13 ± 1 L were utilized, all of which were double-jacketed to control temperature, with the exception of STR_UAF, which was operated at room temperature. The LBR temperatures were maintained between 51 and 56 °C, and the temperature of both the UAF and CSTR was 36.5 °C. Pictures of the two-stage plant, including detailed views of the different reactors, pumping and sampling devices are shown in Appendix II-A.

The LBRs were allocated about one meter higher than the STRs. A sieve plate with bores of 1.5 mm diameter followed by a filter mat (30 dpi; 40 mm thickness) retained the particulate material of the LBR. The sieve plate was fixed in a polyvinyl chloride (PVC) structure, allowing for easy removal of the digestate. The leachate from the STR was pumped into the LBR with a ceramic centrifugal pump (Universal 1250; Eheim, Germany) and percolated back by gravity flow. A gas bag (2 L) was connected to each STR to compensate the rapid volume variations during the leachate recirculation pump operation. The internal recirculation rate was controlled by the OLR to approximately 100 L gVS_{added}⁻¹ d⁻¹ (see Table 3-2 for more details), according to Buschmann and Busch (2009). The LBRs were operated in batch-fed mode with an SRT of 21 days, with an LBR
being fed every seventh day. A portion of digestate mass, equivalent to 20% of the new substrate charge, was retained in each cycle.

A three-channel peristaltic pump (313FDM; Watson Marlow, Germany) recirculated 4.4 L d\(^{-1}\) from the STR\(_{UAF}\) to each LBR semi-continually (three cycles per hour), and the exceeding liquid from the three LBRs overflowed back into the STR\(_{UAF}\). Figure 2-1 shows all reactors, recirculation flows and sampling points in detail. Small openings to the atmosphere of about \(\sim 1\) mm diameter were placed in the overflow connection from the thermophilic STRs to the STR\(_{UAF}\) to allow \(\text{CH}_4\) to be degassed from the leachate, thus minimizing the transport of dissolved \(\text{CH}_4\) from the STRs to the UAF. This thermophilic-originated \(\text{CH}_4\) could potentially interfere with the measurement of the stable carbon signatures from the UAF.

![Diagram of two-stage experimental plant](image)

**Figure 2-1:** Schematic representation of the two-stage experimental plant, recirculation flows and sampling points.

A constant recirculation of 50 mL min\(^{-1}\) was maintained between the UAF and STR\(_{UAF}\) with a peristaltic pump (Pumpdrive 5201; Heidolph, Germany). This resulted in an upflow velocity of 0.125 m h\(^{-1}\). The UAF was designed to allow for a simple biofilm carrier (BC) sampling strategy to regularly characterize the sessile biomass. A total of 50 PVC strips (395 x 18 x 3 mm) were used as BCs, resulting in a surface area of 0.83 m\(^2\). These strips were fixed in a radial disposition from the top of the reactor and could be separately removed without disturbing the remaining biomass of the system.
A numeration system was utilized to identify the position of each BC. The carrier support system was divided into four quarters (A, B, C and D), that were further divided into four rings (I, II, III, IV) which could support two to five BCs (1, 2, 3, 4 and 5). Thus, each BC was identified by its quarter-ring position number, e.g. “B-IV-4.” Figure 2-2 shows a picture from the BC support system and the systematic of the BCs’ identification. A sampling point for the suspended biomass fraction was arranged at the bottom of the reactor.

**Figure 2-2:** Biofilm carrier support structure. Schematic view (a) and a picture before the UAF inoculation (b). The red BC is mentioned in the text to exemplify the numeration system (BC: B-IV-4).

The stirring system from the CSTR was maintained continually at about 10 RPM. The CSTR was operated at a constant HRT of 15 days. The liquid volumes, the solids retention times (SRT) and HRTs of all reactors are provided in Table 2-2. On average, samples of approximately 1 L were taken per week and were replaced with tap water, resulting in an HRT of 380 days for the entire system (total liquid volume of 54 L).
Table 2-2: Operational parameters from the two-stage experimental plant.

<table>
<thead>
<tr>
<th>Reactors</th>
<th>$T_{\text{liq.}}$</th>
<th>$V_{\text{liq.}}$</th>
<th>Rec. Flow</th>
<th>Flow rate</th>
<th>Retention times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>L</td>
<td>[-]</td>
<td>L d$^{-1}$</td>
<td>HRT$^a$ d</td>
</tr>
<tr>
<td>LBR$01$</td>
<td>52.0 ± 1.0</td>
<td>9.0</td>
<td>$a_{01}$</td>
<td>24 – 48</td>
<td>2.0</td>
</tr>
<tr>
<td>STR$01$</td>
<td></td>
<td></td>
<td>$b_{01}+d_{01}$</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>LBR$02$</td>
<td>53.5 ± 1.0</td>
<td>9.0</td>
<td>$a_{02}$</td>
<td>24 – 48</td>
<td>2.0</td>
</tr>
<tr>
<td>STR$02$</td>
<td></td>
<td></td>
<td>$b_{02}+d_{02}$</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>LBR$03$</td>
<td>55.0 ± 1.0</td>
<td>9.0</td>
<td>$a_{03}$</td>
<td>24 – 48</td>
<td>2.0</td>
</tr>
<tr>
<td>STR$03$</td>
<td></td>
<td></td>
<td>$b_{03}+d_{03}$</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>UAF</td>
<td>24.3 ± 2.1</td>
<td>7.0</td>
<td>$b_{01-03}$</td>
<td>13.2</td>
<td>1.5</td>
</tr>
<tr>
<td>CSTR</td>
<td>36.5 ± 0.5</td>
<td>9.0</td>
<td>$d_{01-03}$</td>
<td>0.6</td>
<td>15</td>
</tr>
</tbody>
</table>

$^a$ Hydraulic retention times are calculated for the pairs LBR-STR and for the UAF-STR$_{UAF}$ pair;

$^b$Average retention time for the removable BCs.

2.2.3 Monitoring parameters, plant operation and sampling

All the reactors were provided with online temperature sensors (Pt1000; Fühlersystem, Germany). The gas production and composition was individually determined for each LBR-STR pair, for the UAF and for the CSTR. The gas flows were measured through Milligascounters MGC-1 (Ritter, Germany) and the gas composition in an Awiflex gas analyzer (Awite, Germany) with the following relative measuring uncertainties: CH$_4$ ± 2 %, CO$_2$ ± 2 %, O$_2$ ± 2 %, H$_2$ ± 5 % and H$_2$S ± 5 %. The H$_2$S was not measured in the gas flow from the LBRs. Much higher H$_2$S partial pressures were expected for the operation at thermophilic condition, low pH values and high OLRs in the LBRs, which could damage the online measuring device. Accumulated gas in the headspace from the STR$_{UAF}$ was collected in a gas bag (10 L), and the composition and volume were determined at weekly intervals. Glass sample pipes (500 mL) with a PTFE septum were allocated behind the gas counting devices in the UAF and in the CSTR (see Appendix II-B). Gas-tight glass valves on the samples pipes allowed the transport of the gas samples for the further analysis of stable carbon isotopes. The glass collecting tubes were flushed with nitrogen before each sampling and a new septum was used for each sample.

The plant operation involved a daily routine with visual control of: i) reactor fill volumes, ii) clogging in tubes and connections, iii) pump operation, iv) reactor temperatures and v) gas flow rates. The daily control sheet is shown in Appendix II-A. Additionally, an online system allowed for a remote control of all reactor temperatures and the fill level of the STR$_{UAF}$. The other daily operational tasks included the CSTR feeding and sampling (see sampling points in the Figure 2-1) for the measurement of pH, conductivity and/or
laboratory analytics. The CSTR was fed six days per week by pumping 600 mL of leachate from the LBR-STRs, and the effluent was returned to the UAF-LBR system. A sample from each LBR and from the UAF and CSTR effluents was taken for pH and conductivity measurements. These determinations were conducted with a hand device Multi-3430 (WTW, Germany) using a WTW SinTex-980 pH sonde (pH 0 – 14, 0 – 100 °C, 3 Mol) and a WTW TetraCin-925 conductivity sonde (10 – 2000 µS/cm, 0 – 100 °C). The sample could be returned to the STRUAF after the measurements or stored for further analysis at -20 °C.

2.2.4 Organic loading rates calculation

The OLR calculation from the LBR-STR system was based on the liquid volume from the reactors (\(V_{\text{liq}}\); Table 2-2) and their respective SRT, and was defined on a COD basis:

\[
\text{OLR}_{\text{LBRs}} = \frac{M_{\text{in}} \cdot TS \cdot i_{\text{COD}}}{V_{\text{liq}} \cdot SRT} \quad (\text{g COD L}^{-1} \text{ d}^{-1})
\]

where \(M_{\text{in}}\) is the weight of the given substrate (g), TS is the total solid contents from the substrate (%) and \(i_{\text{COD}}\) the COD content of the substrate (g COD gTS\(^{-1}\)). Different approaches to define the OLRs for LBRs exist in the literature. The utilization of the working volume from the LBRs (\(V_{\text{LBR}}\)) has been reported (Cysneiros et al., 2011; Nizami and Murphy, 2011; Schönberg and Linke, 2012). Thus, this alternative OLR calculation was also utilized to provide a wider comparison basis with literature data:

\[
\text{OLR}_{\text{LBRs}}^* = \frac{M_{\text{in}} \cdot TS \cdot i_{\text{COD}}}{V_{\text{LBR}} \cdot SRT} \quad (\text{g COD L}^{-1} \text{ d}^{-1})
\]

A detailed overview of the reactors’ volume is given in Appendix II-A. The OLR for both mesophilic reactors was determined as a function of the solubilization of organic matter in the LBR system, thus, it varied dynamically and depended on COD measurements from the leachate. The OLR for the UAF was determined as:

\[
\text{OLR}_{\text{UAF}} = \frac{C_{\text{COD,LBRmix}} \cdot Q_{b1,2,3}}{V_{\text{liq,UAF}}} \quad (\text{g COD L}^{-1} \text{ d}^{-1})
\]

and for the control CSTR:

\[
\text{OLR}_{\text{CSTR}} = \frac{C_{\text{COD,LBRmix}} \cdot Q_{d1,2,3}}{V_{\text{liq,CSTR}}} \quad (\text{g COD L}^{-1} \text{ d}^{-1})
\]

with \(Q_{b1,2,3}\) and \(Q_{d1,2,3}\) as the flow rates from the recirculation flows b and d, respectively (Table 2-2) and \(C_{\text{COD,LBRmix}}\) the COD concentration measured from the leachate mixture from the three STRs-LBRs.
2.3 Batch assays for determination of acetate degradation pathways

2.3.1 Batch assays conduction

The contribution of both acetate degradation pathways was quantified through batch assays. Samples from the UAF (sludge and biofilm), from the control CSTR and from a full-scale WWTP digester were utilized as inoculum. The batch assays were conducted in glass bottles with 300 +/- 1 mL total volume (nominal volume of 250 mL; Schott Duran® Pressure Plus, Germany) with online manometric determination of the gas production. A pressure transmitter (ATM.ECO; STS, Switzerland) was fixed in a stainless steel connector which had a manual valve for gas sampling. Manometric data was recorded at 5 min intervals. All the components from a batch assay flask are depicted in Figure 2-3.

![Figure 2-3: Experimental vessel for the batch assays. 1: 250 mL DURAN® laboratory bottle pressure plus (Schott, Germany); 2: manometric transmitter ATM.ECO (STS, Switzerland); 3: custom-made stainless steel connection system with one G 1/4 and one G 1/8 threaded joints; 4: silicon seal; 5: polypropylene screw cap GL 45; and 6: gas-tight valve.]

Start acetate concentrations ranged from 1.3 to 3.3 g L\(^{-1}\). Acetate concentrations around 1 g L\(^{-1}\) are recommended for acetoclastic activity tests (Angelidaki et al., 2007) and the higher dosage should reproduce the overload conditions in the parent reactors. The liquid fill volumes from the assays, which varied from 100 to 160 mL, were adjusted to obtain the maximal gas production for sampling without exceeding 1.5 bar of overpressure. This limit avoids high CO\(_2\) partial pressures on the system, which could affect the pH values (Vavilin et al., 1995). No significant effects on the isotopic equilibrium for the CO\(_2\) and CH\(_4\) gases exists for this pressure range (Harting, 1978; Szaran, 1997). A 10 g L\(^{-1}\) glacial acetate solution (99 – 100 % pure, 60.05 g Mol\(^{-1}\), diluted with tap water; J. Baker, Germany) was the only added carbon source. An amount of [2\(^{-13}\)C]acetate (99 % pure,
61.04 g Mol⁻¹; Sigma Aldrich, USA) was added in the labeled acetic acid assays, comprising 1.3 – 1.5 and 2.6 – 3.2 % of the total acetic acid added.

A quantity of 1.0 to 2.0 L effluent of the UAF was collected (sampling point number 5 from Figure 2-1) for the batch assays preparation; thereafter, the internal recirculation was stopped for at least 15 min to allow a partial sedimentation of the suspended biomass at the bottom of the reactor and a sludge sample was taken (sampling point number 6 from Figure 2-1). One BC was utilized for each batch assay with sessile biomass. They were rapidly removed from the reactor and immediately cut into pieces of 3 – 5 cm with sharp scissors directly into a glass flask containing the acetate solution. A similar approach for acetate specific activity assays was utilized by Gárcia-Morales et al. (2003), who utilized sessile biomass attached in sintered-glass BCs. The UAF effluent collected previously was used to refill the flask up to the desired fill volume. The utilization of the UAF effluent in the assays guaranteed the reproduction of the same nutrient availability found in the UAF. The BC was exposed to atmospheric oxygen for 3 – 5 min during this preparation process. This short exposure time should not affect the activity of the methanogenic biofilm biomass (Kato et al., 1993). The settled sludge at the bottom of the UAF was collected and mixed directly with the acetate solution in the flask in the assays with suspended biomass.

Immediately after refilling the bottles, their headspace was flushed with nitrogen gas for 1 – 2 minutes and afterwards they were tightly sealed. Blank assays with solely UAF effluent and UAF sludge were conducted to determine the gas production from dissolved substrates other than the added acetate. The preparation of the different assays is illustrated in Figure 2-4. The same procedure concerning the assays with UAF sludge was utilized for batch assays with effluent of the control CSTR as inoculum. The bottles were placed in a water bath regulated at 36.5 ± 0.1 °C and were shaken gently once a day. Preceding the gas sampling, the bottles were vigorously shaken in order to enforce/ensure the equilibrium between the carbon isotopes from CO₂ and CH₄ in the liquid and in the headspace (Conrad, 2005). Gas samples were taken in a 50 mL glass sample pipe with two PTFE gas-tight valves and a PTFE septum. Pressurized gas from the batch flasks flowed through the gas pipes into a gas bag (to avoid any contact with the atmosphere). The glass flasks were flushed with nitrogen before each sampling and the septum was replaced each time.
Material and methods

Figure 2-4: Schematic visualization of the assay procedure. a) Assays with suspended biomass inoculum; b) assays with biofilm biomass (for more details see the Appendix III-C).

2.3.1 Calculation of the specific methanogenic activity

The specific methanogenic activity (SMA) was determined through the maximal slope of the gas production from each assay (Batstone and Jensen, 2011):

\[
SMA = \frac{\Delta Q_{CH_4,max}}{\Delta t \times X_{BM}} \left( g_{CH_4,COD} \frac{g_{COD}}{L} \frac{d}{d} \right)
\]

where \( X_{BM} \) is the biomass concentration (g COD L\(^{-1}\)) and \( \Delta Q_{CH_4,max} \) is the maximal specific methane production (g COD L\(^{-1}\) reactor\(^{-1}\) d\(^{-1}\)) for the predefined time interval \( \Delta t \). The determination of the biomass concentration in batch assays is usually defined in terms of volatile suspended solids (VSS; Angelidaki and Sanders, 2004). However, in the experiments in this study, the protein contents were utilized to determine the biomass concentrations. The low sample amount required for the protein determinations, about 1 mL, defined this parameter choice. The VSS determination could require a sample of up to 40 mL from a total sampling volume that was usually below 80 mL. Moreover, it can be assumed that the hydrolyzed proteins from the MS are completely converted into VFAs in the LBRs (with a hydrolysis rate for the MS proteins of 0.3 d\(^{-1}\) according to Lübken et al. 2015a and a SRT of 21 d a degradation degree of over 99 % is calculated) and, thus, that the proteins in the UAF and control CSTR are exclusively from bacterial and archaeal origins.

Protein determination according to the Lowry method (Lowry et al., 1951) is a widespread methodology for analysis of protein mixtures (Waterborg, 2002) and, despite its simplicity, a high correlation to biomass activity is reported (Lazarova and Manem, 1995). Biomass
concentrations were estimated from the particulate (>0.45 μm) protein fraction, $X_{PR}$ (g L$^{-1}$):

$$X_{BM} = \frac{i_{N,PR}}{i_{N,XBM}} \cdot X_{PR} \text{ (g COD L}$^{-1}$)$$

(2-6),

where $i_{N,PR}$ (g N g$^{-1}$) is the average nitrogen content of the proteins, assumed to be 0.16 (Fang et al., 1998), and $i_{N,XBM}$ (g N g$^{-1}$) is the nitrogen content of the biomass. The elementary composition of the biomass was assumed to be C$_5$H$_7$O$_2$N (Lübken et al., 2015b), or normalized to one mol carbon: CH$_{1.4}$O$_{0.4}$N$_{0.2}$, hence, $i_{N,XBM}$ is 0.124 g N g$^{-1}$ and 1 g of biomass corresponds to 1.42 g COD (see Equation 2-9 below).

### 2.3.2 Biofilm characterization

Physical properties and specific concentrations from methanogenic biofilms are seldom reported, as these determinations are complicated due to difficulties in sampling and due to the heterogeneity of biofilm structures. The uniform surface together with the removable BCs in the UAF allowed for estimation of some biofilm properties. The biofilm volume ($V_{biofilm}$) was estimated through the volume displacement:

$$V_{biofilm} = V_{fill} - V_{BC} - V_{UAF,eff} - V_{ac,added} \text{ (mL)} \quad (2-7),$$

where $V_{fill}$ (mL) is the calibrated fill level, $V_{BC}$ (mL) is the BC volume (each BC has a volume of 22 mL), $V_{UAF,eff}$ (mL) is the amount of UAF effluent and $V_{ac,added}$ (mL) is the amount of added acetate solution. The $V_{fill}$, $V_{UAF,eff}$ and $V_{ac,added}$ were gravimetrically determined assuming that 1 g = 1 ml for all the solutions. Distilled water was utilized for the fill level calibration.

At the end of the assays, the biofilm was manually completely scraped off the BC into the liquid phase of the assay obtaining a homogeneous solution. The analysis of this solution was utilized to calculate the start concentrations of COD, TOC, TN and protein in the biofilm:

$$C_{i,biofilm} = \frac{c_{i,end}(V_{fill} - V_{BC}) - c_{i,UAF,eff}.V_{UAF,eff}}{V_{biofilm}} \text{ (mg L}_{biofilm}^{-1})$$

(2-8),

where $C_{i,j}$ (mg L$^{-1}$) is the concentration from the parameter “i” for the solution “j”. Naturally, variations on these parameters during the assay conduction cannot be determined.

### 2.4 Mass balance calculations

#### 2.4.1 Chemical oxygen demand and nitrogen balance determination

Mass balances on the basis of COD and TN measurements were defined for the two-stage experimental plant and for the batch assays. Chemical oxygen demand was measured directly in the liquid samples, while the theoretical oxygen demand (ThOD) for
the gas (CH₄ and H₂) and solid samples (MS and digestate) was calculated as follows (Koch et al., 2010):

\[
\text{ThOD} \ (C_a H_b O_c N_d) = 16 \cdot (2 \cdot a + 0.5 \cdot (b - 3 \cdot d) - c) \ (\text{gO}_2 \ \text{mol}^{-1}) \quad (2-9)
\]

The gas flows or partial gas pressures were converted to the mol number (n) according to the ideal gas constant for standard conditions. The balance for the operational data of the two-stage experimental plant was determined on a weekly basis, accompanying the feeding interval. The inflow was relatively simple, consisting only of the new MS charge. In the output, however, more mass flows needed to be considered: digestate removal, liquid samples, BC removal, and CH₄ and H₂ flows from five measuring points (see Figure 2-5). Moreover, the accumulated masses of N and COD also needed to be considered. In the gas outputs, the volume of gas lost during the opening of the reactor (i.e. the volume of the opened LBR, ~ 13 L) for a new feeding was additionally considered. In the liquid samples, the amount of dissolved CH₄ from the experimental plant and from the batch assays was calculated with the Henry coefficient:

\[
S_{\text{CH}_4} = p_{\text{CH}_4} \cdot K_{\text{HCH}_4} \ (\text{mol}^{-1}) \quad (2-10),
\]

where \(p_{\text{CH}_4}\) is the methane partial pressure (bar) and \(K_{\text{HCH}_4}\) is the Henry coefficient (mol L⁻¹ bar⁻¹).

![Figure 2-5: Schematic description of the mass balances. a) Two-stage experimental plant; b) batch assays.](image)

**2.4.1 **¹³C recovery calculations for batch assays with labeled [¹³C]acetate

The recovery of ¹³C atoms was calculated for each assay with labeled [¹³C]acetate (Figure 2-5b). The balance included only the ¹³C available for degradation during the assays. Hence, the mass from organic matter not degraded present in the inoculum was not included in these calculations. The isotopic balance was defined on the basis of
absolute $^{13}$C concentrations (in µmol$^{13}$C units). Therefore, all the $\delta^{13}$C measurements were first converted into $^{13}$C atom percentage (AP) and later into absolute molar masses. The start $^{13}$C from inorganic carbon (IC) was defined on the basis of $\delta^{13}$CCO$_2$, pH and NH$_4$-N concentration measurements from the parent reactor. With help of the pH and NH$_4$-N, the initial total bicarbonate concentration (HCO$_3^-$) was determined (see Appendix II-D for the calculation of the initial S$_{HCO_3}$ concentration). The $^{13}$C abundance in the HCO$_3^-$ was calculated through the Equation 1-19. The $\delta^{13}$C from the degradable substrate in the inoculum was estimated according to data from Londry et al. (2008), assuming that $\delta^{13}$C$_{inoculum} = \delta^{13}$C$_{biomass}$.

The start $^{13}$C mass in the experiment ($M_{13C,t0}$) resulted from the sum of the $^{13}$C atoms in the added acetate solution ($M_{13C,ac}$), in the dissolved methane from the inoculum ($M_{13C,diss.CH4}$), in the degraded inoculum fraction ($M_{13C,inoc}$) and in the total inorganic carbon ($M_{13C,TIC}$):

$$M_{13C,t0} = \sum M_{13C,ac,t0} + M_{13C,inoc,t0} + M_{13C,TIC,t0} + M_{13C,diss.CH4,t0} \quad (2-11).$$

The masses in all the calculations were determined in µmol $^{13}$C. At the end of the assays, the heavier carbon masses should be found again in the headspace as CH$_4$ ($M_{13C,CH4}$) and CO$_2$ ($M_{13C,CO2,end}$), and dissolved as CH$_4$ ($M_{13C,sol.CH4}$) and total inorganic carbon:  

$$M_{13C,end} = \sum M_{13C,CH4,end} + M_{13C,CO2,end} + M_{13C,TIC,end} + M_{13C,sol.CH4,end} \quad (2-12).$$

Alternatively, a more detailed balance was utilized including the $^{13}$C masses from the acetate which was not degraded (assuming that $\delta^{13}$C$_{ac,t0} = \delta^{13}$C$_{ac,end}$) and the $^{13}$C incorporated in the biomass (calculated directly proportional to $\delta^{13}$C$_{ac,t0}$ and the biomass growth yield without any fractionation):

$$M_{13C,end} = \sum M_{13C,t_end} + M_{13C,ac,t_end} + M_{13C,biomass,t_end} \quad (2-13).$$

### 2.5 Quantification of the methanogenic pathways

#### 2.5.1 Carbon isotope fractionation flow chart

The amount of CH$_4$ produced from CO$_2$ through the analysis of stable carbon isotopes were determined following the calculations proposed in Conrad (2005). The stable carbon isotope ratio of CH$_4$ derived from the acetoclastic methanogenesis, $\delta^{13}$C$_{ma}$, results from the acetate-methyl stable carbon isotope ratio, $\delta^{13}$C$_{ac-methyl}$, and the acetoclastic fractionation factor $\alpha_{ma}$:

$$\delta^{13}C_{ma} = \frac{1}{\alpha_{ma}} \cdot (\delta^{13}C_{ac-methyl} + 10^3 - \alpha_{ma} \cdot 10^3) \quad (\%) \quad (2-14).$$

Analogously, the carbon isotope ratios of CH$_4$ derived from CO$_2$, $\delta^{13}$C$_{mc}$, can be calculated:
\[
\delta^{13}C_{mc} = \frac{1}{\alpha_{mc}} \cdot (\delta^{13}C_{CO2} + 10^3 - \alpha_{mc} \cdot 10^3) \quad (\%) \tag{2-15},
\]

where \(\delta^{13}C_{CO2}\) is the stable carbon isotope ratio for the CO\(_2\) and \(\alpha_{mc}\) is the fractionation factor for the hydrogenotrophic methanogenesis. The active species of carbon utilized by hydrogenotrophic methanogenesis is CO\(_2\) rather than HCO\(_3^{-}\) (Conrad, 2005), therefore, the CO\(_2\) values measured in the gas phase are utilized in Equation 2-15. The reduced isotopic equilibrium effect between the dissolved and gaseous CO\(_2\) (see Table 1-5) is not considered in the calculations. Considering acetate and CO\(_2\) as the only methanogenic precursors, the CH\(_4\) fraction originating from the hydrogenotrophic methanogenesis, \(f_{mc}\), is calculated as:

\[
f_{mc} = \frac{\delta^{13}C_{CH4} - \delta^{13}C_{ma}}{\delta^{13}C_{mc} - \delta^{13}C_{ma}} \quad (2-16),
\]

where \(\delta^{13}C_{CH4}\) is the stable carbon isotope ratio for the CH\(_4\). A visualization of this calculation procedure is given in Figure 2-6. Data from \(\delta^{13}C\) from the acetate and CO\(_2\) is utilized to calculate the theoretical \(\delta^{13}C_{CH4}\) resulting from the microbial fractionation of both methanogenic pathways. The \(\delta^{13}C_{CH4}\) measured as the mixture of both CH\(_4\) sources allow for the determination of the contributions of each pathway.

**Figure 2-6**: Schematic flow chart of the carbon isotope-based methanogenic pathway determination. Measured variables (fully filled circles) and calculated (without filling) relative to the acetoclastic (black arrows and circles) and hydrogenotrophic (gray arrows and circles) methanogenesis are given.
2.5.2 Thermodynamic calculations

Thermodynamic calculations were included to determine the constraints for both methanogenic pathways. The actual Gibbs energy change (ΔG) values were determined on the basis of the activities of substrates and products according to Kleerebezem and Van Loosdrecht (2010):

\[
\Delta G = \Delta G^0 + R \cdot T \cdot \sum_{i=1}^{n} Y_{si} \cdot \ln(a_{si}) \text{ (kJ reaction}^{-1})
\]  

(2-17),

where \( R \) is the ideal gas constant (bar mol\(^{-1}\) K\(^{-1}\)), \( T \) the temperature (K), \( Y_{si} \) the stoichiometric coefficient of the compound Si, \( a_{si} \) its activity and \( \Delta G^0 \) the standard Gibbs free energy calculated, as follows:

\[
\Delta G^0 = \sum_{i=1}^{n} Y_i \cdot G_{f_i}^0 \text{ (kJ reaction}^{-1})
\]  

(2-18).

Values for the standard formation values of the Gibbs energy (\( G_{f_i}^0 \)) were obtained from Kleerebezem and Van Loosdrecht (2010) and Thauer et al. (1977).

2.5.3 Stoichiometric determination of the maximal hydrogen yields

In addition to the \( f_{mc} \) calculations from the stable carbon isotope signatures, calculations were performed to determine the maximum hydrogen yields from the combined syntrophic VFAs’ oxidation, analogously to Jeris and McCarty (1965) and Weng and Jeris (1976). The stoichiometry suggested in the ADM1 for acetogenic processes (Batstone et al., 2002) defined the propionate (pro), butyrate (but) and valerate (val) oxidation products, however, for caproate (cap), the β-oxidation route was assumed (Jeris and McCarty, 1965). The VFA oxidation reactions and the calculated yields for acetate and hydrogen are listed in detail in Table 2-3.

Table 2-3: VFA oxidation reactions and calculated acetate and hydrogen yields according to the ADM1 (Batstone et al., 2002).\( ^a \)

<table>
<thead>
<tr>
<th>Acetogenic reactions</th>
<th>( Y_{\text{ac,VFA}} ) ( \text{gCOD}^{-1} )</th>
<th>( Y_{\text{H2,VFA}} ) ( \text{gCOD}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate</td>
<td>( \text{C}_3\text{H}_5\text{O}_2^- + 3 \text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_3\text{O}_2^- + 3 \text{H}_2 + \text{HCO}_3^- + \text{H}^+ )</td>
<td>0.569</td>
</tr>
<tr>
<td>Butyrate</td>
<td>( \text{C}_4\text{H}_7\text{O}_2^-+2 \text{H}_2\text{O} \rightarrow 2 \text{C}_2\text{H}_3\text{O}_2^- + 2 \text{H}_2 + \text{H}^+ )</td>
<td>0.752</td>
</tr>
<tr>
<td>Valerate</td>
<td>( \text{C}_5\text{H}_9\text{O}_2^-+2 \text{H}_2\text{O} \rightarrow \text{C}_3\text{H}_5\text{O}_2^- + \text{C}_2\text{H}_4\text{O}_2 + 2 \text{H}_2+ \text{H}^+ )</td>
<td>0.547</td>
</tr>
<tr>
<td>Caproate</td>
<td>( \text{C}<em>6\text{H}</em>{11}\text{O}_2^- + 4 \text{H}_2\text{O} \rightarrow 3 \text{C}_2\text{H}_3\text{O}_2^- + 4 \text{H}_2 + 2 \text{H}^+ )</td>
<td>0.670</td>
</tr>
</tbody>
</table>

\( ^a \) Biomass growth yields were considered in the calculations, thus, \( Y_{\text{CH}_4,\text{ac}} + Y_{\text{CH}_4,\text{H}_2} < 1. \)

The resulting total yields of the acetate and hydrogen from the inflow VFA loads, \( b_{\text{ac,VFA}} \) and \( b_{\text{H2,FA}} \) (both in \( \text{gCOD d}^{-1} \)), respectively, are calculated considering a complete VFA and hydrogen uptake:
Material and methods

\[ b_{ac,VFA} = b_{ac} + \sum b_{pro} \cdot Y_{ac,pro} + b_{but} \cdot Y_{ac,but} + b_{val} \cdot Y_{ac,val} + b_{cap} \cdot Y_{ac,cap} \]  \hspace{1cm} (2-19)

\[ b_{H2,VFA} = \sum b_{pro} \cdot Y_{H2,pro} + b_{but} \cdot Y_{H2,but} + b_{val} \cdot Y_{H2,val} + b_{cap} \cdot Y_{H2,cap} \]  \hspace{1cm} (2-20),

where \( b_i \) stands for the acid \( i \) load (in \( g_{COD} \) d\(^{-1} \)) and \( Y_{j,i} \) for the yields from the substrate \( i \) to the product \( j \) (in \( g_{COD} g_{COD}^{-1} \)). The hydrogen to acetate ratio, \( f_{H2,VFA} \), in the UAF is calculated as:

\[ f_{H2,VFA} = \frac{b_{H2,VFA}}{b_{ac,VFA}+b_{H2,VFA}} \]  \hspace{1cm} (2-21)

\( f_{H2,VFA} \) and \( f_{mc} \) can be directly compared, assuming that \( CH_4 \) production on the UAF derives exclusively from the VFA and that no substrate accumulation occurs. Considering the limitation of the VFA measurement precision, \( f_{H2,VFA} \) was not calculated when the total VFA concentration was below 0.1 \( g_{COD} L^{-1} \).

2.5.4 Implementation of the calculation routines

The calculations of the methanogenic pathways, the \( \Delta G \) values and the maximal \( H_2 \) yields were implemented in an Excel sheet. A second implementation of the methanogenic pathway calculations in the software Matlab R2009a (Mathworks, USA) was utilized for sensitivity analysis. Equations 2-14, 2-15 and 2-16 were calculated including variations in the input data measured to account for the analytical uncertainties or in the fractionation factors. A stochastic calculation routine evaluated the error propagation of the analytical uncertainties for the \( f_{mc} \) calculation. Each point measured was taken randomly inside its standard error interval to calculate the \( f_{mc} \) value, the process was repeated \( n \)-times (the adopted value for \( n \) was 1000) and the averages, minimal and maximal values for each \( f_{mc} \) point were recorded. The sensitivity of the fractionation factors was determined by a parameter substitution routine. The codes implemented for both routines can be found in Appendix II-C.

2.6 \( ^{12}C^{13}C \) Model for acetate degradation

2.6.1 \( ^{12}C^{13}C \) Model structure

The model, implemented on a molar basis, differentiates carbon atoms in both of its stable isotopes: \( ^{12}C \) and \( ^{13}C \). This approach differs from the isotopic models of Vavilin (2012a, 2012b), which included the isotopic ratio as an additional variable calculated for the organic substrates and \( CO_2 \) and \( CH_4 \). This simplifies the implementation, as only new calculations are included without interfering in the existing processes. Nevertheless, the isotopic differentiation through all the model variables allows for a more detailed balance of both carbon isotopes. Moreover, the utilization of the C-atoms in molar units avoids typical rounding problems that arise in the more common COD-based models.
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(Kleerebezem and Van Loosdrecht, 2006). A precise representation of the C-concentrations is especially important in the calculations using natural isotopes, i.e. unlabeled substrates, considering the very fine variations that are analytically determined.

The $^{12}$C$^{13}$C Model includes three biological processes: acetoclastic and hydrogenotrophic methanogenesis and acetate oxidation. A single biomass group counts for each of the processes. A pair of variables accounting for the light and heavy carbon isotopes is necessary for the model variables which include carbon atoms. A further distinction is necessary for the acetate to determine the $^{13}$C position. This leads to three acetate (acetate is defined here as the sum of both undissociated acetic acid and acetate ion) variables: light acetate ($S_{ac,12}$): CH$_3$-COOH; heavy [2-$^{13}$C]acetate ($S_{ac,12}$): CH$_3$-$^{13}$COOH; and heavy [1-$^{13}$C]acetate ($S_{ac,12}$): CH$_3$-$^{12}$COOH. The model does not account for acetate with two heavy carbon isotopes. All variables were utilized in mmol L$^{-1}$ to avoid numerical problems with the very low concentrations of the heavy isotope species.

2.6.2 Microbial and hydrolytic processes

Biomass growth processes follow Monod kinetics, including a minimal substrate threshold, $S_m$, according to Panikov (1995). An empirical lower limit pH inhibition ($I_{pH}$), nitrogen limitation ($I_N$) and free ammonia inhibition ($I_{NH3,ma}$) for acetoclastic methanogens are implemented as described in Batstone et al. (2002). The maximal uptake rates, $k_{m_j}$ (j identifies one of the three different biomass groups), are calculated on the basis of the total substrate ($S_{ac} = S_{ac,12} + S_{ac,13}$) and total biomass ($X_j = X_{j,12} + X_{j,13}$) concentrations. Maximal uptake rates for acetoclastic methanogens ($X_{ac}$), acetate oxidizers ($X_{ca}$) and hydrogenotrophic methanogens ($X_{mc}$) are described below:

$$k_{m_{ma}} = k_{m_{max,ma}} \cdot \frac{S_{ac,sum}\cdot S_{m,ac,ma}}{K_{S,ac,ma}+S_{ac,sum}} \cdot X_{ma,sum} \cdot I_{pH,ma} \cdot I_{NH3,ma} \cdot I_N \quad (d^{-1}) \quad (2-22)$$

$$k_{m_{ca}} = k_{m_{max,ca}} \cdot \frac{S_{ac,sum}\cdot S_{m,ac,ca}}{K_{S,ac,ca}+S_{ac,sum}} \cdot X_{ao,sum} \cdot I_{pH,ca} \cdot I_N \quad (d^{-1}) \quad (2-23)$$

$$k_{m_{mc}} = k_{m_{max,mc}} \cdot \frac{S_{h2,mc}\cdot S_{m,h2}}{K_{S,h2,mc}+S_{h2}} \cdot X_{mc,sum} \cdot I_{pH,mc} \cdot I_N \quad (d^{-1}) \quad (2-24),$$

where $\mu_{max}$ is the maximal growth rate and $K_{S,ac,j}$ and $K_{S,h2,mc}$ describe the half-saturation constants for acetate and hydrogen. The assumption that hydrogenotrophic methanogenesis is always the fast step on the SAO is also the reason for not including any $H_2$-related inhibition term for the acetate oxidizers, i.e. $H_2$ is assumed to be readily consumed. Otherwise, the inclusion of a thermodynamic constraint in the acetate oxidation process is necessary.
The stoichiometric reduction of the light and heavy substrates is proportional to their abundance in the total substrate and dependent on the fractionation factor involved. The consumption of the light and heavy acetate forms, $S_{ac,i}$ (i identifies one of both carbon isotopes), and light and heavy carbon dioxide, $S_{co2,i}$, are calculated as:

$$\frac{\Delta S_{ac,i}}{\Delta t} = - \frac{S_{ac,i}}{S_{ac,sum}} \cdot \left( \frac{km_{ma}}{\alpha_{ma,i}} + \frac{km_{ca}}{\alpha_{ca,i}} \right) \text{ (mmol L}^{-1} \text{ d}^{-1})$$

$$\frac{\Delta S_{co2,i}}{\Delta t} = - \frac{S_{co2,i}}{S_{co2,sum}} \cdot \frac{km_{mc}}{\alpha_{mc,i}} \text{ (mmol L}^{-1} \text{ d}^{-1})$$

where $\alpha_{ca,i}$, $\alpha_{ma,i}$ and $\alpha_{mc,i}$ stand for the fractionation factors for acetate oxidizers, and acetoclastic and hydrogenotrophic methanogens, respectively. By definition, the fractionation factors for the light carbon isotopes, $^{12}$C, is always equal unity. The isotopic distribution of the products formed corresponds to the substrates consumed. Therefore, a consumption of light carbon yields light biomass, $^{12}$CH$_{1.4}$O$_{0.4}$N$_{0.2}$, and heavy carbon yields heavy biomass, $^{13}$CH$_{1.4}$O$_{0.4}$N$_{0.2}$. Acetate with light and heavy carbon is assumed to have equal yields for light and heavy biomass, $Y_{j,12}$ and $Y_{j,13}$, respectively:

$$Y_{j,12} = Y_{j,13} = \frac{Y_j}{2} \text{ (mmol mmol}^{-1})$$

where $Y_j$ stands for the biomass yield of the biomass group $j$. In the acetoclastic methanogenesis, the position of the heavy carbon of the acetate consumed defines whether a heavy or light CO$_2$ and CH$_4$ are formed (see Equations 1-21 and 1-22). A consumption of a mol heavy methyl acetate results in $^{13}$CH$_4$ and $^{12}$CO$_2$ as products, and $^{12}$CH$_4$ and $^{13}$CO$_2$ result from the degradation of heavy carboxyl acetate.

A first-order kinetic process describes the biomass decay. Each mol of decayed biomass results in a mol biomass decay product, $X_P$, which is considerably degradable, at a rate, $k_{hyd,XP}$, which turns out to be much slower than the microbial growth process (Aquino and Stuckey, 2008; Noguera et al., 1994). After the hydrolysis, the decay products are available as acetate with same ratio of heavy carbon ($\delta^{13}C_{XP} = \delta^{13}C_{ac,XP}$) uniformly distributed in the methyl and carboxyl positions. This simplified approach of adopting an acetate-equivalent stoichiometry for the biomass decay products maintains the isotopic mass balance of the model without the inclusion of any new variables. Nevertheless, the influence of the $X_P$ hydrolysis on the model outputs is very low, as the assays had a short running time (between two and ten days) and $k_{hyd,XP}$ is very slow. Additionally, the fraction of degradable COD from the batch assays was included by the variable $X_C$. The hydrolysis of one mol of $X_C$ results directly in acetate, as described for the hydrolysis for the biomass decay products.
2.6.3 Physicochemical processes

The physicochemical processes implementation is analogous to Batstone et al. (2002), with the inclusion of the EIE related to the inorganic carbon and CH$_4$. Nitrogen gas, N$_2$, is included in the model as an inert gas variable (S$_{N2}$) in order to adequately describe the headspace pressure increase during the batch assays. The protons concentration (S$_{H+}$) results from the charge balance for the following ionic variables: ammonium nitrogen (S$_{NH4-}$), bicarbonate (S$_{HCO3-}$), acetate ion (S$_{ac-}$), cations (S$_{cat+}$), anions (S$_{an-}$) and water hydroxide (S$_{OH-}$). A set of algebraic equations calculate the equilibrium concentrations between S$_{NH4-}$ and free ammonia nitrogen (S$_{NH3}$), and between acetate (S$_{ac-}$) and acetic acid (S$_{ac-}$). The equilibrium from dissolved CO$_2$ (S$_{CO2}$) and S$_{HCO3-}$ is implemented as a set of two differential equations including the fractionation factor, $\alpha_{AB,CO2,13}$, between both species (Mook et al., 1974). The exchange rates from liquid to gas phases are calculated as a diffusive mass transfer (Reichert, 1998). The liquid-gas diffusive transport includes two fractionation factors to account for the higher solubility of the heavy S$_{CO2,13}$ and S$_{CH4,13}$ isotopes (see Table 1-5), namely $\alpha_{D,CO2,13}$ and $\alpha_{D,CH4,13}$.

2.6.4 Model implementation

The model was implemented in the software AQUASIM 2.1d (Computer Program for the Identification of Aquatic Systems; Reichert, 1994). The AQUASIM is widely used for the simulation of biochemical processes in bioreactors and natural aquatic systems, and the software and its source codes have been freely available from EAWAG (Switzerland) since 2013. The AQUASIM solves systems of algebraic and differential equations (DAE) simultaneously which allows for a robust solution of processes with very different kinetics, as in biological growth and the much faster ionic equilibria.

In total, the model included 32 state variables, 16 biological/hydrolytic processes, 9 ionic equilibria and 5 gas diffusive gas exchange ($^{12}$CH$_4$, $^{13}$CH$_4$, $^{12}$CO$_2$, $^{13}$CO$_2$ and H$_2$) processes. Notably, the number of state variables and processes is comparable to the ADM1. Nonetheless, in the ADM1, seven different biomass groups are included instead of three, as implemented in this $^{12}$C$^{13}$C Model. This highlights the complexity that arises with the differentiation of the carbon isotopes. A flow chart including all the C-fractionation processes is depicted in Figure 2-7. The Gujer-Petersen matrix for all biological/hydrolytic and physicochemical processes of the model is in Table 2-4 and Table 2-5, respectively. The balances of the elements C, H, O and N, and of COD and charge is assured in all the processes. A detailed description of the Gujer-Petersen matrix notation and balance calculation can be found elsewhere (e.g. Gernaey et al., 2006; Gujer and Larsen, 1995; Wichern et al., 2011). A relation of all physicochemical parameters and coefficients is depicted in Appendix II-D.
Figure 2-7: Carbon fractionation flowchart in the $^{12}$C-$^{13}$C Model for anaerobic acetate degradation. Black (full) lines stand for variables and processes related only to $^{12}$C; black dashed and dash-dot lines for variables and processes including $^{13}$C; gray lines are for carbon-free variables and processes; gray and white field indicates the gas and liquid phases, respectively. KIEs: kinetic isotope effects; EIEs: equilibrium isotope effects.

Simulated ratios between the heavy and light forms ($S_{i,H}$ and $S_{i,L}$) of the variables including carbon atoms are converted to $\delta^{13}$C values against the PDB standard as follows:

$$\delta^{13}C = \left( \frac{S_{i,H}}{S_{i,L}} - 1 \right) \times 1000 \quad (\%o) \quad (2-28)$$

The calculation of the fraction of anaerobically oxidized acetate ($f_{ac,ox}$) considers the dynamic rates of both acetate-consuming organisms:

$$f_{ac,ox} = \frac{\sum_{t=start}^{t=end} km_{ca,t_i}}{\sum_{t=start}^{t=end} km_{ma,t_i} + km_{ca,t_i}} \quad (2-29),$$

where $km_{ca,t_i}$ and $km_{ma,t_i}$ are the overall process rates of the acetate oxidation and acetoclastic methanogenesis processes, respectively; both processes consume 1 mmol acetate within a defined period of time ($t_{start}$ to $t_{end}$).

The batch assay vessels were implemented as liquid and a gas CSTR connected by a diffusive link. The mass flow in the gas-liquid interface of the compound, $i (J_i)$, is calculated as follows
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\[ J_i = k_{La} \cdot \left( KH_i' \cdot \alpha_{D,i} \cdot C_{i,\text{gas}} - C_{i,\text{liquid}} \right) \quad \text{(mmol d}^{-1}) \quad (2-30), \]

where \( k_{La} \) is the gas exchange coefficient, \( KH_i' \) the non-dimensional Henry coefficient, \( \alpha_{D,i} \) the EIE related to solubility of the heavy compound and \( C_i \) the compound concentration in both compartments. A gas outflow is considered only when a gas sample are taken during the experiment. The gas flow rate results from the pressure gradient between reactor and atmosphere for a restricted orifice (Batstone et al., 2002; Rosen and Jeppsson, 2006):

\[ Q_{\text{Gas}} = V_{\text{headspace}} \cdot \frac{(p_{\text{headspace}} - p_{\text{atm}})}{p_{\text{atm}}} \cdot k_p \quad \text{(ml d}^{-1}) \quad (2-31) \]

with

\[ p_{\text{headspace}} = p_{\text{CH}4} + p_{\text{CO}2} + p_{\text{H}2} + p_{\text{N}2} + p_{\text{H}2O} \quad \text{(bar)} \quad (2-32), \]

where \( k_p \) is the pipe resistance coefficient (d\(^{-1}\)), \( p_i \) is the gas pressure of \( i \) (bar; atm stands for atmosphere) and \( V_{\text{headspace}} \) is the volume of the gas phase. Equation 2-30 is only valid if \( p_{\text{headspace}} \) is larger than \( p_{\text{atm}} \). The value of \( k_p \) always equaled zero, i.e. without gas flow, assuming positive values only during the sampling time period. A dynamic gas exchange coefficient, \( k_{La} \), depending on the total gas flow velocity was considered (Merkel and Krauth, 1999):

\[ k_{La} = K \cdot u_{\text{gas}}^\beta \quad \text{(d}^{-1}) \quad (2-33), \]

where \( u_{\text{gas}} \) is the gas velocity (m d\(^{-1}\)) and \( \beta \) and \( K \) are empirically determined constants. The gas velocities are calculated from the experiments (gas production divided by the superficial area) and values of 8 and 0.93 were assumed for \( K \) and \( \beta \), respectively (Merkel and Krauth, 1999). Biofilm effects on mass transfer were not considered in the present simulations, assuming that i) hydrogen transfer occurs without diffusion limitations between acetate oxidizers and hydrogenotrophic methanogens; and ii) acetate is readily accessible to all microorganisms after the initiation of the experiments. In fact, even considering a biofilm thickness up to 1.5 mm, the biofilm base reaches 90 % of the acetate concentration from the bulk volume in less than one hour after the assay start, assuming an acetate effective diffusion coefficient of 139\( \cdot 10^{-6} \) m d\(^{-1}\) (Stewart, 2003).
<table>
<thead>
<tr>
<th>Process</th>
<th>Xa</th>
<th>Xb</th>
<th>Xc</th>
<th>Xd</th>
<th>Xe</th>
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</table>

**Composition matrix of the state variables for the elemental composition (mol)**

- C: Carbon
- N: Nitrogen
- O: Oxygen
- H: Hydrogen
- S: Sulphur

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Table 2.1: Giljé-Reesén matrix for the microbiological processes (p1 to p3) and hydrolytic processes (p4 and p5) from the 12C12C Model. Stoichiometric coefficients are given in mmol units and rates in 1/d (continues on the next page).
Table 2-4: (continuation).

<table>
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<th>Rate</th>
<th>Overall kinetic rate*a</th>
<th>$^{12}$C$^{13}$C factors</th>
<th>Equations for limitation and inhibition factors</th>
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<td>Sac,12,12/Sac</td>
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<tr>
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<td>1/2a$ma$</td>
<td>Sac,13,12/Sac</td>
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<tr>
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<td>1/2a$ma$</td>
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<td>Xma,13</td>
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<td>Sac,12,12/Sac</td>
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<td>1/2a$ca$</td>
<td>Sac,13,12/Sac</td>
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<td>Sac,12,13/Sac</td>
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<td>Sco,12,13/Sco2</td>
</tr>
<tr>
<td>$p_{dec}$3</td>
<td>Xmc,12</td>
<td>Xmc,12-KSac,mc</td>
<td></td>
</tr>
<tr>
<td>$p_{dec}$3</td>
<td>Xmc,13</td>
<td>Xmc,13-KSac,mc</td>
<td></td>
</tr>
<tr>
<td>p4</td>
<td>Xc$^d$</td>
<td>Xc$^d$Kexp,XC</td>
<td></td>
</tr>
<tr>
<td>p5</td>
<td>Xa$^e$</td>
<td>Xa$^e$Kexp,XP</td>
<td></td>
</tr>
</tbody>
</table>


*b: p4: degradable organic matter from the inoculum and p5: microbial product decay.

*c: Water (H$_2$O) is included in the matrix for balance calculation purposes, but is not implemented as a state variable. The factor multiplying the biomass yields is defined depending on the biomass composition.

*d: $f_{XC,12}$, $f_{XC,13}$, $f_{XP,12}$ and $f_{XP,13}$ are dynamic factors indicating the ratios of light and heavy substrates.

*e: The overall kinetic is multiplied through the specific isotope kinetic effect (KIE) and through the substrate relative abundance in the processes where carbon isotope fractionation occurs.

f: These equations are valid if the pH is lower than pH$_{H_{2}O,ma}$, otherwise no pH inhibition is considered.
Table 2-5: Gujer-Petersen Matrix for the ionic equilibrium processes from the $^{12}$C$^{13}$C-Model. In the algebraic equilibrium equations (AEE) the state variable which defines the equilibrium, i.e. value for which the AEE results in zero, is indicated by “EV”. Stoichiometric coefficients are given in mmol units and rates in d$^{-1}$.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Process</th>
<th>Rate / AEE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{CO2,12}$</td>
<td>Eq1</td>
<td>$S_{hCO3-,12}$: $k_{A,CO2}$·($S_{hCO3-,12}/1000$·$S_{h+}$) - $k_{A,CO2}$·$S_{hCO3-,12}$/$1000$</td>
</tr>
<tr>
<td>$S_{CO2,13}$</td>
<td>Eq1</td>
<td>$S_{hCO3-,13}$: $k_{A,CO2}$·($S_{hCO3-,13}/1000$·$S_{h+}$) - $k_{A,CO2}$·$S_{hCO3-,13}$/$1000$</td>
</tr>
<tr>
<td>$S_{ac-,1212}$</td>
<td>Eq2</td>
<td>$S_{nh4+}$: $S_{nh4+} - S_{nh3}/1000$</td>
</tr>
<tr>
<td>$S_{ac-,1213}$</td>
<td>Eq2</td>
<td>$S_{nh3}$: $S_{nh3}$ + $S_{nh4+} - S_{in}/1000$</td>
</tr>
<tr>
<td>$S_{ac-,1312}$</td>
<td>Eq3</td>
<td>$S_{h2O}$: $S_{oh-}$ = $S_{h+}$</td>
</tr>
<tr>
<td>$S_{ac-,1313}$</td>
<td>Eq3</td>
<td>$S_{h2O}$: $S_{oh-}$ = $S_{h+}$</td>
</tr>
<tr>
<td>$S_{ac-,1213}$</td>
<td>Eq4</td>
<td>$S_{ac-,1213}$: $S_{ac-,1213}$: $K_{A,CO2}$·$S_{ac-,1213}$/$1000$ - ($K_{A,CO2}$ + $S_{h+}$)·$S_{ac-,1213}$/$1000$</td>
</tr>
<tr>
<td>$S_{ac-,1312}$</td>
<td>Eq4</td>
<td>$S_{ac-,1312}$: $S_{ac-,1312}$: $K_{A,CO2}$·$S_{ac-,1312}$/$1000$ - ($K_{A,CO2}$ + $S_{h+}$)·$S_{ac-,1312}$/$1000$</td>
</tr>
<tr>
<td>$S_{ac-,1313}$</td>
<td>Eq4</td>
<td>$S_{ac-,1313}$: $S_{ac-,1313}$: $K_{A,CO2}$·$S_{ac-,1313}$/$1000$ - ($K_{A,CO2}$ + $S_{h+}$)·$S_{ac-,1313}$/$1000$</td>
</tr>
<tr>
<td>Charge</td>
<td>Eq5</td>
<td>Charge: $S_{h+}$ = $S_{oh-}$ + $S_{nh4+}$ - ($S_{hCO3-,12}$ + $S_{hCO3-,13}$ + $S_{nh4+}$)/1000</td>
</tr>
</tbody>
</table>

Composition matrix of the state variables for the elemental composition (n mol$^{-1}$), charge (e-equivalent mol$^{-1}$) and COD content (gCOD mol$^{-1}$).

<table>
<thead>
<tr>
<th>Variable</th>
<th>8</th>
<th>9</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>b</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{12}$C</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>H</td>
<td>3</td>
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<td>3</td>
<td>3</td>
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<td>1</td>
<td>4</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>64</td>
<td>64</td>
<td>64</td>
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<td>64</td>
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<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

---

* Implemented as a dynamic process for numerical stiffness; $k_{A,CO2}$ was set to $10^{14}$, hence, still much faster than the biological processes.  
** $S_{nh4+}$ and $S_{nh3}$ are implemented in Mol units.
3. RESULTS AND DISCUSSION

3.1 Performance of the two-stage experimental plant under increasing loading rates

3.1.1 Substrate characterization and organic loading rates

Two different charges of MS from the same crop field were utilized during the experimental period overviewed here. The substrate was stored frozen at -20 °C. Parameters determined for both MS charges are depicted in Table 3-1. On average, the substrate had 33.8 % TS and 95.7 %TS VS. The δ¹³C values are in good agreement with literature data for *zea mays* (see Table 2-1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elemental composition</td>
<td></td>
<td>C₃.₇₅H₆.₂₀O₂₅₆N₀.₀₇S₀.₀₁ MS²</td>
</tr>
<tr>
<td>Total solids (TS)</td>
<td>%</td>
<td>35.1 MS¹ / 32.5 MS²</td>
</tr>
<tr>
<td>Volatile Solids (VS)</td>
<td>%TS</td>
<td>95.3 MS¹ / 96.0 MS²</td>
</tr>
<tr>
<td>Ethanol</td>
<td>gCOD kgMS⁻¹</td>
<td>2.1 MS¹</td>
</tr>
<tr>
<td>Acetate</td>
<td>gCOD kgMS⁻¹</td>
<td>2.2 MS¹</td>
</tr>
<tr>
<td>Propionate</td>
<td>gCOD kgMS⁻¹</td>
<td>0.2 MS¹</td>
</tr>
<tr>
<td>Lactate</td>
<td>gCOD kgMS⁻¹</td>
<td>22.5 MS¹</td>
</tr>
<tr>
<td>Stable carbon isotope ratio (δ¹³C)</td>
<td>% PDB</td>
<td>-12.7 ± 0.3 MS²</td>
</tr>
</tbody>
</table>

a Analysis conducted by the ATB.
b Carbon isotope analysis conducted by Christiane Wenske, Institute for Geology, Leibniz Universität Hannover (Hannover, Germany), n = 3; elemental composition analysis conducted by Wessling (Bochum, Germany).

The investigation under increasing OLR comprises a period of 196 days. Here, are overviewed the results of the operation of the LBRs under three OLR stages: OLR-S1, OLR-S2 and OLR-S3 (see Figure 3-1). The first MS was utilized until day 50; thereafter, only the second charge was given to the plant. Prior to the OLR-S1, the LBRs were operated for 195 days under low OLRs, 1.0 gCOD L⁻¹ d⁻¹ (OLR-S0), after inoculation with digestate from a two-stage agricultural biogas plant (with swine manure and MS as the main substrates). This initial period at the OLR-S0 was utilized to establish the
Results and discussion

methodologies of biogas sampling for the $\delta^{13}$C measurements. In addition, other operational aspects, such as internal recirculation rates and biofilm removal strategies, were tested and optimized at this period. After the operation at the OLR-S3, the plant was returned to the OLR-S2 for 110 days more to provide stable conditions for the UAF biomass, which was utilized for the batch assays. The OLRs calculated for the reaction volume instead of the liquid volume of the LBRs are shown in in Table 3-3.

Figure 3-1: The OLRs for the LBR-01 (white circles), LBR-02 (gray squares) and LBR-03 (black triangles) and the average for the three LBRs (black line).

3.1.2 Thermophilic solids degradation

The minor temperature differences between the LBRs (Table 2-2) led to slight differences in the VS and TS degradation rates (Table 3-2). The lower average temperatures from the LBR$_{01}$-STR$_{01}$ pair, 52 °C, resulted in lower VS and TS degradation rates. The 95% confidence interval (CI$_{95}$) deviations from the degradation rates of the other two LBRs systems, average temperatures of 53.5 °C and 55.0 °C, were very similar. The higher solids degradation for all reactors was observed at the OLR-S1. The reduction of the VS degradation rates after the OLR-S1 are probably due to design deficiencies of the LBRs. At the OLR-S2 and OLR-S3, the fresh MS in the LBRs stacked vertically – height to radius ratio of 2:1. This did not always result in a homogeneous leachate distribution on the substrate, which could lead to an incomplete degradation of the substrate (see Appendix III-A). The utilization of reactors with a larger surface area, instead of the great height utilized here, is, therefore, recommended for LBRs.

The higher solids degradation rates in the OLR-S3 resulted from the increased SRTs from the last three feedings. After three weeks of operation at the OLR-S3, a start of acidification of the UAF reactor was observed (see a more detailed discussion in Section 3.1.3) and the MS feeding was firstly reduced to the levels of OLR-S1, and thereafter, interrupted for two weeks (see Appendix III-A for additional information of the OLR in the
Therefore, the MS retention time was temporally increased to 35 days, and this explains the increase of the VS degradation rates to 65 ± 2 % in the OLR-S3.

**Table 3-2**: Internal recirculation rates of the LBRs and their respective STR and degradation rates for VS (%VS) and TS (%TS) at the three OLR stages and digestate elemental composition.

<table>
<thead>
<tr>
<th>Reactors</th>
<th>OLR-S1</th>
<th>OLR-S2</th>
<th>OLR-S3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L d⁻¹</td>
<td>L d⁻¹ g⁻¹ VS</td>
<td>L d⁻¹</td>
</tr>
<tr>
<td>LBRs ↔ STRs</td>
<td>24</td>
<td>106</td>
<td>36</td>
</tr>
<tr>
<td>VS-degradation</td>
<td>av.</td>
<td>CI₉₅</td>
<td>av.</td>
</tr>
<tr>
<td>LBR₀₁-STR₀₁</td>
<td>71</td>
<td>1</td>
<td>51</td>
</tr>
<tr>
<td>LBR₀₂-STR₀₂</td>
<td>77</td>
<td>2</td>
<td>58</td>
</tr>
<tr>
<td>LBR₀₃-STR₀₃</td>
<td>74</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>Average-VS</td>
<td>74</td>
<td>2</td>
<td>58</td>
</tr>
<tr>
<td>TS-degradation</td>
<td>av.</td>
<td>CI₉₅</td>
<td>av.</td>
</tr>
<tr>
<td>LBR₀₁-STR₀₁</td>
<td>75</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>LBR₀₂-STR₀₂</td>
<td>76</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>LBR₀₃-STR₀₃</td>
<td>73</td>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>Average-TS</td>
<td>74</td>
<td>1</td>
<td>61</td>
</tr>
</tbody>
</table>

**Digestate elemental composition**

| LBR₀₁-STR₀₁    | C₃.₉₆H₆.₂₀O₂.₁₉N₀.₁₄ |
| LBR₀₂-STR₀₂    | C₄.₀₀H₆.₀₀O₂.₄₆N₀.₁₄ |
| LBR₀₃-STR₀₃    | C₄.₀₀H₆.₀₀O₂.₂₂N₀.₁₄ |

The elemental composition (for carbon, hydrogen, oxygen and nitrogen) of the digestate was determined for three samples in the OLR-S3. The samples from the three LBRs presented a very similar composition with identical C to N ratios. Compared to the fresh MS composition, C₃.₇₅H₆.₂₀O₂.₅₈N₀.₀₇, it is possible to observe an increase in the nitrogen concentrations. Nitrogen in MS is mostly found in proteins, which are found to have hydrolysis rates in the order of 0.3 d⁻¹ (Lübken, 2009; Lübken et al., 2015a). Hence, a virtually complete protein hydrolysis can be expected for retention times of 21 days. Although part of the proteins may be not readily available for hydrolysis through the lignocellulosic polymeric structures disposition (Lynd et al., 2002), this increase in the nitrogen must have been due to the growth of sessile biomass onto the solid substrate. Indeed, the hydrolysis rate for cellulose substrates is found to be directly correlated to the amount of fixed biomass in the material (Jensen et al., 2009; Song et al., 2005). On average, the digestate had a COD content of 1.5 gCOD gTS⁻¹; this value is similar to values reported for lignin of 1.56 gCOD gTS⁻¹ (Koch et al., 2010).
3.1.3 Methane and volatile fatty acids production

The operation of batch-fed LBRs combined with mesophilic methanogenic reactors results in variable daily gas production rates and leachate characteristics that depend on the feeding intervals (Nizami and Murphy, 2011). In OLR-S1 and OLR-S2, the maximal leachate VFA concentrations were observed for the two days after a LBR feeding (Figure 3-2). Thereafter, the VFA levels decreased until a new substrate charge was added to the system, resulting in a reproducible weekly CH$_4$ production pattern for each OLR stage. Table 3-3 lists the minimum and maximum OLRs applied to the LBRs and the resulting OLR ranges of the UAF from leachate recirculation. Additionally, the specific VFA production (Y$_{\text{VFA,LBRs}}$) and the CH$_4$ yields for all the reactors of the plant (Y$_{\text{CH4,sum}}$) are given.

Table 3-3: Maximum and minimum OLRs for the LBRs and UAF, specific production yields of methane of the complete plant (Y$_{\text{CH4,sum}}$) and VFAs in the LBRs (Y$_{\text{VFA,LBRs}}$) at the three OLR stages. The Y$_{\text{CH4,sum}}$ values are given with the 95% confidence interval ranges.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>OLR-S1</th>
<th>OLR-S2</th>
<th>OLR-S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of feeding</td>
<td>(–)</td>
<td>11</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>OLR$_{\text{LBRs}}$</td>
<td>g$_{\text{VS}}$ L$^{-1}$ d$^{-1}$</td>
<td>0.7 – 1.4</td>
<td>1.6 – 2.1</td>
<td>2.3 – 2.7</td>
</tr>
<tr>
<td></td>
<td>g$_{\text{COD}}$ L$^{-1}$ d$^{-1}$</td>
<td>1.0 – 2.0</td>
<td>2.3 – 3.0</td>
<td>3.2 – 3.9</td>
</tr>
<tr>
<td>OLR$_{\text{LBRs}}$$^a$</td>
<td>g$_{\text{COD}}$ L$^{-1}$ d$^{-1}$</td>
<td>0.8 – 1.5</td>
<td>1.7 – 2.3</td>
<td>2.4 – 2.9</td>
</tr>
<tr>
<td>OLR$_{\text{UAF}}$</td>
<td>g$_{\text{COD}}$ L$^{-1}$ d$^{-1}$</td>
<td>1.7$^b$ – 3.7</td>
<td>2.0$^b$ – 5.9</td>
<td>5.7$^b$ – 16.8</td>
</tr>
<tr>
<td>Y$_{\text{VFA,LBRs}}$$^c$</td>
<td>g$<em>{\text{VFA,COD}}$ g$</em>{\text{VS}}^{-1}$</td>
<td>0.25</td>
<td>0.28</td>
<td>0.73</td>
</tr>
<tr>
<td>Y$_{\text{CH4,sum}}$</td>
<td>L$<em>{\text{CH4}}$ kg$</em>{\text{VS}}$^{-1}</td>
<td>274 ± 12</td>
<td>252 ± 17</td>
<td>283 ± 11</td>
</tr>
</tbody>
</table>

$^a$ Alternative calculation considering the LBR’s working volume (Equation 2-3).

$^b$ Minimal values after the three LBRs were fed on the maximal OLR of the specific OLR stage.

$^c$ The interpolated average VFA production curves (Figure 3-2a) were utilized to calculate Y$_{\text{VFA}}$ for the OLR-S1 and OLR-S2; only the VFA production before the UAF acidification was considered for OLR-S3.

The two-stage plant had an average CH$_4$ yield of 271 ± 25 L$_{\text{CH4}}$ kg$_{\text{VS}}$^{-1} (SD for n = 29) over the three OLR stages, which represents 85% of the theoretical maximal CH$_4$ yield based on stoichiometric calculations (Equation 2-9) with the average VS degradation of 67%. It is very likely that higher CH$_4$ yields would be achieved with better substrate distribution within the LBRs. Ficara and Malpei (2011) and Busch et al. (2008) report yields of 303 and 330 L$_{\text{CH4}}$ kg$_{\text{VS}}$^{-1}, respectively, for two-stage plants fed with MS. Nevertheless, high specific VFA production rates were achieved. At the OLR-S3, the maximal values were over 0.8 g$_{\text{COD}}$ g$_{\text{VS}}$^{-1}, in good accordance with literature data from previous studies with LBRs (Cysneiros et al., 2012). These high Y$_{\text{VFA,LBRs}}$ resulted in an OLR for the UAF up to 16.8 g$_{\text{COD}}$ L$^{-1}$ d$^{-1}$. This is almost three times higher than the maximal OLR for the UAF at the OLR-S2. During all OLR stages, the control CSTR was...
operated at low OLRs, below 0.7 g COD L\(^{-1}\) d\(^{-1}\), with maximal specific CH\(_4\) production rates of 0.3 L CH\(_4\) L\(_{\text{reactor}}\)\(^{-1}\) d\(^{-1}\).

**Figure 3-2:** Weekly dynamics for the LBRs and the UAF during the OLR-S1 (white circles), OLR-S2 (gray squares) and OLR-S3 (black triangles). Error bars gives the 95% confidence intervals (n ≥ 4). An LBR is fed on the first week day (1 d\(_{\text{week}}\)); a) daily leachate VFA concentrations (inflow to the UAF); b) average leachate VFA distribution in COD equivalent; c) daily methane production rates for the sum of the three LBRs; d) daily methane production rates of the UAF.

The average weekly CH\(_4\) production for the UAF and for the sum of the three LBRs are shown in Figure 3-2c and Figure 3-2d, respectively. An LBR was fed on the first day of the weekly operational cycle (day 1 in Figure 3-2). The UAF CH\(_4\) production rates also include the gas collected from the STR\(_{\text{UAF}}\) (≈ 10% of the total UAF CH\(_4\) production). In the LBRs, a small increase of CH\(_4\) production from 57 to 64 L week\(^{-1}\) occurred from OLR-S1 to OLR-S2. At OLR-S3, the CH\(_4\) production decreased substantially to 42 L week\(^{-1}\) in response to the lower pH values from the leachate (Figure 3-3c). By contrast, the CH\(_4\) production increase in the UAF at each OLR stage was high, from 13 L week\(^{-1}\) at OLR-S1 to 73 L week\(^{-1}\) at OLR-S3. The bell-shaped curve for CH\(_4\) production at OLR-S1 and OLR-S2 is not observed at OLR-S3 (Figure 3-2d), indicating that the UAF operated
Results and discussion

continually at its maximal CH\textsubscript{4} production rates. In fact, after three weeks of operation at OLR-S3, a light acidification of the UAF reactor occurred, and the average pH value dropped to 6.9 for 21 days. Otherwise, the UAF pH values were continually between 7.2 and 7.4 (see Appendix III-B). The pH dynamics of the mixed leachate and in the LBRs that became the weekly feeding are depicted in Figure 3-3c and Figure 3-3d, respectively.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure3}
\caption{Fractions of acetate (f\textsubscript{ace}) and butyrate (f\textsubscript{but}) VFA distribution according to the pH values from samples of the STR\textsubscript{01} (white circles), STR\textsubscript{02} (gray squares) and STR\textsubscript{03} (black triangles). Fractions calculated on COD basis. Dashed lines were arbitrarily defined to illustrate the correlation tendencies between both parameters. Weekly dynamics of the pH for the mixed leachate in the inflow to the UAF (c) and for the leachate of the more recently fed LBR (d). Error bars give the 95 % confidence intervals (n ≥ 4).}
\end{figure}

The daily VFA leachate concentrations had a higher variation range than the CH\textsubscript{4} production rates; hence, no confidence interval was determined. This large variation can be mainly explained through the discrete (daily) VFA sampling in contrast to the online biogas measurements, which yield a more precise determination of LBR dynamic outflows. Nevertheless, the average CH\textsubscript{4} production for OLR-S1 and OLR-S2 are in accordance with the average VFA loads. This correlation cannot be established
adequately for OLR-S3 due to the UAF acidification. The increased acidifying conditions also led to higher variations for LBR CH₄ production during OLR-S3 (Figure 3-2c). However, the COD distribution among the VFAs (acetate, propionate, butyrate, valerate and caproate) only have small variations (Figure 2b). Acetate and butyrate together comprise more than 60 % of the COD from the leachate VFA during the three OLRs, characterizing a clostridial butyric-type fermentation that is typical for silages (Sträuber et al., 2012).

The formation of butyric and acetic acids was related to the pH values: in more basic conditions, acetic acid production was favored (Figure 3-3a) and butyric acid production increased for pH values below 7.0 (Figure 3-3b). This result corroborates with other investigations on the pH influence on the formation of acidogenic products (Horiuchi et al., 2002). Moreover, a significant increase of caproate formation was observed in the OLR-S3, reaching almost 10 % of the VFA production. This is very likely a result from a reverse β oxidation, through which a butyrate mol (four C atoms) can be elongated to a mol of caproate (six C atoms; Spirito et al., 2014). Indeed, the combined formation of acetate, butyrate and caproate was reported in fermentation of maize fibers in thermophilic conditions (Agler et al., 2012).

The NH₄-N concentrations and biogas composition of the UAF did not exhibit significant variations during the increasing OLRs. The NH₄-N concentrations increased from averages of 350 mg L⁻¹ at OLR-S1 to 500 mg L⁻¹ at OLR-S3 (see Figure 3-4 and Table 3-4, respectively). The average CH₄ contents of the UAF were high at 77 ± 3 % (SD for 196 days), similar to the values reported for high-rate AFs (Busch et al., 2008; Nizami and Murphy, 2011; Schönberg and Linke, 2012). The H₂ partial pressures increased marginally in response to the increasingly high VFA concentrations. The average partial pressures were 22, 34 and 44 ppm for OLR-S1, OLR-S2 and OLR-S3, respectively (Table 3-4). Differently, a significant increase was observed for the hydrogen sulfide, H₂S, at the OLR-S3 with an average of 111 ppm, against 19 ppm in the OLR-S2. High H₂S concentrations may indicate a high acetate oxidation through sulfate-reducing Bacteria:

\[
C_2H_3O^- + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O
\]  

(3-1)

This could lead to a significant influence on the carbon stable isotope analysis, as acetate fractionation also occur in this process (Goevert and Conrad, 2008). Yet, calculations considering the H₂S outflow as gas and the dissolved H₂S load from STR_UAF to the LBRs (recirculation b in Figure 2-1; dissolved H₂S concentration determined with the Henry coefficient obtained from Sander, 1999) indicates that sulfate oxidation was not relevant, accounting for less than 0.1 % of the total degraded acetate.
Table 3-4: Average gas compositions and 95 % confidence intervals for the UAF and for the three LBRs at the three OLR stages.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>OLRS1</th>
<th>OLRS2</th>
<th>OLRS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBRs</td>
<td></td>
<td>CI95</td>
<td>CI95</td>
<td>CI95</td>
</tr>
<tr>
<td>CH$_4$ [%]</td>
<td>%</td>
<td>47</td>
<td>2</td>
<td>45</td>
</tr>
<tr>
<td>H$_2$ [ppm]</td>
<td>Ppm</td>
<td>1972</td>
<td>653</td>
<td>3971</td>
</tr>
<tr>
<td>UAF</td>
<td></td>
<td>CI95</td>
<td>CI95</td>
<td>CI95</td>
</tr>
<tr>
<td>CH$_4$ [%]</td>
<td>%</td>
<td>78</td>
<td>1</td>
<td>79</td>
</tr>
<tr>
<td>H$_2$ [ppm]</td>
<td>Ppm</td>
<td>22</td>
<td>5</td>
<td>34</td>
</tr>
<tr>
<td>H$_2$S [ppm]</td>
<td>Ppm</td>
<td>16</td>
<td>6</td>
<td>19</td>
</tr>
</tbody>
</table>

The increasing OLRs in the LBRs resulted in lower CO$_2$ and higher CH$_4$ and H$_2$ partial pressures (Table 3-4). Actually, the H$_2$ pressures at the OLR-S3 exceeded the detection limit, 70,000 ppm (i.e. 7 %), in the hours after a new substrate charge was given. Considering the complete gas flow of the two-stage experimental plant, the average content of CH$_4$ was found to be equal 51 %, hence, very similar to the fermentation of maize in a single stage plant (Gehring et al., 2013; Lübken et al., 2015a). The daily gas flows and contents for the UAF and LBRs together with pH and conductivities measurements is given in Appendix III-B.

3.1.4 Mass balances

The mass balance of the plant was calculated for the days 0 to 217, which includes the complete operational period depicted in Figure 3-1 and the following three weeks, in which the plant recovered from the overload conditions. During this period, there was a significant CH$_4$ outflow resulting from the accumulated VFA degradation (Figure 3-4a). The masses of nitrogen and COD accumulated and removed in the biofilm were estimated with average data of the BCs sampled (see Section 3.3.1). The overall importance of the biofilm for the mass balances was reduced. Differently, the increasing concentrations of total dissolved nitrogen in the period responded to 26 % of the nitrogen added (considering the 54 L total liquid volume of the plant) and, in addition, 13 % was removed through the liquid samples. The increase of biomass and its decay products, as well as NH$_4$-N (Figure 3-4b), were responsible for the augmentation of the TN concentrations. By contrast, although the dissolved concentrations of COD also increased in the liquid phase over the 196 days (Figure 3-4), they were not relevant for the mass balance, neither was the amount of COD removed in the liquid samples. Actually, this continual increase of NH$_4$-N and COD is typical for the continual operation of two-stage systems with LBRs (Buschmann and Busch, 2009; Lübken et al., 2015c). The more accentuated COD increase at the OLR-S3 resulted from the UAF acidification;
nonetheless, it did not affect the mass balance as the accumulated VFA was degraded before day 217.

Figure 3-4: UAF effluent concentrations of COD (a) and NH$_4$-N (b) over 217 days.

The distribution of the inflow COD and nitrogen is depicted in Figure 3-5. Digestate removal was an important component for both balances, accounting for 51 and 32 % of the masses of nitrogen and COD added, respectively. In total, 58 % of the COD added was removed in form of CH$_4$, and only very low amounts of H$_2$. The deficit in the nitrogen balance, 6 %, was lower than for COD, 9 %. Similar deficit values were reported for the fermentation of MS in mixed tank reactors (Lübken, 2009). A possible source of both these deficits was the biomass growth in the tubes and connections; especially in the overflow connection from the LBRs to the UAF reactor, where biofilm biomass was removed to avoid clogging approximately every second week. The rapid biomass growth was possible due to the contact with the atmospheric oxygen. The mass losses due to the changes of the filter material from the LBRs (each LBR filter mat material was changed once in this 217-day period) was also not quantified.
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Figure 3-5: Mass balance for the two-stage plant for COD (black bars) and total nitrogen (gray bars). Values are given as fractions of the total substrate added. The following mass flows are included in the calculations: i) digestate removal; ii) biogas flow (CH$_4$ and H$_2$ as COD); iii) liquid sampling; iv) BC sampling and biofilm biomass growth; and v) the accumulation of dissolved components in the liquid phase of the reactors.

3.1.5 Biomass characterization

The total amounts of Bacteria and Archaea were determined using quantitative real time PCR (Figure 3-6). In the OLR-S1, a proportion of archaeal to total 16S rRNA gene copies in the biofilm of 25 % was found. This corresponds to the same range reported for newly formed biofilm in PVC carriers (Habouzit et al., 2011). In the OLR-S3, the Archaea gene copies proportion reduced to 14 %, reaching the same value found for both sludge samples that were analyzed. The relative abundance of the archaeal community was detected with the molecular fingerprinting method TRFLP in combination with a cloning/sequencing approach for TRF identification (Figure 3-6a). The most dominant TRF in all UAF sessile and suspended biomass samples, with abundances between 55 and 84 %, was the TRF-106bp, which was identified as a member of the acetoclastic genus *Methanosaeta* (order *Methanosarcinales*). The *Methanosaeta* abundance in the sludge samples increased progressively, reaching its maximum at OLR-S3 (84 %).

An inverse effect was observed regarding the other acetoclastic genera, *Methanosarcina* (TRF-627bp), which had a start abundance of 10 % and decreased over time until it completely disappeared at OLR-S3. The TRF’s identification clearly indicated a *Methanosaeta* dominance in the UAF throughout the experiment. The low acetate concentrations in the UAF effluent, always below 150 mg L$^{-1}$ (excluding the acidification period), as well as the low NH$_4$-N concentrations, under 510 mg L$^{-1}$ (see Figure 3-4b), agrees well with the *Methanosaeta* dominance observed (De Vrieze et al., 2012; Fotidis et al., 2013; Karakashev et al., 2005). Two TRFs were identified as hydrogenotrophic Archaea belonging to the orders *Methanobacteriales* (TRF-342bp) and
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*Methanomicrobiales* (TRF-428bp). The total abundance of both hydrogenotrophic Archaea varied from 7 – 11 % in the biofilm and 8 – 21 % in the sludge. The unidentified TRFs (TRF-86bp, TRF-98bp and TRF-470bp) comprised 8 – 21 % of the TRF copies. *Methanosaeta* was also found to dominate the archaeal population in the control CSTR (data not shown), with an average of 62 % of the archaeal TRFs.

**Figure 3-6:** TRFLP and qPCR results for biofilm and sludge samples at the experiment start (S0) and at the end of the three OLR stages (S1, S2 and S3). Analysis conducted by the ATB. a) Distribution of archaeal TRFs: TRF-106bp *Methanosaeta* (black), TRF-627bp *Methanosarcina* (dark gray), TRF-342bp *Methanobacteriales* (light gray), TRF-428bp *Methanomicrobiales* (white). The average value for three measurements is given (three technical replicates, i.e., TRFLP analyses). A value of 100% refers to the sum of fluorescence intensity of all TRFs with a relative fluorescence intensity above 3% of the total fluorescence. TRFs with minor fluorescence were excluded from the analysis. b) Proportion of Archaea 16S rRNA gene copies (black bars) and number of archaeal 16S rRNA gene copies.

In the Figure 3-7a,b maximum confocal laser scanning microscopic images of sessile biomass samples are shown, the rod-shaped *Methanosaeta* structure is clearly identifiable. In Figure 3-7a the EPS fluorescence (green) overlays most of the Bacteria and Archaea (in red), while in the Figure 3-7b the rod-shaped biomass is visible through the red SYTO60 DNA dye. The white-gray structures in Figure 3-7b indicates inert solid material located in the deeper levels of the biofilm. A progressive biofilm growth at the end of each OLR has being visually observed reaching a biofilm thickness around 1.2 mm in the OLR-S3 (Figure 3-7c,d).
Figure 3-7: Maximum confocal laser scanning microscopic images and biofilm biomass on BCs. Extracellular polymeric structure (EPS) is indicated in green (dyed with Aleuria aurantia Lectin labeled with AlexaFluor488). Microorganisms were marked with red SYTO60 DNA dye. a) Image of a z-stack from a biofilm sample at the end of OLR-S2. Because the micrograph is a maximum projection of a z-stack from the biofilm, the EPS (green) overlays most of the microorganisms (red). Bar scale equals 100 µm; b) Overlay images of biofilm biomass in the OLR-S1. A reflection scan was utilized to indicate the solid, not fluorescent areas. Sequential scans were performed and scan parameters were calibrated to obtain the best images. Bar scale equals 75 µm; c) BC removed after 96 days; d) BC removed after 404 days.
3.2 Estimation of the anaerobic degradation pathways through $\delta^{13}$C measurements

3.2.1 Stable isotope measurements and methanogenic pathways calculation

Figure 3-9 provides an overview of the $\delta^{13}$C biogas data measured for the UAF at the three OLRs and for the control CSTR. A constant increase in $\delta^{13}$C from carbon dioxide ($\delta^{13}$C$_{CO2}$) occurred at an approximate rate of 1.5 ‰ every 50 days during the complete carbon isotope measurement phase (Figure 3-8). This increase was similar for the UAF and control CSTR reactor, indicating that the rise of $\delta^{13}$C$_{CO2}$ values was due to variations in the leachate derived from the LBRs. The constant fractionation through methanogenic processes, in both LBRs and methanogenic reactors, is attributed to this continual rise in the $\delta^{13}$C$_{CO2}$ values. Additionally, through the long leachate HRT (380 days for the entire plant) high amounts of dissolved inorganic carbon are retained within the system, enhancing the $^{13}$C accumulation, as bicarbonate is more enriched in $^{13}$C than the outgassing CO$_2$ (Mook et al., 1974). Due to this enrichment of $\delta^{13}$C$_{CO2}$ isotopes, the apparent fractionation from CO$_2$ to CH$_4$ ($\Delta^{13}$C$_{CO2-CH4} = \delta^{13}$C$_{CO2} - \delta^{13}$C$_{CH4}$) is a more adequate parameter to compare the variations in isotope signatures. Furthermore, $\delta^{13}$C$_{CH4}$ exhibited a high sensitivity to changes in the substrate charge during the eighth week at OLR-S1 (operational day 50). The minimal $\delta^{13}$C$_{CH4}$ values for the first substrate charge were approximately 2.0 ‰ lower than for the second charge, causing the higher variation range observed for $\Delta^{13}$C$_{CO2-CH4}$ during OLR-S1 (Figure 3.9b). Despite these variations in $\delta^{13}$C$_{CO2}$ and $\delta^{13}$C$_{CH4}$, the $\Delta^{13}$C$_{CO2-CH4}$ distributions were relatively similar for OLR-S1 and OLR-S2. At OLR-S3, the average $\Delta^{13}$C$_{CO2-CH4}$ increased above 6.0 ‰, indicating a potential change in the fractionation processes and, thus, in the methanogenic pathways.

The $\delta^{13}$C$_{CH4}$ values in the control reactor (CSTR) increased virtually at the same rate as the $\delta^{13}$C$_{CO2}$, resulting in invariant $\Delta^{13}$C$_{CO2-CH4}$ values, with an average of 42.3 ‰ and a difference of only 0.9 ‰ between the first and third quartiles. Thus, aside from the increase in $\delta^{13}$C$_{CO2}$, it is possible to assume that the leachate isotopic composition was nearly constant. Additionally, $\delta^{13}$C values for acetate, $\delta^{13}$C$_{Ac}$, were determined for leachate samples ($\delta^{13}$C$_{Ac}$ measurements were a courtesy of Prof. R. Conrad and P. Claus, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany). The calculation of f$_{mc}$ values proceeded with each set of $\delta^{13}$C$_{CO2}$, $\delta^{13}$C$_{CH4}$ and $\delta^{13}$C$_{Ac}$ data measured. The $\delta^{13}$C for methyl-acetate was determined assuming the following intramolecular acetate isotopic distribution: $\delta^{13}$C$_{Ac-methyl} = \delta^{13}$C$_{Ac} - 10.0$ ‰ (Conrad et al., 2012). This value corresponds well with the reported $\delta^{13}$C$_{Ac}$ and $\delta^{13}$C$_{Ac-methyl}$ differences of -9.2 ‰ for ethanol-derived acetate (Meinschein et al., 1974) and of -11.4 ‰ for
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Glucose-derived acetate (Blair et al., 1985). The average δ^{13}C_{ac-methyl}, value of -6.9 ‰, was assigned to calculate f_{mc} in cases in which δ^{13}C_{ac} was not measured. No distinction between suspended and sessile biomass was necessary in the UAF, according to TRF identification. The acetoclastic activity was assumed to be exclusive to the Methanoseta genera, as Methanosarcina abundance was always extremely low (Figure 3-6). This distinction is important, as the acetoclastic stable carbon isotope fractionation, α_{ma}, through Methanoseta is considerably lower than that for Methanosarcina (Goevert and Conrad, 2009). In the calculations, an α_{ma} of 1.010 was adopted according to reported values for Methanoseta (see Table 1-5).

![Variation of δ^{13}C in the biogas samples in both mesophilic reactors, the UAF (black circles) and the control CSTR (gray squares).](image)

**Figure 3-8:** Variation of δ^{13}C in the biogas samples in both mesophilic reactors, the UAF (black circles) and the control CSTR (gray squares). Error bars indicates the standard errors. The linear regression was defined for both UAF and CSTR data measured.

Reported values for the hydrogenotrophic methanogenesis fractionation factor, α_{mc}, vary between 1.031 and 1.077 (Table 1-5), presenting a wider range than for acetoclastic methanogenesis fractionation (Conrad, 2005). Indeed, α_{mc} depends on the Gibbs free energy (ΔG) available in the methanogenic environment (Penning et al., 2005). High free energy availability (low ΔG values) facilitates CO₂ activation and results in a reduced fractionation (low α_{mc}); hence, α_{mc} increases under energetically limited environments. Unfortunately, the possibility of an exact calculation of ΔG for hydrogenotrophic methanogenesis based on H₂ partial pressures (p_{H₂}) measured in the UAF headspace is restricted. Firstly, due to mass transfer limitations, p_{H₂} cannot be used to calculate the dissolved hydrogen concentrations (Pauss and Guiot, 1993). Secondly, ideal conditions for direct interspecies hydrogen transfer are available in the biofilm (Stams et al., 2012), and hydrogen may be consumed before reaching the bulk liquid. In fact, the constant and low hydrogen partial pressures in the UAF are characteristics of an obligate syntrophic growth of methanogens and VFA degraders (Stams and Plugge, 2009).

Thus, a high carbon isotopic fractionation is implied, as this syntrophic growth leaves minimal amounts of energy for the methanogen partner (Penning et al., 2005; Schink,
Hence, a high $\alpha_{mc}$ value of 1.070 was assumed. The distributions of the $f_{mc}$ values calculated for each OLR in the UAF and for the control CSTR are shown in Figure 3-9d, together with the corresponding $f_{H2,VFA}$ values. The results from both methods differ significantly, with higher hydrogenotrophic methanogenesis contributions determined through the isotope-based approach. The lower $f_{H2,VFA}$ at OLR-S3 are likely a result of the UAF acidification mentioned previously, which increased the acetate concentrations in the system.

![Figure 3-9](image)

**Figure 3-9:** Measured $\delta^{13}C$ for the biogas and calculated methanogenic pathways for the control CSTR ($n = 10$) and UAF during OLR-S1 ($n = 8$), OLR-S2 ($n = 8$) and OLR-S3 ($n = 14$). The boxes indicate the first and third quartiles; the horizontal line indicates the median; the circles indicate the mean; and the whiskers indicate the minimal and maximal values. a) $\delta^{13}C_{CO2}$; b) $\Delta^{13}C_{CO2-CH4}$; c) $\delta^{13}C_{CH4}$; d) calculated $f_{mc}$ (black box plots) and $f_{H2,VFA}$ (gray box plots) values.

### 3.2.2 Methanogenic pathways short term dynamics

The dynamics of the methanogenic pathways were investigated in detail for two exemplary weeks, one at the OLR-S2 and one at the OLR-S3. Operational data for both weeks is depicted in Figure 3-10. Despite the difficulties of determining the dissolved $H_2$ concentrations mentioned above, the $\Delta G$ values were estimated for the hydrogenotrophic methanogenesis for comparison purposes. In order to account for the higher dissolved...
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H₂ concentrations, additional calculations were performed considering an oversaturation factor of 20, according to the data reported in Pauss and Guiot (1993) for anaerobic upflow sludge-bed filters. The normal dissolved concentrations (S\textsubscript{H₂}) and with an oversaturation of 20 times are depicted below (S\textsubscript{H₂,oversaturated}; Equations 3-2 and 3-3, respectively):

\[ S_{H_2} = K_{H_2} \cdot P_{H_2} \quad (\text{mol}) \]  
\[ S_{H_2,\text{oversaturated}} = 20 \cdot S_{H_2} \quad (\text{mol}) \]

where \( K_{H_2} \) is the Henry coefficient for hydrogen gas and \( P_{H_2} \) is the measured hydrogen gas pressure in the UAF headspace. Without considering the oversaturation, the \( \Delta G \) values are too high for a microbial growth, while more reliable values were obtained with the utilization of an oversaturation factor. Regardless of the absolute values obtained, it is very likely that the conditions for the hydrogenotrophic methanogens were more favorable in the OLR-S3 week (Figure 3-10e). The \( \Delta G \) values for acetoclastic methanogenesis were also lower at the OLR-S3 as a result of the high VFA loadings (Figure 3-10f). The high VFA concentrations are also visible for the UAF pH values which fall below 7.0. Conversely, at the OLR-S2, the VFAs are virtually completely degraded and the low acetate levels maintained the \( \Delta G \) values below -20 kJ mol\(^{-1}\).

Figure 3-11 depicts the results from daily carbon isotope analysis for the two exemplary weeks from OLR-S2 and OLR-S3 and the corresponding methanogenic pathways calculated. A \( \delta^{13}C_{\text{ac-methyl}} \) uncertainty range of ± 1.0 ‰ was considered for \( f_{mc} \) calculations in addition to the standard errors of the \( \delta^{13}C \) biogas measurements. The weekly variations in the \( f_{mc} \) values were approximately 10 % at all OLRs, as depicted for both exemplary weeks (Figure 3-11d). The \( f_{H_2,VFA} \) determined exhibited no direct correlation with \( f_{mc} \). The resulting specific CH\(_4\) production rates for both methanogenic pathways, acetoclastic (\( Q_{CH_4,ma} \)) and hydrogenotrophic (\( Q_{CH_4,mc} \)), were calculated as follows:

\[ Q_{CH_4,ma} = Q_{CH_4} \cdot (1 - f_{mc}) \quad (\text{L CH}_4 \text{ d}^{-1}) \]  
\[ Q_{CH_4,mc} = Q_{CH_4} \cdot f_{mc} \quad (\text{L CH}_4 \text{ d}^{-1}) \]

The maximal acetoclastic CH\(_4\) production rate at OLR-S2, 5.9 L d\(^{-1}\), is close to the maximal production rates nearly 100 days later at OLR-S3, 6.5 L d\(^{-1}\). The hydrogenotrophic CH\(_4\) production rates nearly doubled in the same period, from 2.4 – 4.6 L d\(^{-1}\) (Figure 3-11e,f).
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Figure 3-10: Operational parameters for the weekly dynamics of the UAF at the OLR-S\textsubscript{2} (gray; first values in the x-axis) and OLR-S\textsubscript{3} (black; second values in the x-axis). a) Inflow (triangles) and outflow (circles) VFA concentrations; b) Leachate (triangles) and UAF (circles) pH values; c) Biogas (dashed line) and methane production (continuous line) at 4-h intervals; d) H\textsubscript{2} partial pressures in the UAF; e) $\Delta G$ values for the hydrogenotrophic methanogenesis, white circles indicate calculation points considering a 20 times higher concentration of dissolved H\textsubscript{2}; f) $\Delta G$ values for the acetoclastic methanogenesis.
Figure 3-11: Weekly dynamics for the UAF during the OLR-S2 (gray; first values in the x-axis) and OLR-S3 (black; second values in the x-axis). Error bars indicate the standard errors (n ≥ 3). a) $\delta^{13}$C$_{CH4}$ data measured; b) $\delta^{13}$C$_{CO2}$ data measured; c) $\delta^{13}$C$_{ac-methyl}$ calculated from $\delta^{13}$C$_{ac}$ data measured or from the average $\delta^{13}$C$_{ac}$ (white fill); d) $f_{mc}$ calculated (circles) and $f_{H2,VFA}$ (diamonds) values; e) specific acetoclastic methane production calculated; f) specific hydrogenotrophic methane production calculated.
An LBR was fed during experimental days 99 and 190; the following day, a decrease in δ\(^{13}\)C\(_{\text{CH}_4}\) of approximately 2.0 ‰ in both curves was observed (Figure 3-11a), similar to previous observations for C4 plant silage fermentation (Laukenmann et al., 2010; Nikolausz et al., 2013). In both experimental weeks, the highest δ\(^{13}\)C\(_{\text{ac-methyl}}\) values were recorded immediately before a new feeding (first day of the week; Figure 3-11c). A significant δ\(^{13}\)C\(_{\text{ac-methyl}}\) decrease accompanying the drop in δ\(^{13}\)C\(_{\text{CH}_4}\) was observed after feeding. The discrimination of light substrates in acetoclastic methanogenesis leads to \(^{13}\)C enrichment of the acetate pool. Thus, δ\(^{13}\)C\(_{\text{ac-methyl}}\) increases when the acetate consumption is higher than the acetate load, which potentially explains the higher δ\(^{13}\)C\(_{\text{ac-methyl}}\) at days 99 and 190, where the VFA production rate of the LBRs is the lowest of the week. Moreover, the α\(_{\text{ma}}\) fractionation factor diminishes under low acetate concentrations (Goevert and Conrad, 2009). Hence, the f\(_{\text{mc}}\) value at day 99 (Figure 5d), where the inflow acetate concentration was below 0.1 g\(_{\text{COD}}\) L\(^{-1}\), may be overestimated. This low effect of acetate concentration on α\(_{\text{ma}}\) probably influenced the f\(_{\text{mc}}\) calculations in the control CSTR, which was maintained continually at low OLRs and, thus, with low VFA concentrations. Therefore, the control CSTR is omitted in further discussions of the methanogenic pathway contributions.

### 3.2.3 Dependence of methanogenic pathways on the organic loading rates

The f\(_{\text{mc}}\) values determined are consistent with the high Methanosaeta abundance. At OLR-S1 and OLR-S2, the contribution of hydrogenotrophic methanogenesis was responsible for 28 – 37 % (the variation range gives the first and third quartile limits) of the total CH\(_4\) production in the UAF. Hence, for OLRs between 1.7 and 5.9 g\(_{\text{COD}}\) L\(^{-1}\) d\(^{-1}\), the methanogenic pathway distribution in the UAF was in the same range as reported for sewage sludge digesters (Jaris and McCarty, 1965; Smith and Mah, 1966). During OLR-S3, the hydrogenotrophic contribution increased to 39 – 44 % in response to the abrupt increase in VFA loads at OLR-S3. A higher variation range for f\(_{\text{mc}}\) was observed in the mesophilic degradation of municipal and cellulosic solid wastes (Qu et al., 2009b; Vavilin et al., 2008b). In these experiments, Methanosarcina was the dominant methanogenic Archaea and was considered to solely promote this pathway shift because some Methanosarcina species are able to metabolize both hydrogen and acetate (Thauer et al., 2008).

In view of the relative low variation in the degradation pathways determined here, i.e. a 10 % variation between the averages from OLR-1 and OLR-S3 (Figure 3-11d), it is difficult to obtain a direct correlation with TRF abundance. Furthermore, not only a qualitative variation of the methanogenic pathways occurred, but also the amounts of CH\(_4\) produced varied. Nearly all of the higher maximal CH\(_4\) production rates at OLR-S3
can be attributed to an increase in hydrogenotrophic methanogenesis capacity, as shown in Figure 3-11e,f. Conversely, the specific acetoclastic CH4 production increased only marginally in the observational period.

This rise of syntrophic hydrogenotrophic communities and maintenance of the acetoclastic methanogens suggest the formation of a layered sessile biofilm structure (Satoh et al., 2007). Acetoclastic methanogens can be maintained in deeper biofilm layers, as the high acetate concentrations did not result in diffusion limitations, and the syntrophic communities benefit from the high VFA concentrations near the bulk liquid. Alternatively, this observed increase in Δ^{13}C_{CO2-CH4} values and high Methanoseta abundance could also be interpreted as an indicator of DIET-mediated processes. Recently, the Methanoseta capability to reduce CO2 into CH4 accepting electrons from Geobacter was reported for microbial aggregates from a UASB reactor treating brewery waste (Rotaru et al., 2014). Methanoseta activates CO2, forming formyl methanofuran, similar to hydrogenotrophic methanogens (Penning et al., 2005; Rotaru et al., 2014). Hence, both carbon reduction methanogenic processes probably have similar CO2 fractionation factors. Thus, the determination of carbon fractionation has the potential to reveal more details of Methanoseta-dominated systems.

3.2.4 Sensitivity analysis for the methanogenic pathways calculations

A sensitivity analysis was conducted considering variations in both methanogenic fractionation factors according to reported literature values (Conrad, 2005; Penning et al., 2006a, 2005; Valentine et al., 2004) to determine the maximal variations ranges of the f_{mc} values. In addition to the f_{mc} values, Figure 3-12 shows the average f_{H2,VFA}, 16 %, for the three OLR stages. The variations ranges were between 1.006 and 1.012 for α_{ma} and between 1.060 and 1.080 for α_{mc}. Simultaneous high α_{ma} and α_{mc} fractionation factors resulted in the lowest f_{mc} values. Conversely, the highest values of f_{mc} were observed for low acetoclastic and hydrogenotrophic fractionation factors. The variations between both extremes of f_{mc} are considerably large, with an almost 30 % variation, e.g. the maximal average f_{mc} in the OLR-S3 varies from 30 – 60 %. Nevertheless, the relative f_{mc} for the three OLR stages is maintained for every fractionation factor combination, whereby the lower the α_{mc} values, the more accentuated the differences of f_{mc} are between the different OLR stages.
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Figure 3-12: Sensitivity analysis of $\alpha_{ma}$ and $\alpha_{mc}$ for the $f_{mc}$ calculations at the OLR-S1 (white circles), OLR-S2 (gray squares) and OLR-S3 (black triangles). The gray dashed line is the average of $f_{H_2,VFA}$ for all three OLRs. The boxes indicate the first and third quartiles, the horizontal line the median, the circles the mean and the whiskers the minimal and maximal values. a) $\alpha_{ma}$ of 1.006; b) $\alpha_{ma}$ of 1.008; c) $\alpha_{ma}$ of 1.010, the dotted arrow points to a possible tendency of the $f_{mc}$ values at OLR-S3 considering lower ΔG values; d) $\alpha_{ma}$ of 1.012.

It is important to note that constant fractionation factors were adopted for these calculations without considering the variations of substrate abundance or of the thermodynamic constraints (Goevert and Conrad, 2009; Penning et al., 2005). It is possible to assume that the lower ΔG values for the carbon reduction during the OLR-S3 (see Figure 3-10e) results in a lower $\alpha_{mc}$ value and, consequently, in different $f_{mc}$ values. The dashed arrow in the Figure 3-12c indicates the probable tendency of $f_{mc}$ in the OLR-S3 under a variable $\alpha_{mc}$ and an $\alpha_{ma}$ of 1.010. Hence, it is possible to hypothesize that a larger contribution of the methanogenic pathways occurred at the OLR-S3. Nonetheless, further conclusions require reliable measurements of the $H_2$ concentrations available for the microorganisms to allow for a precise ΔG determination, and/or detailed data of the $\alpha_{mc}$ variations for the environment investigated (Penning et al., 2006b). Penning et al. (2005), for instance, suggest that the ΔG available to the methanogens can be predicted from the $\alpha_{mc}$ values.
They demonstrated the potential of this fractionation-based $\Delta G$ estimation under controlled experimental conditions. Therefore, further investigations with the specific inhibition of trophic groups as the acetoclastic methanogens could be very helpful (Florencio et al., 1994; Conrad, 2005).

### 3.2.5 Carbon isotope fractionation for thermophilic acetate formation

According to the $\delta^{13}C$ data measured shown here, homoacetogenic acetate formation in the LBRs can be discarded. The average $\delta^{13}C_{ac}$ measured in the leachate was $+3.1\%$, and the homoacetogenic CO$_2$ fractionation to acetate ($\alpha_{CO2,ac}$ values of 1.040 – 1.068; Blaser et al., 2013) would lead to $\delta^{13}C_{ac}$ ranging from -52 to -27\%. Moreover, this average enrichment of 15.8\% in acetate formation ($\delta^{13}C_{maize} = -12.7\%$) is considerably higher than the acetate enrichment reported for fermentation processes in pure cultures, in the range of 0 – 3\% (Penning and Conrad, 2006). The mechanisms of this high enrichment are not investigated here, nor, to the best of my knowledge, are other published data for acetogenic isotope effects in anaerobic digesters available in the open literature at present. However, the invariant $\Delta^{13}C_{CO2-CH4}$ values determined in the control CSTR present a preliminary line of evidence of constant fractionation in thermophilic reactors at different OLRs. A better understanding of $\delta^{13}C_{ac}$ during VFA formation in bioreactors is essential for further anaerobic pathway determination through carbon isotope analysis.

### 3.2.6 Oxidation pathways of volatile fatty acids

The maximal hydrogenotrophic methanogenesis calculated through VFA oxidation yields differed significantly from the calculations from $\delta^{13}C$ measurements (Figure 3-9d and Figure 3-11d). The inflow VFA at OLR-S1 and OLR-S2 was completely degraded and correlated well with the CH$_4$ production rates. Nevertheless, on average, stoichiometric calculations of hydrogenotrophic methanogenesis, f$_{H2,VFA}$, were approximately 10 – 15\% lower than the values derived from isotope fractionation (Figure 3-9d). An inadequacy of the fractionation factors, $\alpha_{ma}$ and $\alpha_{mc}$, chosen could lead to an over- or underestimation of f$_{mc}$. Nevertheless, the uncertainty analysis results indicate that the f$_{H2,VFA}$ yields are always below the f$_{mc}$ values for a wide range of fractionation parameters (Figure 3-12). Thus, a partial degradation of acetate through syntrophic oxidizing bacteria (Hattori, 2008) can explain the discrepancies between the two calculation methods (see discussion on Section 3.4.1). There is also evidence that an analysis of fractionation in the acetate-carboxyl group may allow for a more direct differentiation between the acetate oxidation and acetoclastic pathways (Conrad and Klose, 2011).
3.3 Acetate degradation assays

3.3.1 Inocula characterization

A total of four different kinds of inoculum were utilized for the acetate degradation activity tests. Besides the samples from the two-stage experimental plant, UAF (BCs and sludge) and control CSTR, inoculum from a full-scale WWTP sludge digester (Ölbachtal, Ruhrverband, Bochum) was utilized. The inoculum of the UAF and the control CSTR were sampled between the operational days 137 and 303. The sample from the sludge digester was collected on January 13, 2014. The main properties of the different inocula are depicted in Table 3-5 and Table 3-6. The high variation of the biomass concentrations from the three suspended biomass assays can be explained by the operational conditions of the different reactors. The low OLRs applied to the control CSTR resulted in a low biomass density, while the more intensely loaded UAF presented much higher biomass concentrations. In the full-scale digester, the feeding substrate, sewage sludge, is much more complex than the VFA mixture from the LBRs’ leachate. Consequently, a more complex microbial community is required for the substrate degradation, which makes the comparison with the biomass amounts from the VFA-fed reactors difficult. Moreover, proteins are an important fraction of the inflow COD from waste-activated sludge (Ramirez et al., 2009), hence, it is very likely that the biomass fraction from the WWTP is overestimated.

Table 3-5: Inoculum characterization from the suspended biomass samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>UAF Sludge</th>
<th>Control CSTR</th>
<th>WWTP digester</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_{BM}$</td>
<td>g COD L$^{-1}$</td>
<td>4.2 – 4.6</td>
<td>0.7 – 1.2</td>
<td>7.9</td>
</tr>
<tr>
<td>COD</td>
<td>g COD L$^{-1}$</td>
<td>5.0 – 7.9</td>
<td>1.8 – 2.5</td>
<td>18.2</td>
</tr>
<tr>
<td>Acetate</td>
<td>mg COD L$^{-1}$</td>
<td>0 – 50</td>
<td>&lt; 10</td>
<td>50</td>
</tr>
<tr>
<td>NH$_4$</td>
<td>mg N L$^{-1}$</td>
<td>480 – 600</td>
<td>500 – 600</td>
<td>860</td>
</tr>
<tr>
<td>TOC</td>
<td>mg C L$^{-1}$</td>
<td>1530 – 3510</td>
<td>700 – 885</td>
<td>3900</td>
</tr>
<tr>
<td>TIC</td>
<td>mg C L$^{-1}$</td>
<td>650 – 740</td>
<td>650 – 770</td>
<td>780</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>7.4 – 7.8</td>
<td>7.4 – 7.5</td>
<td></td>
</tr>
<tr>
<td>$\delta^{13}$C$_{ac}$ $^a$</td>
<td>% PDB</td>
<td>2.0</td>
<td>2.0</td>
<td>-23.0$^d$</td>
</tr>
<tr>
<td>$\delta^{13}$C$_{IC}$ $^b$</td>
<td>% PDB</td>
<td>18.9 to 21.9</td>
<td>19.4 to 21.0</td>
<td>10.8</td>
</tr>
<tr>
<td>$\delta^{13}$C$_{XB}$ $^c$</td>
<td>% PDB</td>
<td>-1.9</td>
<td>-1.9</td>
<td>-26.9</td>
</tr>
</tbody>
</table>

$^a$ From $\delta^{13}$C$_{ac}$ measurements in the UAF inflow (see Section 3.2.1).

$^b$ Calculated from $\delta^{13}$C$_{CO2}$ measured in the gas phase and parameters from Mook et al. (1974).

$^c$ Calculated from $\delta^{13}$C$_{ac}$ values and biomass fractionation factors (Londry et al., 2008).

$^d$ Value for $\delta^{13}$C reported for wastewater (Gearing et al., 1991; Griffin et al., 1998).
The assays with the BCs presented a higher variability in comparison to the suspended biomass assays for the UAF. While the calculated biomass concentration in the sludge varied within a slight range, between 4.2 and 4.6 g_{COD} L^{-1}, the assays with the sessile biomass assays varied from 2.8 – 8.9 g_{COD} L^{-1}. It is possible to observe a parallel between the calculated biomass concentrations and the visual observations of the biofilm, with the higher concentrations corresponding to thick and well-structured biofilms. Some biofilm pictures with their respective biomass densities calculated are shown in Appendix III-C. The biofilm density calculated is in good agreement with other literature values (Lazarova and Manem, 1995; Wichern et al., 2008a). Moreover the mean C:N ratio found on the biofilm, 5.1 ± 1.2 (SD for n = 12), is consistent with the elemental composition of C_{1.0}H_{1.4}O_{0.4}N_{0.2} assumed for the biomass. The UAF_{eff} biomass concentrations were negligible.

Table 3-6: Inoculum characterization from the UAF effluent (UAF_{eff}) and biofilm samples, and properties from the mixture of effluent and biofilm.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>UAF_{eff}</th>
<th>UAF_{eff} + Biofilm</th>
<th>Biofilm^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>X_{BM}</td>
<td>g_{COD} L^{-1}</td>
<td>0.0 – 0.3</td>
<td>2.8 – 8.9</td>
<td>23.9 – 45.0</td>
</tr>
<tr>
<td></td>
<td>mmol_C L^{-1}</td>
<td>0 – 11</td>
<td>52 - 167</td>
<td>215 – 842</td>
</tr>
<tr>
<td>COD</td>
<td>g_{COD} L^{-1}</td>
<td>1.7 – 3.3</td>
<td>5.6 – 10.6</td>
<td>26.2 – 57.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>mg_{COD} L^{-1}</td>
<td>0 – 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH_{4}</td>
<td>mg_N L^{-1}</td>
<td>470 – 580</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOC</td>
<td>mg_C L^{-1}</td>
<td>700 – 940</td>
<td>2380 – 4500</td>
<td>8760 – 25,040</td>
</tr>
<tr>
<td>TIC</td>
<td>mg_C L^{-1}</td>
<td>500 – 1040</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>7.4 – 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ^{13}C_{ac}^b</td>
<td>%o PDB</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>δ^{13}C_{bc}^c</td>
<td>%o PDB</td>
<td>18.9 to 21.9</td>
<td>18.9 to 21.9</td>
<td></td>
</tr>
<tr>
<td>δ^{13}C_{XB}^d</td>
<td>%o PDB</td>
<td>-1.9</td>
<td>-1.9</td>
<td></td>
</tr>
<tr>
<td>Biofilm volume</td>
<td>mL BC^{-1}</td>
<td></td>
<td></td>
<td>10 – 19</td>
</tr>
<tr>
<td>Biofilm thickness</td>
<td>mm</td>
<td></td>
<td></td>
<td>0.6 – 1.2</td>
</tr>
</tbody>
</table>

^a Data for BCs with retention time over 400 days.
^b From δ^{13}C_{ac} measured in the UAF inflow (see Section 3.2.1).
^c Calculated from δ^{13}C_{CO2} measured in the gas phase and parameters from Mook et al. (1974).
^d Calculated from δ^{13}C_{ac} values and biomass fractionation factors (Londry et al., 2008).

The biomass concentrations calculated in the UAF sludge and biofilm samples were very near to the total COD concentrations measured. The ratio from the biomass COD and the total COD concentrations was about 80 % in the sludge and 90 % in the biofilm. It is important to note that proteins are one of the main, or the main, fraction composing the
extra polymeric substance (EPS; Laspidou and Rittmann, 2002), hence, the definition of biomass defined here also accounts for this biomass material which is not metabolically active.

### 3.3.2 Specific methanogenic activity determination

The maximal CH$_4$ production rate (MCP) calculations for the different assays depending on the biomass concentrations are depicted in Figure 3-13 (time interval, \( \Delta t \), in Equation 2-5 was defined as 1 hour). In order to obtain a better comparison between the assays with suspended and sessile biomass, the MCPs are given as CH$_4$ production per unit of liquid volume. The regression curves obtained for the UAF biomass indicated a high correlation between the MCPs and the sludge biomass, \( R^2 \) of 0.97. The data in the biofilm assays point toward a reduced activity of the biofilms by increasing biomass superficial densities (see Figure 3-13b). It is common to observe a substantial inert fraction in the deeper biofilm layers when the biofilm is thicker (Satoh et al., 2007; Wanner and Gujer, 1986; Zhang and Bishop, 1994), as observed in Figure 3-7. The specific methanogenic activities (SMA) can be calculated from the linear regressions in Figure 3-13a. In order to calculate the SMA values, a biomass concentrations of 2.5 g VSS L$^{-1}$ (1 g VSS $X_B M = 1.42$ g COD $X_B M$) was adopted, which is the proposed concentration to define the yields coefficients for anaerobic activity assays (Angelidaki et al., 2007). The SMA for the sludge and biofilm are 0.5 and 0.7 g CH$_4$ COD g VSS$^{-1}$ d$^{-1}$, respectively. Hence, both are within the activity ranges for high-rate reactors inocula (Angelidaki et al., 2007; Batstone and Jensen, 2011). Additionally, the calculated CH$_4$ production per biofilm volume yields values in the range of 5 – 10 $\mu$mol CH$_4$ cm$^{-3}$ h$^{-1}$, which is the same range reported for anaerobic granular sludge (Satoh et al., 2007).

Considering the average biofilm density of 30 g COD m$^{-2}$ (BCs with over 400 days retention time and sampled after the OLR-S2) and the total biofilm superficial area of 0.83 m$^2$, a production rate of 6.9 L CH$_4$ d$^{-1}$ can be expected from the biofilms. A potential of 2.6 L CH$_4$ d$^{-1}$ is calculated for the sludge, assuming an average biomass concentration of 4.5 g COD L$^{-1}$ in a sludge layer with 4 L of volume. These both CH$_4$ flow rates were determined with a MCP for a biomass concentration of 1 g COD (i.e. for a high substrate to biomass ratio). This results in a total CH$_4$ flow rate very similar to that recorded at the end of the OLR-S3 (Figure 3-10). Thus, in spite of the assumptions adopted for these calculations, this highlights the potential of the batch assays to investigate metabolic processes in the UAF.
Results and discussion

Figure 3-13: Correlation between biomass and MCPs. a) Suspended (white triangles for the UAF sludge, black triangles for the control CSTR and gray triangles for the WWTP digester) and biofilm biomass (circles) concentrations; b) superficial biomass distribution for the biofilm assays. MCPs for suspended biomass assays are given for the average of triplicates, error bars indicate the 95 % confidence intervals.

3.3.3 Reproducibility of the $\delta^{13}$C measurements and $[2-^{13}$C]acetate recovery

A high reproducibility of the $\delta^{13}$C measurements was observed during the acetate degradation assays when the suspended biomass was utilized as inocula (see Table 3-7). The 95 % confidence intervals for the $\delta^{13}$C$_{\text{CO}_2}$ and $\delta^{13}$C$_{\text{CH}_4}$ varied from 0.6 – 1.4 ‰ in the assays without labeled acetate. This range is considerably near to the standard errors of the single samples observed (on average 0.3 ‰). Higher CI$_{95}$ ranges were observed in the assays with dosage $[2-^{13}$C]acetate, with a variation range up to ± 5.0 ‰ in the assays with UAF sludge and 3 % labeled acetate.

A much wider variation range was observed in the CI$_{95}$ intervals for the $\delta^{13}$C$_{\text{CO}_2}$ and $\delta^{13}$C$_{\text{CH}_4}$ data in the assays with biofilm biomass. This heterogeneity converges with the variability of the biomass concentrations and of the biogas production kinetics in the biofilm assays. Hence, it is very likely that the different biomass compositions were present in each biofilm assay, resulting in variations of the acetate degradation pathways. Consequently, each BC was evaluated as a single experiment and statistical measures were applied only for the assays with suspended biomass. The heterogeneity of the biofilm biomass is not surprising considering the long residence time and the distribution of the BCs. The inflow distribution at the bottom of the reactors does not guarantee a uniform flow through the cross-sectional area of the reactor. Preliminary investigation in the UAF utilizing fluorescein as a tracer pointed to a preferential flow of substrate near the reactor walls (see Appendix III-C).
Table 3-7: Averages and 95 % confidence intervals (CI\textsubscript{95}) for the batch assays with biofilm (B) and suspended biomass (S) inocula for different dosages of [2-\textsuperscript{13}C]acetate.

<table>
<thead>
<tr>
<th>Assay</th>
<th>n</th>
<th>[2-\textsuperscript{13}C] (%)</th>
<th>(\delta^{13}\text{C}_{\text{CH}_4}) (%)</th>
<th>(\text{CI}_{95})</th>
<th>(\delta^{13}\text{C}_{\text{CO}_2}) (%)</th>
<th>(\text{CI}_{95})</th>
</tr>
</thead>
<tbody>
<tr>
<td>B\textsubscript{UAF}</td>
<td>4</td>
<td>0</td>
<td>-50.0</td>
<td>-11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S\textsubscript{UAF}</td>
<td>3</td>
<td>0</td>
<td>-52.3</td>
<td>0.6</td>
<td>-12.8</td>
<td>1.4</td>
</tr>
<tr>
<td>S\textsubscript{CSTR}</td>
<td>3</td>
<td>0</td>
<td>-51.7</td>
<td>1.0</td>
<td>-14.1</td>
<td>1.1</td>
</tr>
<tr>
<td>B\textsubscript{UAF}</td>
<td>3</td>
<td>1</td>
<td>331.9</td>
<td>11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S\textsubscript{UAF}</td>
<td>4</td>
<td>1</td>
<td>342.4</td>
<td>1.3</td>
<td>1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>S\textsubscript{CSTR}</td>
<td>3</td>
<td>1</td>
<td>388.7</td>
<td>1.9</td>
<td>-5.7</td>
<td>0.1</td>
</tr>
<tr>
<td>S\textsubscript{WWTP}</td>
<td>3</td>
<td>1</td>
<td>319.5</td>
<td>1.3</td>
<td>-4.3</td>
<td>0.3</td>
</tr>
<tr>
<td>B\textsubscript{UAF}</td>
<td>4</td>
<td>3</td>
<td>2262</td>
<td>73.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B\textsubscript{UAF}</td>
<td>6 (8)</td>
<td>3</td>
<td>2271</td>
<td>103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S\textsubscript{UAF}</td>
<td>3</td>
<td>3</td>
<td>1974</td>
<td>(44)\textsuperscript{a}</td>
<td>68</td>
<td>5.5</td>
</tr>
<tr>
<td>S\textsubscript{UAF}</td>
<td>3</td>
<td>3</td>
<td>2363</td>
<td>4</td>
<td>66</td>
<td>5.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} One sample with very high standard errors was considered as an outlier, thus, this CI\textsubscript{95} value is not considered in the further discussions.

Data from the assay series without labeled substrate and with 1 % [2-\textsuperscript{13}C]acetate was the basis for the \textsuperscript{12}C\textsuperscript{13}C Model development. In the assays series with 3 % [2-\textsuperscript{13}C]acetate, the simulations with the \textsuperscript{12}C\textsuperscript{13}C Model were utilized to improve the experimental design of the assays. The main aim of this latter assay series was the determination of the acetate fractions degraded through the SAO pathway. In view of the heterogeneity between the BCs, a higher number of biofilm samples was utilized. A total of ten BCs were sampled for the SAO determination, this corresponds to 20 % of the biofilm biomass in the UAF. In four assays, the initial acetate concentration was approximately 1.5 g L\textsuperscript{-1} (assays Ba) and in the other six, 3.1 ± g L\textsuperscript{-1} (assays Bb). Analogously, two triplicate series of assays were conducted for the sludge inocula with two different acetate start concentrations (assays Sa and Sb). After 0.92 days, a gas sample from two experiments from the series Bb was taken without interrupting the assays. Approximately one day later, other two Bb assays and two Ba assays were sampled for gas and liquid probes (the assays were interrupted). The objective of these intermediate samples were to indicate a possible short-time pathway variation. The maximal rates of the SAO pathway, for instance, are expected at the beginning of the assays, assuming that acetate oxidizing bacteria have a reduced affinity to acetate than the \textit{Methanoaeta}. 
In the Table 3-8 a detailed overview of these assays is given. The BCs retention times varied between 445 – 575 days, while an accurate SRT estimation of the sludge is more complicated. Moreover, a continuously exchange between both biomass pools is expected through attachment and detachment processes. The recovery rates of \(^{13}\)C atoms are high, between 95 and 102 \%, for almost all assays. The exceptions are the sludge assays, with low acetate concentrations (Sa) and the assays with less than 70 \% acetate degradation (Ba-1, Bb-4 and Bb-5). In the Sa assays, 29 \% of the CH\(_4\) originated from the degradable organic matter in the sludge, hence, diluting the concentrations of labeled \(^{13}\)C and potentially increasing the amount of \(^{13}\)C assimilation in the biomass. Variations of the biomass isotopic composition were not analyzed in this work. Conversely, the \(^{13}\)C recovery in the early interrupted assays was higher than expected from the acetate degradation fraction (e.g. in Bb-5, 53 \% of the \(^{13}\)C was found in the biogas, while only 40 \% of the acetate was degraded). This could be the result of an inverse fractionation (see discussion in Section 3.4.2).
Table 3-8: Description, degradation (Deg.) and $^{13}$C recovery (Rec.) rates for batch assays with 3 % [2-$^{13}$C]acetate. SE (standard errors) for single assays and CI$_{95}$ (95 % confidence intervals) are given for triplicates experiments.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Assay Nr.</th>
<th>Biofilm Age</th>
<th>Biofilm Position</th>
<th>Acetate $C_{ac}$ mol</th>
<th>Deg.</th>
<th>Inoculum $V_{inoc.}$ mL</th>
<th>Assay duration D</th>
<th>$\delta^{13}$C$_{CH4}$ %</th>
<th>$\delta^{13}$C$_{CO2}$ %</th>
<th>$^{13}$C Rec.</th>
<th>$^{13}$C Rec.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biofilm low acetate concentrations (Ba)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm Ba-1</td>
<td>553</td>
<td>A</td>
<td>III</td>
<td>4</td>
<td>24.0</td>
<td>1.4</td>
<td>67</td>
<td>119</td>
<td>4</td>
<td>2.04</td>
<td>2323.4</td>
</tr>
<tr>
<td>Biofilm Ba-2</td>
<td>553</td>
<td>B</td>
<td>IV</td>
<td>1</td>
<td>24.0</td>
<td>1.4</td>
<td>99</td>
<td>119</td>
<td>3</td>
<td>2.04</td>
<td>2334.0</td>
</tr>
<tr>
<td>Biofilm Ba-3</td>
<td>575</td>
<td>C</td>
<td>IV</td>
<td>1</td>
<td>24.2</td>
<td>1.5</td>
<td>99</td>
<td>118</td>
<td>6</td>
<td>5.92</td>
<td>2214.7</td>
</tr>
<tr>
<td>Biofilm Ba-4</td>
<td>545</td>
<td>D</td>
<td>III</td>
<td>3</td>
<td>24.2</td>
<td>1.5</td>
<td>99</td>
<td>118</td>
<td>6</td>
<td>5.92</td>
<td>2174.0</td>
</tr>
<tr>
<td><strong>Biofilm high acetate concentrations (Bb)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilm Bb-1</td>
<td>575</td>
<td>A</td>
<td>IV</td>
<td>1</td>
<td>50.3</td>
<td>3.0</td>
<td>100</td>
<td>58</td>
<td>3</td>
<td>5.92</td>
<td>2325.7</td>
</tr>
<tr>
<td>Biofilm Bb-2</td>
<td>575</td>
<td>C</td>
<td>III</td>
<td>3</td>
<td>53.6</td>
<td>3.2</td>
<td>100</td>
<td>53</td>
<td>2</td>
<td>5.92</td>
<td>2285.1</td>
</tr>
<tr>
<td>Biofilm Bb-3</td>
<td>553</td>
<td>D</td>
<td>II</td>
<td>3</td>
<td>52.9</td>
<td>3.2</td>
<td>100</td>
<td>54</td>
<td>2</td>
<td>5.92</td>
<td>2165.3</td>
</tr>
<tr>
<td>Biofilm Bb-4</td>
<td>553</td>
<td>D</td>
<td>II</td>
<td>2</td>
<td>54.9</td>
<td>3.3</td>
<td>18</td>
<td>51</td>
<td>4</td>
<td>2.04</td>
<td>2217.9</td>
</tr>
<tr>
<td>Biofilm Bb-5</td>
<td>445</td>
<td>D</td>
<td>III</td>
<td>2</td>
<td>53.5</td>
<td>3.2</td>
<td>40</td>
<td>53</td>
<td>3</td>
<td>2.04</td>
<td>2404.1</td>
</tr>
<tr>
<td>Biofilm Bb-6</td>
<td>575</td>
<td>A</td>
<td>III</td>
<td>2</td>
<td>53.5</td>
<td>3.2</td>
<td>100</td>
<td>53</td>
<td>2</td>
<td>5.92</td>
<td>2278.1</td>
</tr>
<tr>
<td><strong>Sludge assay low and high acetate concentrations (Sa and Sb triplicates)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sludge Sa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Sludge Sb</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a Inoculum volume ($V_{inoc.}$) for the biofilm assays includes the biofilm and the UAF effluent and CP is the inoculum CH$_4$ production (in % of the total produced CH$_4$); b Recovery rates of $^{13}$C without considering the $^{13}$C from the remaining acetate; c Recovery rates of $^{13}$C considering the $^{13}$C from the remaining acetate, assuming that: $\delta^{13}$C$_{ac,start}$ = $\delta^{13}$C$_{ac,end}$; d One outlier. The average is given for the other two measured points.
3.4 Stable isotopes based simulations through the $^{12}\text{C}^{13}\text{C}$-Model

3.4.1 Determination of the syntrophic acetate oxidation rates

According to the archaeal community analysis results (Section 3.1.4), the acetoclastic biomass in the model, $X_{\text{ma}}$, was defined only on the basis of data reported for *Methanosaeta*. No specific parameters were defined for either hydrogenotrophic methanogenic genera identified, *Methanobacteriales* or *Methanomicrobiales*, and they were lumped together into one state variable $X_{\text{mc}}$. Actually, kinetic parameters for the hydrogenotrophic methanogens are not expected to present a significant sensitivity for these simulations as their metabolic rate is limited by the slower acetate oxidation rates. The definition of the parameters for acetate oxidizers was based of the few available data on the literature for models including the SAO process (Shimada et al., 2011; Vavilin, 2012a, 2012b). An overview of all kinetic and isotopic fractionation parameters utilized for the simulations is depicted on Table 3-9. A conversion of all parameters to a COD-basis is provided to allow for a directly comparison with the ADM1 and other COD-based models. Analogously to the ADM1, the free ammonia inhibition was considered exclusively for the acetoclastic methanogens. Parameters for the hydrogenotrophic methanogens are implemented identically to the suggested values for high rate mesophilic digesters in the ADM1. For the acetoclastic methanogens, a lower maximal uptake rate ($K_{m,max}$) and half-saturation constant ($K_S$) are considered for the *Methanosaeta* population (Straub et al., 2006).

The kinetic parameters from Table 3-9 were maintained constant for all simulation runs and the fractions of active biomass in the inocula were utilized to fit the simulations to the data measured. Using this simulation approach, all uncertainties in the parameters’ identification are lumped together in the estimated biomass concentrations and a straightforward comparison of the different assays’ simulations is possible. Moreover, this methodology initially avoids the difficulties in determining the specific initial biomass concentrations in anaerobic assays (Donoso-Bravo et al., 2013). Nonetheless, the validity of these estimated biomass concentrations may be restricted to the assay vessels investigated. Sections 3.4.5 and 3.4.6 refer to further aspects of the uncertainties in the parameter identification and biomass concentrations estimations.
Table 3-9: Kinetic parameters for the simulations at 37 °C. Values given in molar concentrations as implemented in the $^{12}$C$^{13}$C-Model and in a COD-basis to facilitate the comparison to other models, as the ADM1.

<table>
<thead>
<tr>
<th>Biomass groups</th>
<th>Parameter</th>
<th>Description</th>
<th>Unit</th>
<th>Acetoclastic methanogens ($X_{ma}$)</th>
<th>Hydrogenotrophic methanogens ($X_{mc}$)</th>
<th>Acetate oxidizing Bacteria ($X_{ca}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{m,max}$</td>
<td>Monod maximum specific uptake rate</td>
<td>mmol mmol$^{-1}$bio$^{-1}$d$^{-1}$</td>
<td>2</td>
<td>70</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COD COD$^{-1}$bio$^{-1}$d$^{-1}$</td>
<td>4</td>
<td>35</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$Y$</td>
<td>Biomass yield</td>
<td>mmol$^{-1}$bio mmol$^{-1}$</td>
<td>0.1</td>
<td>0.12$^e$ (0.03)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COD COD$^{-1}$bio$^{-1}$</td>
<td>0.05</td>
<td>0.06</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$K_S$</td>
<td>Half-saturation constant</td>
<td>mmol L$^{-1}$</td>
<td>0.8</td>
<td>1.56·10$^{-3}$</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kgCOD m$^{-3}$</td>
<td>0.05</td>
<td>2.5·10$^{-5}$</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$S_{min}$</td>
<td>Threshold concentration for growth</td>
<td>mmol L$^{-1}$</td>
<td>0.12$^f$</td>
<td>4.0·10$^{-4}$</td>
<td>0.12$^f$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>kgCOD m$^{-3}$</td>
<td>0.007</td>
<td>6.4·10$^{-6}$</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$K_{I,NH3}$</td>
<td>Free ammonia inhibition constant</td>
<td>mmol$^{-1}$L$^{-1}$</td>
<td>1.8</td>
<td>1.8·10$^{-3}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>kmol$^{-1}$m$^{-3}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$k_{dec}$</td>
<td>Biomass decay rate</td>
<td>d$^{-1}$</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>$pH_{ll}$</td>
<td>Lower level for pH inhibition</td>
<td>(-)</td>
<td>5.5</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>$pH_{ul}$</td>
<td>Upper level for pH inhibition</td>
<td>(-)</td>
<td>7.0</td>
<td>6.0</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>$\alpha_{13}^d$</td>
<td>Biological fractionation factor</td>
<td>(-)</td>
<td>1.01</td>
<td>1.07</td>
<td>1.01</td>
</tr>
</tbody>
</table>

$^a$ Batstone et al. (2002), Conklin et al. (2006).
$^c$ Shimada et al. (2011); Vavilin (2012a, 2012b).
$^e$ Value given for 4 Mol of H$_2$. The value for 1 Mol H$_2$ is given in parenthesis.

The same value was necessary for both acetate degrading biomass groups to avoid numerical problems that arise if $S_{min}$ becomes higher than $S$. This parameter is not present in the ADM1. A review of $S_{min}$ values is given in Pavlostathis and Giraldo-Gomez (1991), while Panikov (1995) provides a detailed discussion of its significance and implementation within kinetics models.
The simulation runs were set on the basis of data measured from the gas composition (CO₂ and CH₄ contents; and δ¹³C_CO₂ and δ¹³C_CH₄ abundances), dissolved organic matter (COD and VFA), total inorganic carbon (TIC; equivalent to the state variable S_{HCO₃} in the $^{12}$C-$^{13}$C Model) and nitrogen (NH₄) concentrations. Using blank batch assays, the concentration of degradable substrate from inocula, X_C, and the correspondent hydrolyses rate, k_{hyd,X_C}, were determined a priori. A k_{hyd,X_C} of 0.28 d⁻¹ was estimated and the hydrolysis of microbial decay products, k_{hyd,XP}, was much slower, 0.0008 d⁻¹. The X_C fraction was more significant in the assays with sludge; its contribution to the CH₄ production in the biofilm assays was between 2 and 6 % (Table 3-8). The initial conditions for the simulations were defined according to the inoculum properties in Table 3-5 and Table 3-6. Additionally, the δ¹³C from the glacial acetic acid was determined as -44 ‰ (courtesy of Prof. R. Conrad and P. Claus, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany), hence, the acetate solution with the 3 % $[^{2-13}C]$acetate has a calculated δ¹³C_ac of 1311 ‰ (δ¹³C_{ac-methyl} = 2732 ‰). The detailed calculations for the initial concentrations of each model state variable are depicted in Appendix I-D.

The overview of the measured and simulated biogas production and isotopic ratios for the assays with the addition of 3 % $[^{2-13}C]$acetate are shown in Figure 3-14 (detailed assay information is in Table 3-8). The production of CO₂ and CH₄ in all the simulations is adequately represented (Figure 3-14c). The fitting of the initial acetate oxidizers fraction allows for a precise correspondence of the simulated and measured δ¹³C_CO₂ (Figure 3-14b). This also resulted in a satisfactory accordance of the simulated δ¹³C_CH₄ values (Figure 3-14a). The fractions of oxidized acetate, f_{ac,ox}, were calculated for the complete assay duration and also for the first 12 hours of the experiment. f_{ac,ox} values ranging from 7 – 18 % were determined. It is important to note that a marginal fraction of the methyl acetate group, 1.2 – 3.1 %, is converted to CO₂ in the acetate cleavage by Methanosaeta. This range indicates values determined for pure Methanosaeta concilli and Methanosaeta soenhgenii cultures (Patel, 1984; Zehnder et al., 1980). Hence, the acetate oxidation rates reported here may be slightly overestimated. According to these calculated f_{ac,ox} and to the previously obtained SMA values, the calculated specific acetate oxidation rates stand between 2.9 – 3.8 mg_{ac,COD} gVS⁻¹ h⁻¹ for the biofilm assays. The sludge assays resulted in lower values for both parameters SMA and f_{ac,ox}. Hence, the average specific acetate oxidation rates were considerably lower (1.4 – 2.5 mg_{ac,COD} gVS⁻¹ h⁻¹).

Very similar acetate oxidation contributions were found for the sessile and suspended biomass assays with the lower acetate concentrations. In this case, an average 10 % of the acetate start concentration was oxidized in the biofilm assays; the same value was found for the sludge assays (with a CI₉₅ of 1 %). The f_{ac,ox} values in the first 12 hours
Results and discussion

were only slightly higher ~ 1 – 2 % in both low acetate concentrations assays. Comparing the biofilm assays with low and high acetate concentrations, a higher SAO contribution of the latter assays was observed, with an average of 11 % (13 % after 12 hours). Although this SAO dependence on acetate concentrations is in line with the investigations of Hao et al. (2011), they found a more explicit pathway variation in investigations using anaerobic granula at 55 °C with considerably higher acetate concentrations. The authors reported, for instance, SAO contributions of 13 – 41 % and of 55 – 72 % for acetate concentrations below 45 and between 65 and 100 mmol L⁻¹ (i.e. < 2.7 and 3.9 – 6.0 g L⁻¹), respectively.

![Graphs and Data](image1)

**Figure 3-14:** Measured and simulated data for assays with labeled [2,¹³C]acetate and UAF inocula: sludge (squares) and biofilm (circles for low and triangles for high acetate concentrations). a) δ¹³CCH₄ values; b) δ¹³CCO₂ values; c) CO₂ (gray) and CH₄ (black) partial pressures; d) acetate oxidation rates in the first 12 hours (black bars) and after the assay end (gray bars). Data with "x" markers identify samples taken after 0.92 and 2.04 days. Error bars gives the 95 % confidence intervals (for n = 3).

Conversely, lower SAO rates in the sludge, 7 ± 1 %, were observed in the assays with high acetate concentrations. One possible explanation for the lower f_{ac,ox} values for the
sludge assays is that the stress conditions caused by the high acetate concentration, such as the initial lower pH values (Figure 3-15f and Figure 3-16f), have different impacts on the suspended and sessile syntrophic acetate-oxidizing communities. Although biofilms are well-known for their capabilities of supporting better stress conditions (Rajeshwari et al., 2000), further assays under controlled pH values would be necessary for a more conclusive discussion on the mechanisms controlling the SAO variations in sludge and biofilm systems. A further study from Hao et al. (2012) obtained a higher SAO contribution at lower initial pH values for thermophilic granular sludge.

The higher SAO contributions determined for the biofilm assays are in a comparable range to mesophilic digesters operated at high ammonia concentrations. Werner et al. (2014) determined that 16 – 23 % of the acetate was oxidized in swine waste digesters at a NH₄-N concentration of 4.4 – 4.8g L⁻¹. Nonetheless, in their investigation, Methanosarcina dominated the acetoclastic population, which are more tolerant to NH₃ than Methanosaeta (De Vrieze et al., 2012) and can take part in the SAO pathway. Thus, this confirms that, besides the NH₃ inhibition, the SRT is an important factor for the SAO pathway. In fact, although Methanosaeta and the SAO pathway were suggested to be mutually exclusionary characteristics by Karakashev et al. (2006), their investigations included only mixed tank reactors, with HRT between 15 and 30 days (Karakashev et al., 2005). In addition, studies focusing on dynamic acetate degradation pathway shifts concentrated mostly on thermophilic conditions (Hao et al., 2011; L.-P. Hao et al., 2012; Lü et al., 2013), hence, further investigations into mesophilic high-rate reactors are necessary to determine the SAO importance in these systems.

3.4.2 Stable carbon isotope dynamics in the biofilm assays with labeled acetate addition

The dynamics involving the acetate degradation for three biofilm batch assays are depicted in detail in Figure 3-15, and Figure 3-16 shows the results for two assays interrupted after 2.04 days. The headspace pressure increase ceases around the third day of the assays (Figure 3-15a). The rapid pressure increase directly after the experiments start results mostly from the headspace temperature increase (22 °C to 37 °C) and could be correctly described in the model through a detailed definition of the initial conditions in the simulations and a dynamic gas exchange coefficient, kₖA, implementation (see Appendix III-D for simulation results with an invariant kₖA coefficient). The headspace gas pressure in the assay with two gas samplings is also consistently reproduced in the model (Figure 3-15a). This first gas sampling results in notable effects on the pH value (Figure 3-15f), which immediately increases in response to the CO₂ loss through the gas outflow. The CO₂ and CH₄ contents agreed well for the sampling point after 2.04 and 5.92 days (Figure 3-15b and Figure 3-16b). In the samples
after 0.92 days, a gas sample pipe of 10 mL was utilized due to the reduced volume of biogas and only the isotope abundances were determined.

The δ\(^{13}\)C\(_{\text{CO}_2}\) values, as discussed above, were utilized to fit the initial biomass concentrations, thus, deviations below ±1.0 ‰ exist between the simulations and the data measured (Figure 3-15c and Figure 3-16c). This was also possible for the assays with two δ\(^{13}\)C\(_{\text{CO}_2}\) measuring points (Figure 3-15c). In general, the δ\(^{13}\)C\(_{\text{CO}_2}\) has a linear increase accompanying the headspace pressure and, thereafter, stays mostly constant. Conversely, the δ\(^{13}\)C\(_{\text{CH}_4}\) values increase much faster and, by the end of the first day, they were near their maximum. This results from the high rates of conversion of the labeled methyl group (82 – 93 ‰) to \(\text{CH}_4\). The conversion rates of \(^{13}\)C methyl are much lower for \(\text{CO}_2\) and are diluted by the conversion of the unlabeled carboxyl to \(\text{CO}_2\) (see Equations 1.21 and 1.22). In addition, there is also a dilution of the \(^{13}\)C labeled atoms in the dissolved TIC pool. The HCO\(_3^-\) concentration increases accompanying the pH values (Figure 3-15e,f), affecting the δ\(^{13}\)C distribution. The simulated end pH values are, on average, 0.2 units lower than the values measured. These effects result from the high pressures in the simulations, 1.5 – 2.0 bar. The pH measurements occurred after the assay vessels were opened, i.e. at atmospheric pressure.

The δ\(^{13}\)C\(_{\text{CH}_4}\) results marked with an “x” in Figure 3-14a correspond to the samples taken after 0.92 and 2.04 days. The simulated δ\(^{13}\)C\(_{\text{CH}_4}\) underestimated the measured values, especially for the samples with high acetate concentrations (Figure 3-16c). Even simulation runs considering no \(^{13}\)C fractionation or higher gas to liquid exchange rates were unable to reproduce the high δ\(^{13}\)C\(_{\text{CH}_4}\) values observed (data not shown). Actually, in both high acetate concentrations the \(^{13}\)C recovery rates after 2 days were of 38 and 53 ‰. Thus, higher than expected as only 18 and 40 ‰ of the acetate, respectively, was degraded by the sampling time (Table 3-8). Notably, the \(f_{\text{ac,ox}}\) determination for these both assays resulted in the lowest and highest values for the biofilm assays, 7 and 18 ‰. A possible mechanism to explain this phenomenon may be related to an EIE between acetate ion (\(\text{C}_2\text{H}_3\text{O}^-\)) and undissociated acetic acid (\(\text{C}_2\text{H}_4\text{O}_2\)), which is not accounted for in the model. The higher molecular weight (64 against 63 g mol\(^{-1}\)) of the undissociated acetic acid indicates that it would get enriched in heavy atoms against the acid base equilibrium:

\[
\text{C}_2\text{H}_4\text{O}_2 \cdot \text{H}^+ \leftrightarrow^{13} \text{C} \leftrightarrow K_{a, \text{ac}} \cdot \text{C}_2\text{H}_4\text{O}_2^-
\]

(3-6)

However, lower pH values result in higher ratios of undissociated acetic acid to acetate. Consequently, if undissociated acetic acid is assumed to be a direct substrate for the acetoclastic methanogenesis (e.g. Andrews, 1969; Wilson et al., 2012), the low pH values would imply a substrate more enriched in \(^{13}\)C atoms at the start of the assay. This would
result in a higher $^{13}$C output than observed in the assays without complete acetate degradation (Table 3-8). This EIE related to the acetate consumption was utilized by Goevert (2008) and Goevert and Conrad (2008) to describe an “inverse” fractionation, i.e. an isotopic discrimination favoring the heavier isotope, via sulfate reducers. Investigation focusing on the pH effects for the fractionation of acetotrophic organisms may therefore, be necessary in further studies. Nevertheless, this EIE effect in assays with complete acetate is negligible, as the total availability of $^{13}$C atoms is not affected, but only temporally shifted. It is important to note that this is only valid assuming that both acetotrophic organisms consume the same acetate specie, i.e. acetoclastic methanogens, and acetate oxidizers consumes undissociated acetic acid.

Nitrogen concentrations were almost completely invariant throughout the duration of the assays. It is possible to see the very continuous simulated NH$_4$-N values in Figure 3-15e. The differences in the concentrations of the different assays resulted in the different start ratios of acetate solution and inocula. However, the effects of a NH$_3$ inhibition on the acetoclastic methanogenesis is reduced for all assays (Figure 3-20).
Figure 3-15: Exemplary dynamical simulation results for assays with labeled $[2^{13}\text{C}]$acetate with BCs as inoculum with low (gray lines and symbols) and high (black lines and symbols) acetate concentrations. Assays with two gas samplings (after 0.92 and 5.92 days) are indicated by dashed lines and symbols with white centers. a) headspace pressure; b) CH$_4$ and CO$_2$ partial pressures; c) $\delta^{13}\text{C}_{\text{CH}_4}$ values; d) $\delta^{13}\text{C}_{\text{CO}_2}$ values; e) NH$_4$ (circles and continuous lines) and TIC concentrations (squares and dotted lines); f) pH values.
Figure 3-16: Exemplary dynamical simulation results for assays with labeled [2-$^{13}$C]acetate with BCs as inoculum with low (gray lines and symbols) and high (black lines and symbols) acetate concentrations. Both assays were interrupted after two days. a) headspace pressure; b) CH$_4$ and CO$_2$ partial pressures; c) $\delta^{13}$C$_{CH_4}$ values in gas (continuous line) and liquid (dotted line) phases; d) $\delta^{13}$C$_{CO_2}$ values in gas (continuous line) and liquid (dotted line) phases; e) acetate concentrations; f) pH values.
3.4.3 Stable carbon isotope dynamics in the sludge assays with labeled acetate addition

The dynamic simulations for the sludge assays with 3 % [2-13C]acetate are depicted in Figure 3-17 and Figure 3-18. In addition to the gas production in the low and high acetate concentrations assays, the pressure increase from the blank assays is depicted. Approximately 1 mL CH₄ for each 6 g inocula were produced. The correct representation of the gas production in the blank assays is not only important to determine the total CH₄ output, but also for the δ¹³C in the biogas. Due to higher ratios of inocula in the assays with low acetate concentrations, there is a higher dilution of the labeled acetate. Hence, considerably lower δ¹³C(CH₄) values were recorded for the low acetate concentration assays. However, the δ¹³C(CO₂) values were almost the same in both assays triplicates, pointing to a higher SAO for the more diluted assays. The ranges given for the δ¹³C in Figure 3-17 are the 95 % confidence interval boundaries estimated from the δ¹³C(CO₂) data measured, resulting from variations in the start acetate oxidizers fraction, fX₀,ca, of about ± 1 %. These variations fX₀,ca have no significant effects on the other simulated variables.

Nevertheless, is important to note that a significant deviation of the simulated to the measured HCO₃⁻ assay exists for the high acetate concentration assays (Figure 3-18e). However, this overestimation of the dissolved TIC prejudices the determination of the δ¹³C abundances in the gas phase and is a possible reason for the inferior simulation results for δ¹³C(CH₄) in the high acetate concentration assays (Figure 3-17d). Thus, a more detailed description of the physicochemical processes can be addressed to improve further simulations. This may include correction of ionic activities and gas solubility coefficients (Batstone et al., 2012) and/or inclusion of further ionic species (Westergreen et al., 2012). Westergreen et al. (2012), for instance, demonstrated the importance of considering the ionic activity of the biomass for the alkalinity determination. In these assays, the different inocula to acetate solution ratios resulted in different biomass concentrations that could, therefore, potentially affect TIC calculation. Contrarily to the biofilm assays, the NH₄-N concentrations presented slight variations as a result of the hydrolysis of the degradable organic matter from the sludge.
Figure 3-17: $\delta^{13}$C$_{\text{CO}_2}$ calibration for the 95 % confidence intervals for the sludge assays with 3 % $[2^{-13}\text{C}]$acetate. a) simulation of the assays with low acetate concentrations; b) simulation of the assays with high acetate concentration; c) $\delta^{13}$C$_{\text{CH}_4}$ values for the calibrated $f_{X_{0,\text{ca}}}$ ranges for low acetate concentration assays; d) $\delta^{13}$C$_{\text{CH}_4}$ values for the calibrated $f_{X_{0,\text{ca}}}$ ranges for low acetate concentration assays.
Figure 3-18: Exemplary dynamical simulation results for assays with labeled [2-\textsuperscript{13}C]acetate and UAF sludge as inoculum with low (gray lines and symbols) and high (black lines and symbols) acetate concentrations. Small dots or error bars indicates the 95\% confidence intervals. a) headspace pressure; b) CH\textsubscript{4} and CO\textsubscript{2} partial pressures; c) NH\textsubscript{4} (circle and continuous lines) and TIC concentrations (squares and dotted lines); d) pH values.
3.4.4 Stable carbon isotope dynamics in the assays without labeled acetate addition

The $^{12}$C/$^{13}$C Model also provides reliable simulation results for the assays without labeled substrates. Results for the UAF inocula, biofilm and sludge, and for the control CSTR (results for the suspended biomass assays from triplicate measurements) are shown in Figure 3-19. The very low biomass densities in the CSTR reactor result in a much slower substrate degradation. Through the lower limit pH inhibition, the $^{12}$C/$^{13}$C Model reproduces the gas production kinetics in the CSTR inoculum assays well (Figure 3-19; the same kinetic parameter for the UAF and CSTR assays were utilized).

Figure 3-19: Exemplary dynamic simulation results for assays without labeled substrates and different inocula: BCs (gray lines and symbols), UAF sludge (black lines and symbols) and control CSTR sample (dashed gray lines and white filled symbols). a) headspace pressure; b) CH$_4$ and CO$_2$ partial pressures; c) $\delta^{13}$C$_{CH_4}$ values; d) $\delta^{13}$C$_{CO_2}$ values.

A very different $\delta^{13}$C distribution in comparison to the values measured from the UAF and CSTR reactors can be observed (see Section 3.2.1). These result from the $^{13}$C abundance in glacial acetate $\delta^{13}$C$_{ac}$ -44 ‰, that is much more depleted in heavy carbon than the MS ($\delta^{13}$C$_{MS}$ -12.7 ‰). A measurement of the $\delta^{13}$C from the acetate methyl group
was not available, therefore, the $\delta^{13}C_{ac,methyl}$ was estimated through the simulations. An internal distribution of $\delta^{13}C_{ac,methyl} = \delta^{13}C_{ac} - 10 \%$ resulted in the best fit of the measured data for the three inocula. Notably, the same value that was adopted for the MS derived acetate (see Section 3.2.1). Nevertheless, the determination of the $\delta^{13}C$ from methylacetate is highly recommendable for further simulations without labeled acetate addition, as it might allow for the estimation of biological fractionation factors (see Section 3.4.6). Further simulation results with the $^{12}C^{13}C$ Model are presented in Appendix III-D.

### 3.4.5 Estimation of the initial biomass concentrations

An overview of the average initial biomass distributions for the simulations with 3 % [2-$^{13}C$]acetate are shown in Table 3-10. A constant distribution of 70 % acetoclastic and 30 % hydrogenotrophic methanogenic Archaea was arbitrarily assumed. So far, the hydrogenotrophic methanogen concentrations were equal or higher than from the acetate oxidizers, they had no significant influence on the simulation results as their growth rate was limited by the $H_2$ supply obtained through the acetate oxidation. 15 % of the calculated biomass fraction in the sludge was involved in the degradation of acetate, while this value was considerably lower for the biofilm assays, between 8 and 9 % on average. The high content of protein in the EPS and the presence of inactive biomass in the biofilm can explain this apparent lower biofilm activity (see discussion above in Sections 3.3.1 and 3.3.2).

**Table 3-10:** Average acetate oxidation fraction ($f_{ac,ox}$) and initial biomass fractions ($f_X$) for the biofilm and sludge assays with low (a) and high (b) acetate concentrations. The $f_{ac,ox}$ values are given for the whole assay and for the first 12 hours ($f_{ac,ox,12h}$). Standard deviation (SD) for the biofilm assays and 95 % confidence intervals (CI$_{95}$) for the sludge assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>n</th>
<th>Acetate oxidation fraction (%)</th>
<th>Initial biomass fractions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$f_{ac,ox}$</td>
<td>SD/CI$_{95}$</td>
</tr>
<tr>
<td>Ba</td>
<td>4</td>
<td>10.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Bb</td>
<td>6</td>
<td>11.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Sa</td>
<td>3</td>
<td>9.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Sb</td>
<td>3</td>
<td>7.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^a$ The ranges given for $f_{X,ca}$ values for the sludge assays corresponds to the 95 % confidence intervals determined for the $\delta^{13}C_{CO_2}$ measurements (Figure 3-17a,b).

Different values were found in the biofilm assay for the initial biomass concentrations, which reflect the heterogeneity of the BCs. Nonetheless, on average, a higher fraction of acetate oxidizers in biofilm, 16.8 – 19.1 % against 7.5 – 12.3 % in the sludge, was defined
through the simulations. However, the different ranges of $f_{X_0,ca}$ found for both sludge assays point to a limitation of the kinetic parameters adopted here. Hence, a further calibration of the microbial kinetics would be required to obtain the equal $f_{X_0,ca}$ ranges for the sludge Sa and Sb assays. Yet, the identifiability of the Monod parameters for growth and the half-saturation substrate concentrations, $\mu_{\text{max}}$ and $K_S$, respectively, is difficult, as both parameters are strongly correlated (Flotats et al., 2003; Holmberg, 1982; Vanrolleghem and Keesman, 1996). Holmberg (1982) demonstrates that for the identification of both Monod parameters, a low ratio $K_S$ to $S_0$ ($S_0 =$ initial substrate concentration) is desirable. Although the ratios for both acetate half-saturation constants, $K_{S,ac,ma}$ and $K_{S,ac,ca}$ (Table 3-11) are adequately low, the activities of both groups is only distinguished by the $\delta^{13}$C data which consist of only one to two points for each assay. Thus, further experimental designs based on online $\delta^{13}$C measurements (Keppler et al., 2010) are very likely to provide adequate conditions for the identifiability of kinetic parameters of acetate oxidizers and acetoclastic methanogens. In addition, the utilization of different substrate to biomass ratios can be defined to a better identification of microbial kinetic parameters, i.e. growth rates and half-saturation constants (Donoso-Bravo et al., 2011; Flotats et al., 2003; Grady et al., 1996; Holmberg, 1982).

**Table 3-11:** Average initial substrate to biomass ($S_0/X_0$) and half saturation constant to initial substrate ratios ($K_{S}/S_0$) for the biofilm (B) and sludge (S) assays with low (a) and high (b) acetate concentrations. $K_{S}/S_0$ values are given for the acetate half-saturation constants of the acetoclastic methanogens (ma) and acetate oxidizers (ca).

<table>
<thead>
<tr>
<th>Assay</th>
<th>N</th>
<th>$S_0/X_0$</th>
<th>$K_{S}/S_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total $X_0$</td>
<td>Active $X_0$</td>
</tr>
<tr>
<td>Ba</td>
<td>4</td>
<td>0.31</td>
<td>3.47</td>
</tr>
<tr>
<td>Bb</td>
<td>6</td>
<td>0.30</td>
<td>3.58</td>
</tr>
<tr>
<td>Sa</td>
<td>3</td>
<td>0.28</td>
<td>1.76</td>
</tr>
<tr>
<td>Sb</td>
<td>3</td>
<td>0.57</td>
<td>3.69</td>
</tr>
</tbody>
</table>

Although the $S_0$ to initial biomass concentration is an important factor for the parameter estimation in batch assays, it is only seldom mentioned (Donoso-Bravo et al., 2011). $S_0/X_0$ values of over 20 are recommended for the determination of intrinsic parameters, i.e. those that are independent of determination method, nonetheless, the larger they become, the greater is the change in the original culture (Grady et al., 1996). Hence, the lower $S_0/X_0$ values (Table 3-11) of this study allow for a better representation of the biomass activity from the parent reactor at the time they were sampled (Grady et al., 1996).
Additional difficulties concerning the estimation arise from the pH and NH₃ inhibitions that affect the different biomass groups. The simulated inhibition factors for pH (I_pH,low,ma) and NH₃ (I_NH3) during assays with low and high acetate concentration (values equal to 1.0 mean no inhibition, and equal to 0.0 mean complete inhibition) are depicted in Figure 3-20. A significant difference between both inhibition factors is evident during the first day of the experiments. Nevertheless, both factors were calculated based on literature parameters. An accurate identification of both inhibition factors requires further assays designed specifically for this objective (e.g., Wilson et al., 2012). There is also evidence that the acetate oxidation may involve a heterogeneous community rather than a defined culture of acetate oxidizers (Werner et al., 2014); this would have important implications for the estimation of the kinetics from the SAO pathway.

![Figure 3-20: Inhibition factors for the acetoclastic methanogenesis for low pH, I_pH,low,ma (black lines), and for free ammonia, I_NH3,ma (gray lines). a) Inhibition factors for the sludge assays with low acetate concentrations; b) inhibition factors for the sludge assays with high acetate concentrations.](image)

### 3.4.6 Sensitivity analysis for the δ¹³C measurements

The sensitivity of different input parameters to the simulated δ¹³C_CH₄ and δ¹³C_CO₂ was assessed. The primary objective of this sensitivity analysis was to orient the design of further experiments for determination of acetate degradation kinetics and/or the determination of fractionation from the different acetate degradation processes. Hence, only parameters affecting the simulated δ¹³C were considered; the sensitivity analysis of the biological kinetic parameters in anaerobic models can be found elsewhere (Koch et al., 2013; Wichern et al., 2009, 2008b). Three different set of parameters were independently evaluated:

1. **Initial concentration of acetate oxidizing Bacteria (fX₀,ca):** variation ranges of ± 10 and 25 % of the original estimated values. The lower variation range corresponds approximately to the fX₀,ca variations determined for 95 % CI intervals.
(Table 3-10) and the higher one was arbitrarily assumed to illustrate the difference in the sensitivity between the labeled and unlabeled acetate assays.

ii. Biological fractionation parameters ($a_{ma}$, $a_{mc}$ and $a_{ca}$): variation ranges of ± 5 and 10 ‰ from the values in Table 3-9 (i.e. $a_i \pm 0.005$ or 0.010). These variation ranges resume most of the literature values (see Table 1-5) analogous to the sensitivity analysis previously presented in Section 3.2.4.

iii. Dissolved start $\delta^{13}C$ values: including the TIC (that was calculated from $\delta^{13}C_{CO2}$ measurements from the parent reactor), the dissolved organic matter (estimated from representative $\delta^{13}C_{ac}$ measurements from the parent reactor) and the methyl carbon from the unlabeled acetate (estimated from the simulations, see Section 3.4.4). Variation ranges from ± 2.5 and 5 ‰ to comprise analytical uncertainties and/or literature variation ranges.

To evaluate the practical aspects of these parameter variations, a relative sensitivity index (RS) was defined based on the $\delta^{13}C$ confidence intervals determined for the triplicate experiments:

$$ RS = \frac{\delta^{13}C_{i,ref} - \delta^{13}C_{i,var}}{CI_{i,ref}} $$

(3-7)

The $\delta^{13}C_{i,ref}$ is the $^{13}C$ abundance in the reference simulation for CO$_2$ or CH$_4$, $\delta^{13}C_{i,var}$ is the maximal/minimal values after the parameter variation, and $CI_{i,ref}$ corresponds to the 95 % confidence intervals from Table 3-7. Hence, an RS value between -1 and 1 indicates a variation within the $CI_{95}$ and, consequently, without practical relevance. Higher or lower RS values indicate parameters with a significant relevance for parameter estimation. The results of the parameter variation and RS calculation for the labeled and unlabeled UAF sludge assays for $\delta^{13}C_{CH4}$ are depicted in Figure 3-21 and for $\delta^{13}C_{CO2}$ in Figure 3-22.
Figure 3-21: Sensitivity analysis for the simulated $\delta^{13}$CH$_4$ values for assay without (black lines) and with 3 % [2-$^{13}$C]acetate (gray lines). a,b) RS and $\delta^{13}$CH$_4$ values for $f_{X_{0,ca}}$ relative variations of $\pm$ 10 % (dashed lines) and $\pm$ 25 % (continuous lines) to the value calibrated; c,d) RS and $\delta^{13}$CH$_4$ values for $\alpha_{ma}$, $\alpha_{mc}$ and $\alpha_{ca}$ absolute variations of $\pm$ 5 ‰ (dashed lines) and $\pm$ 10 ‰ (continuous lines) to the parameters adopted from literature; e,f) RS and $\delta^{13}$CH$_4$ values for TIC, degradable inoculum organic matter and acetate-methyl $\delta^{13}$C absolute variations of $\pm$ 2.5 ‰ (dashed lines) and $\pm$ 5.0 ‰ (continuous lines) to the parameters determined.
Results and discussion

Figure 3-22: Sensitivity analysis for the simulated δ\textsuperscript{13}C\textsubscript{CO2} values for assay without (black lines) and with 3 \% \textsuperscript{2-13}Cacetate (gray lines). a,b) RS and δ\textsuperscript{13}C\textsubscript{CO2} values for \( fX_{0,ca} \) relative variations of ± 10 \% (dashed lines) and ± 25 \% (continuous lines) to the value calibrated; c,d) RS and δ\textsuperscript{13}C\textsubscript{CO2} values for \( \alpha_{ma}, \alpha_{mc} \) and \( \alpha_{ca} \) absolute variations of ± 5 ‰ (dashed lines) and ± 10 ‰ (continuous lines) to the parameters adopted from literature; e,f) RS and δ\textsuperscript{13}C\textsubscript{CO2} values for TIC, degradable inoculum organic matter and acetate-methyl δ\textsuperscript{13}C absolute variations of ± 2.5 ‰ (dashed lines) and ± 5.0 ‰ (continuous lines) to the parameters determined.
The lower relative sensitivity of CO$_2$ observed for the labeled and unlabeled is mostly a result of the larger pool of molecules, which include the dissolved HCO$_3^-$, against the CH$_4$. Hence, the $\delta^{13}C_{CO2}$ was adopted as the reference parameter for the fX$_{0,ca}$ evaluation of the simulations with labeled acetate. Despite the differences in the absolute RS values for $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$, the same tendency is found for both parameters. In the 3 % [2-$^{13}$C]acetate labeled assays, the metabolic pathway is the driving factor for the $\delta^{13}C$ distribution in CO$_2$ and CH$_4$. Variations of fractionation factors or analytical uncertainties in the start isotopic abundances do not affect the $\delta^{13}C$ determinations significantly. Hence, determining $f_{ac,ox}$ is possible even when considering an uncertainty range of $\pm$ 5 ‰ in the initial $\delta^{13}C$ abundances from the unlabeled substrates (which includes the TIC, biomass and internal distribution of the acetate-methyl from the glacial acetic acid). However, despite not including an estimation of specific kinetic parameters, it was possible to reproduce the $^{12}$C and $^{13}$C variations in the biogas and, consequently, calculate the fraction of acetate degraded through the SAO pathway. It is notable that the amounts of labeled acetate here, 3 %, are considerably lower than other studies with [$^{13}$C]acetate (e.g. Hori et al., 2011; Polag et al., 2013; Werner et al., 2014).

Conversely, in the unlabeled assays, the differences between the SAO and the aceticlastic methanogenic pathways is almost unrecognizable through the $\delta^{13}C$ analysis. In fact, as the total amounts of $^{12}$C and $^{13}$C atoms in the batch assays is invariant, differences in the fractionation factors promote a significant variation only at the start period of the substrate degradation. After the complete substrate degradation, the amounts of $^{12}$C and $^{13}$C are mostly independent of the fractionation parameters and the variations in the $^{13}$C abundances accumulated in the biomass are not relevant. Therefore, a sampling at the time when about 50 % is degraded poses the ideal conditions for the determination of fractionation parameters (around day 3 for the unlabeled assay depicted in Figure 3-21 and Figure 3-22), as a high RS is found for both CO$_2$ and CH$_4$ isotopic abundances. This agrees with the recommendation of Goevert and Conrad (2009) for the determination of the fractionation of aceticlastic methanogens using $\delta^{13}C$ analysis for acetate. They found that the acetate fractionation decreases at low substrate concentrations (this mechanism was not included in the model). Although the determination of representative $\alpha$-values may require a more precise characterization of the isotopic abundances of the acetate-methyl in the unlabeled assays (Figure 3-21e,f and Figure 3-22e,f), these results point to the enormous potential of the microbial kinetic simulation to determine both $\alpha$-values, $\alpha_{mc}$ and $\alpha_{ma}$, simultaneously.

Overall there is a clear contrast between the sensitivities of the labeled and unlabeled assays to the three sets of parameters: set i is significantly sensitive only for the labeled assays, while set ii and set iii are relevant for the unlabeled assays. Hence, these specific sensitivities can be explored to define ideal experimental designs to determine both
fractionation, \( \alpha_{mc} \) and \( \alpha_{ma} \), and the fraction of anaerobically oxidized acetate. Parallel assays with and without \([^{13}\text{C}]\)acetate are, therefore, necessary. In the latter assays, the fraction of oxidized acetate can be determined. Thereafter, the fractionation parameters can be estimated in the assay with unenriched acetate. The precision of the fractionation factors determination depends on the data from the isotopic characterization of the substrate. These assays can be utilized to identify aspects of the isotopic fractionation and pathway variation of specific operational variables, such as pH values, inhibitors present and different substrate concentrations. Moreover, the inclusion of \( \delta^{13}\text{C} \) measurements to differentiate degradation pathways could be easily integrated in strategies for multiparameter estimations (e.g., Wichern et al., 2009).
4. **Conclusions and suggestions for future research**

4.1 Conclusions

4.1.1 Utilized $\delta^{13}$C analysis based methods for metabolic pathways quantifications

The utilization of $\delta^{13}$C analysis in biogas plants to determine the dominant methanogenic pathways and to indicate process imbalance has been reported (Laukenmann et al., 2010; Lv et al., 2014; Nikolausz et al., 2013; Polag et al., 2013). There are also examples of methanogenic pathway quantifications in engineered anaerobic systems (Hao et al., 2011; Ho et al., 2014; Qu et al., 2009b; Vavilin et al., 2008b); nonetheless, to the best of my knowledge, this thesis provides the first long-term study confirming the reliability of methanogenic pathway quantifications in a continuously operated biomethanation plant. Additionally, the investigation performed for a two-stage plant allows for a clear differentiation of isotope fractionations related to acetate formation (in the leach bed reactors; LBRs) and consumption (in the upflow anaerobic filter; UAF).

The $\delta^{13}$C data showed a very robust performance over the more than 200 days of investigation in the continuously operated UAF with regular monitoring of the $\delta^{13}$C values in the biogas. Under lower organic loading rates (OLRs), the apparent $^{13}$C fractionation of methane (CH$_4$) to carbon dioxide (CO$_2$), $\Delta^{13}$C$_{CO2-CH4}$, presented low variations for measurements in the UAF and almost no variation in the control CSTR (see the first and third quartiles in the box plots from Figure 3-9). However, a significant $\Delta^{13}$C$_{CO2-CH4}$ variation occurred for higher OLRs in the UAF, which was effectively described through variations in the hydrogenotrophic methanogenic pathway contributions, $f_{\text{mc}}$. Notably, a continual enrichment of approximately 6‰ in $\delta^{13}$C$_{CO2}$ occurred in the leachate within 200 days of operation. However, analysis of $\delta^{13}$C$_{ac}$ from leachate and the invariant $\Delta^{13}$C$_{CO2-CH4}$ in the control CSTR allow for revoke isotope variations in the organic matter from the inflow substrate. Thus, the utilization of control reactors is strongly recommended for further studies in biomethanation plants when continuous $\delta^{13}$C$_{ac}$ data is unavailable. This is especially the case in investigations where plants are operated with different substrates and, consequently, have potentially a variable $\delta^{13}$C inflow.
Conclusions and suggestions for future research

Despite the utilization of $^{13}$C-labeled substrates, which are rather uncommon when compared to $^{14}$C, several investigations adding labeled $[{^{13}}]$acetate exist for the quantification of the syntrophic acetate oxidation (SAO) pathway (e.g., Hori et al., 2011; Shigematsu et al., 2004; Werner et al., 2014). The addition of $^{13}$C-labeled substrates requires a more elaborated experimental design to account for the exchanges of the labeled substrate with the inorganic carbon pool. The current studies utilize multiple parallel assays with different labeled acetate forms ($[^2-^{13}]C$, $[^1-^{13}]C$ and or $[^1,2-^{13}]C$) in order to empirically determine the effects of the $^{13}$C in the background. This thesis approaches the issue differently while presenting a mechanistic model, which includes a differentiation of the $^{12}$C and $^{13}$C carbon compounds species.

The exact distribution of the $^{12}$C and $^{13}$C atoms in the $^{12}C^{13}C$ Model is integrated into the microbial kinetics, while the pathway quantification is obtained directly from the simulations. Kinetic isotope effects (KIE) for each microorganism group implemented are included together with equilibrium isotope effects (EIEs) from acid-base and gas exchange reactions. The two major benefits of this approach are: i) the utilization of the same analytic methods, gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMC), that utilized for $\delta^{13}$C analysis in the investigations without labeled substrates, whereby $^{14}$C assays would require other analytical techniques; ii) following these findings, the novel model presents high potential to determine not only the fraction of oxidized acetate, $f_{ac, ox}$, but also the fractionation factors related to the acetoclastic and hydrogenotrophic methanogenesis and acetate oxidation.

Moreover, an increase in these stable isotope-based methods can be expected with respect to the development of simple analytical methods. The online measurements of $\text{CH}_4$ and $\text{CO}_2$ stable carbon isotopes in biogas plants through near-infrared laser optical spectrometry has already been demonstrated (Keppler et al., 2010). Hence, it is very likely that isotope analytics will be an important parameter in the investigation and operation of anaerobic digesters. The specific methanogenic flows from the acetate cleavage and carbon reduction ($Q_{\text{CH}_4,\text{ma}}$ and $Q_{\text{CH}_4,\text{mc}}$, respectively) are a much broader indicator than the lumped $\text{CH}_4$ production rates, and can potentially serve as a tool to: i) act as an indicator of specific inhibitions of each methanogenic group; ii) optimize biomethanation plants setting the maximal $\text{CH}_4$ production rates for both pathways; and iii) identify kinetic parameters of acetate degradation and methanogenic organisms, potentially allowing for a more accurate implementation of anaerobic digestion models, such as the ADM1 (Batstone et al., 2002).
4.1.2 Acetate oxidation fraction and methanogenic pathways dynamics in the UAF

The methanogenic and acetate degradation pathways were determined for an mesophilic (36.5 °C) anaerobic biofilm reactor, the UAF, with 11 L of reaction volume. The reactor was operated in a closed recirculation cycle with three thermophilic LBRs (51 – 56 °C) fed with maize silage (MS) in batch-feeding modus. The contribution of the carbon reduction for the total CH₄ production, f_{mc}, was determined through δ¹³C data from the inflow (leachate from the LBRs) and outflow of the UAF (CH₄ and CO₂). A direct determination of the SAO contribution, f_{ac,ox}, was possible in batch assays with addition of labeled methyl-acetate. A single biofilm carrier (BC), with ~0.017 m², was utilized for each assay in 300 mL vessels. The same experimental set up was utilized for assays with suspended biomass separately, with 75 – 140 mL sludge of the UAF for each vessel.

Despite the continuous dominance of the acetoclastic Methanosaeta in the UAF, the CH₄ fraction derived from CO₂ reduction, f_{mc}, varied significantly over the investigation period of 200 days. At OLRs below 6.0 g COD L⁻¹ d⁻¹, the average contribution of the hydrogenotrophic methanogenesis, f_{mc}, varied from 28 to 37 % (first and third quartiles in the box plots from Figure 3-9d, respectively), whereas at higher OLRs, with a maximum level of 17.0 g COD L⁻¹ d⁻¹, the f_{mc} ranged from 39 to 44 %, reaching a maximum value of 47 %. Variations of approximately 10 % were found in the daily f_{mc} values recorded during an operational week (i.e. variation of f_{mc} in the days after the weekly MS feeding). Hence, although the results in the lower OLRs converge with the methanogenic pathways found in the literature, the higher OLRs require the consideration of a dynamic f_{mc}, instead of the fixed value of one-third acetoclastic and two-thirds hydrogenotrophic methanogenesis distribution present in most of the models.

The findings point to an inconsistent inflow VFA composition when compared to the methanogenic pathways observed. Even considering the variation of f_{mc} for a wide range of fractionation factors, calculations of H₂ production derived from the VFA oxidation indicate an overestimation of f_{mc} of at least 10 to 15 %. Notably, the fractions of acetate oxidized, f_{ac,ox}, varied from 7 to 18 % in the batch assays with inocula from the UAF. Hence, the CH₄ production through the SAO pathway is a very reliable mechanism to explain the f_{mc} and f_{H2,VFA} differences. The presence of the slow growing syntrophic oxidizers in the UAF is very likely a result of the long hydraulic retention times of biomass in the biofilm and in the sludge. These f_{ac,ox} values, for instance, were determined for biofilm carriers with 445 to 575 days of retention time (see Table 3-8). It is important to note that the NH₄-N concentrations in the UAF (which always remained below 0.6 g N L⁻¹) were much lower than in other studies which identified the SAO in mesophilic reactors, 2.9 – 4.2 (Fotidis et al., 2014; Werner et al., 2014), and insufficient to promote a
substantial NH₃-inhibition for the *Methanosaeta*. Thus, in addition to NH₃, the biomass retention time is probably a driving factor for the SAO pathway.

The batch assay with UAF biofilm and sludge biomass samples presented similar ranges of \( f_{\text{ac,ox}} \) in assays with low and high acetate concentrations, 1.5 and 3.1 g L\(^{-1}\), respectively. The biofilm averages, \( f_{\text{ac,ox}} \), (values in parenthesis were calculated for the first 12 hours of the assays) were 10(11) and 11(13) %; while \( f_{\text{ac,ox}} \) of 7(7) and 10(12) % were determined for the sludge assays. Yet, while higher acetate concentrations resulted in slightly higher \( f_{\text{ac,ox}} \) values for the biofilm assays, an inverse effect was observed with sludge as inoculum. Enhanced stress resistances for the syntrophic communities in the biofilm were considered as a possible explanation for the lower \( f_{\text{ac,ox}} \) value in the high acetate sludge assays. Although the biofilm structure is known as an important factor for syntrophic microbial interactions (Stams et al., 2012), further investigations are required for a deeper understanding about its effects for the SAO communities.

Besides the determination of the CH₄ precursors and acetate degradation pathways, data from the two-stage anaerobic plant indicate constant carbon isotope fractionation for acetate formation at different OLRs within the thermophilic LBRs as well as a negligible contribution of homoacetogenesis. Although a wider dataset would be necessary to confirm this constant acetogenic fractionation in other systems, this suggests that the \( \delta^{13}C_{\text{ac}} \) values can potentially be calculated using literature data or \( \delta^{13}C \) measurements from the substrate, which simplifies the \( f_{\text{mc}} \) calculations.

## 4.1.3 Operational parameters of the two-stage experimental plant

Although the focus of the present work is on the determination of anaerobic metabolic pathways through stable isotope analysis, the operation of the experimental plant was a fundamental component in all steps of these investigations. Controlled biomass growth conditions in this model environment were necessary to determine the influence of operational/environmental factors in the metabolic activities adequately. Moreover, it was especially relevant to obtain representative samples of biofilm biomass throughout the entire investigation period. This was possible through the BC system, which allowed for an adequate sampling of the sessile biomass without disturbing the UAF operation. In addition to the DNA/RNA-based analyses, these samples allowed for microscopic investigations (confocal laser scanning microscopic images) and for batch assays with exclusively sessile biomass.

Three different OLR stages were defined: 2.0 (S1), 3.0 (S2) and 3.9 g\(_{\text{COD}}\) L\(^{-1}\) d\(^{-1}\) (S3). The OLR were defined based on the leachate volume of the storage tank reactor (STR) of each LBR, which were maintained constant at 9 L. The volatile solids (VSs) and total solids (TSs) degradation rates were similar at the OLR three stages, with degradation averages of 68 ± 2 %\(\text{TS} \) and 66 ± 3 %\(\text{VS} \), respectively. A low variation was also observed
for the specific CH₄ yields, which reached average levels of 271 ± 25 L_CH₄ kg_VS⁻¹ for the entire plant. However, an accentuated acidification in all reactors occurred after three weeks at the OLR-S3 operation. This resulted in a reduced CH₄ outflow from the LBRs and much higher VFA flows to the UAF reactor. The specific VFA production yields in the LBRs increased from 0.28 g_COD L⁻¹ d⁻¹ at the OLR-S2 to 0.73 g_COD L⁻¹ g_VS⁻¹ at the OLR-S3; correspondingly, the maximal OLRs for UAF increased from 5.9 g_COD L⁻¹ d⁻¹ to 16.8 g_COD L⁻¹ d⁻¹. In fact, it was only possible to keep the high Y_CH₄ values by incorporating a two-week feeding interruption.

The UAF acidification would certainly be avoided if a high density of BCs was utilized. The removable BC system resulted in a specific biofilm surface area of 75 m² m⁻³, much lower than typical literature values, e.g. 150 m² m⁻³ (Schönberg and Linke, 2012). Consequently, the surface specific organig loading rates (OLRS_A) in this study were also very high: up to ~100 g_COD m⁻² d⁻¹ could be supported without acidification. Yet, according to results from the batch assays, an important contribution to the total CH₄ production from the sludge biomass can be calculated; the real OLRS_A were around 30 % lower. The SMA of the biofilm was higher than in the sludge: 0.7 vs. 0.5 g_CH₄,COD g_VSS d⁻¹. However, increased biofilm thickness points to a reduced SMA. A maximal biofilm thickness of approximately 1.2 mm was determined for BCs with 575 day of retention time. An average biomass density of 30 g_COD,XBM m⁻² for BCs older than 400 days was determined.

Another important aspect in the system investigated here was the constant low hydrogen partial pressure in the UAF. The hydrogen partial pressures in single-stage biogas plants can vary over a wider range (Koch et al., 2010), which may be implied in variations of the α_mc values (Penning et al., 2005). Hence, the determination of α_mc values through acetoclastic methanogenesis inhibition may be required in further studies which consider the methanogenic pathways for biomethanation plants (Conrad, 2005). Alternatively, simulations of the ^12C^13C Model for adequately designed assays can be utilized. Moreover, the acetoclastic methanogenesis was undertaken exclusively by one Archaea genera. The Methanosaeta abundances were much higher for all samples than from Methanosarcina, implying a single fractionation factor α_ma for the f_mc calculation. The possibility of the carbon reduction through Methanosaeta was considered, nevertheless, further studies are necessary to confirm the presence of this novel methanogenic pathway.

### 4.1.4 SAO relevance for design and operation of biomethanation plants

Through the previous calculations of the methanogenic pathways (f_mc values) and the maximal hydrogen availability from the VFA oxidation (f_H2,VFA values) the last steps of the energy flow in Figure 1-1 can be defined for the UAF. Figure 4-1 present the results for the averages contributions from both CH₄ precursors for the UAF at the OLR-S2 and
Conclusions and suggestions for future research

OLR-S3. In the flowchart from Figure 1-1 and Figure 4-1b (OLR-S2) it is possible to observe that the hydrogenotrophic methanogenesis accounts between 32 to 33 % of the total methane production, while at OLR-S3 this values is 10 % higher (Figure 4-1). Nonetheless, the most remarkable difference of both flowcharts is the SAO contributions. Despite of the difficulties for the $f_{\text{H}_2, \text{VFA}}$ determination under acidification conditions (see Section 3.2.1) the SAO contributions must have been over 20% at the OLR-S3. This high percentage, above the SAO contributions determined through the batch assays, is likely a result of an inhibition of the *Methanoseta* due to the high VFA concentrations (over 3 g L$^{-1}$ acetate; De Vrieze et al., 2012); and/or due to a saturation of the maximal acetoclastic methanogenic capacity. Hence, the omission of the SAO pathway might compromise the kinetic parameters identified for the MS fermentation simulations in Figure 1-1 (the kinetic parameter set is presented in Appendix I-A).

![Flowchart](image)

**Figure 4-1:** Average COD-degradation flow for the UAF at the OLR-S2 (a) and OLR-S3 (b). Values are given in %COD of the total produced methane and no biomass growth is considered. Acetate oxidation mass flow are defined as the difference from $f_{\text{mc}}$ and $f_{\text{H}_2, \text{VFA}}$. At the OLR-S3 the $f_{\text{H}_2, \text{VFA}}$ values were likely underestimated (see Section 3.2.1) due the UAF acidification, hence the same value from OLR-S2 was adopted (asterisk markers). Otherwise an acetate oxidation flow of 28% would be obtained.

This SAO presence has ambiguous implications for the design and operation of biomethanation plants. On the one hand, the slower growth rates of the syntrophic communities requires higher biomass concentrations in order to reach degradation rates equivalent to the acetoclastic methanogens. On the other hand, the SAO communities can support much higher free ammonia inhibitions and are potentially suited for substrates with high nitrogen contents. An enhanced resistance of the SAO pathway to other forms of environmental stress, as lower pH and high VFA concentrations, were reported under thermophilic conditions (Hao et al., 2012; Lü et al., 2013) and are also to some extent confirmed for a mesophilic reactor during these investigations. Hence, in spite of lower acetate degradation kinetics, the SAO organisms may allow for a more stable reactor operation, insofar reducing the risks of a system overload. Furthermore, it is possible to improve two-stage plants with solid-phase and high-rate reactors in order
to benefit from both acetate degrading groups. In the hydrolysis of the solids substrates, a leachate with high organics concentrations is preferable as it allows for reduced volumes of storage tanks and of liquids to be pumped. This high strength leachate is better suited for the acetate degradation through SAO organisms. Yet, a further degradation of this substrate through *Methanoseta*, which has a very high affinity to acetate (i.e. acetate degradation to concentrations below 1 mg L\(^{-1}\) are possible; Jetten, 1990), allows for a very high conversion rates to CH\(_4\). Therefore, such a system can potentially be operated at high OLRs with a high efficiency (i.e. high CH\(_4\) yields).

This differentiation of SAO and acetoclastic degradation could be obtained through two high rate reactors in series or through a compartmentalized reactor. For instance, Xing et al. (2014) recently reported such a functional stratification for a high rate upflow anaerobic granula reactor operated with an average OLR of 40 g\(_{\text{COD}}\) L\(^{-1}\) d\(^{-1}\). Although a spatial identification of the microorganisms was not performed in the present investigations, the configuration of the UAF clearly promoted the growth of both acetate degrading groups. Different growth conditions as offered in the sludge bed and in the biofilm along the different reactor depths must have favored the formation of a diverse microbial consortia. Additionally, the presence of a syntrophic methanogenic pathway based on a direct electron transfer (DIET) is conceivable. In spite of the absence of specific investigations on the kinetics of CH\(_4\) formation through DIET processes, Kato et al. (2012) reports that the addition of semi-conductive iron oxides enhances the process rates of this methanogenic pathway. Consequently, the utilization of a conductive biofilm carrier could potentially be utilized to reach higher CH\(_4\) production rates derived from syntrophic DIET consortia. Investigations on DIET based methanogenesis hold promise for the development of novel biomethanation plant designs.

### 4.2 Suggestions for future research

#### 4.2.1 Determination of metabolic pathways through carbon isotope fractionation

Utilizing δ\(^{13}\)C analyses following investigation aspects are suggested in further studies, according to the present results:

- Validation of the quantification methodologies for anaerobic degradation pathways in full-scale plants. The δ\(^{13}\)C dynamics in biogas and an additional characterization of δ\(^{13}\)C in the organic matter (through measurements and/or calculation from literature data) allows for a calculation of the f\(_{\text{me}}\) values in full-scale systems. These calculations can then be validated in batch assays with labeled substrates.
• Utilization of parallel assays with labeled and unlabeled assays for identification of the fractionation parameters, $\alpha_{ma}$ and $\alpha_{mc}$. Ideally, a standardized acetate solution (unlabeled) with previously known values of $\delta^{13}C_{\text{methyl}}$ and $\delta^{13}C_{\text{carboxyl}}$ could be defined. The results from batch experiments with such a solution are easily comparable and reduce the analytical uncertainties and the number of parameters measured. Such assays hold promise for the identification of the kinetic parameters related to the acetate oxidation process.

• In situ determination of $^{13}C$ fractionation parameters in bioreactors through specific inhibition of acetoclastic methanogens. These results can be compared with $\alpha_{ma}$ and $\alpha_{mc}$ determinations suggested in the previous topic.

• A detailed characterization of the Bacteria, additional to the Archaea, presented here, would be beneficial to identify the syntrophic partners involved in the carbon reduction methanogenesis.

4.2.2 Isotope based kinetic modeling

The isotope kinetic model developed here can be further developed; some possible improvements are listed below:

• Implementation of the model for continuously operated reactors. Volatile fatty acid oxidation can be included. Such a model implementation may be helpful for the further experimental design of methanogenic pathway studies and/or evaluation of experimental data.

• Improvement of the acid-base equilibria implementation. Further ionic species can be included, together with correction of ionic activities and gas solubility coefficients. An accurate simulation of the CO$_2$-HCO$_3^-$ system is fundamental for the simulation of the $\delta^{13}C$ distribution in the inorganic carbon.

• Inclusion of variable $\alpha_{mc}$ values according to thermodynamic constraints variations. This could be helpful to determine the $\alpha_m$ dynamics if detailed data of dissolved H$_2$ are available; or, alternatively, simulations with a variable $\alpha_{mc}$ allow for the estimation of the dissolved H$_2$ concentrations.

• Inclusion of EIEs for acetate ion and undissociated acetic acid for the estimation of potential $\delta^{13}C$ variations in the CH$_4$ derived from acetate degradation depending on pH value.
4.2.3 Plant configurations for investigations of methanogenic pathways

The results of this two-stage plant points to potential topics for further investigations of the SAO pathway in mesophilic biofilm reactors:

- Operation of a similar system with a removable BC system with the inclusion of controlled HRT for the sludge. The sludge removal could be utilized to maintain defined SRTs, i.e. sludge ages, through monitoring of the suspended biomass concentrations. It is very likely that a washout point for the acetate oxidizers could be determined through a continual reduction of the SRT. Although operations of UASB or EGSB reactors would reduce the effects of biofilm detachment in the composition of the sludge biomass composition, the reduced SRTs would probably disturb other microbial consortia in the system.

- The system presented is perfectly suited for the investigation of different support materials effects in the SAO pathway, similar to the investigations of Habouzit et al. (2011). Such an investigation could determine the potential contributions of material with high conductivities, as metals and graphite, for DIET-based methanogenesis.
5. Literature


DWA, 2009. Merkblatt DWA-M 380 - Co-Vergärung in kommunalem Klärschlammfaulbehätern, Abfallvergarungsanlagen und landwirtschaftlichen Biogasanlagen. DWA (Deutsche Vereinigung für Wasserwirtschaft, Abwasser und Abfall e. V., Hennef, Germany


flux measurement and formyltetrahydrofolate synthetase (FTHFS) expression profiling. Microbiology 157, 1980–89.


Rademacher, A., Nolte, C., Schönberg, M., Klocke, M., 2012. Temperature increases from 55 to 75 °C in a two-phase biogas reactor result in fundamental alterations within


A – Kinetic parameters for the monofermentation of maize silage

Table I-A1: ADM1 biological kinetic parameters for the monofermentation of MS at 55 °C (Gehring et al., 2013) and suggested values for thermophilic solids degradation (TSD; Batstone et al., 2002).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>MS</th>
<th>TSD</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disintegration and hydrolysis (disintegration is given lumped with the hydrolysis kinetics)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{det},\text{CT}}$</td>
<td>d$^{-1}$</td>
<td>1.05</td>
<td>1.0</td>
<td>Hydrolysis of carbohydrates</td>
</tr>
<tr>
<td>$k_{\text{det},\text{PR}}$</td>
<td>d$^{-1}$</td>
<td>0.6</td>
<td>1.0</td>
<td>Hydrolysis of proteins</td>
</tr>
<tr>
<td>$k_{\text{det},\text{LI}}$</td>
<td>d$^{-1}$</td>
<td>2.0</td>
<td>1.0</td>
<td>Hydrolysis LCFA</td>
</tr>
<tr>
<td>Overall parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{dis}}$</td>
<td>d$^{-1}$</td>
<td>0.2</td>
<td>0.04</td>
<td>Biomass decay</td>
</tr>
<tr>
<td>$K_{\text{S,LI}}$</td>
<td>kmol m$^{-3}$</td>
<td>1$\times$10$^{-4}$</td>
<td>1$\times$10$^{-4}$</td>
<td>Half saturation for NH$_3$</td>
</tr>
<tr>
<td>$pH_{\text{LI,MAXH}}$</td>
<td>-</td>
<td>8.5</td>
<td>8.5</td>
<td>Upper value for pH inhibition</td>
</tr>
<tr>
<td>$pH_{\text{LI,MINH}}$</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>Lower value for pH inhibition</td>
</tr>
<tr>
<td>Sugars fermentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{SU}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>70</td>
<td>70</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,LI}}$</td>
<td>kg COD m$^{-3}$</td>
<td>1</td>
<td>1</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{SU}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.10</td>
<td>0.10</td>
<td>Biomass yield</td>
</tr>
<tr>
<td>Amino acids fermentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{AA}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>70</td>
<td>70</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,AA}}$</td>
<td>kg COD m$^{-3}$</td>
<td>0.3</td>
<td>0.3</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{AA}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.08</td>
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<td>Biomass yield</td>
</tr>
<tr>
<td>LCFA fermentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{FA}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>10</td>
<td>10</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,FA}}$</td>
<td>kg COD m$^{-3}$</td>
<td>0.4</td>
<td>0.4</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{FA}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.06</td>
<td>0.06</td>
<td>Biomass yield</td>
</tr>
<tr>
<td>Disintegration and hydrolysis (disintegration is given lumped with the hydrolysis kinetics)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{C4+}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>18</td>
<td>30</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,C4+}}$</td>
<td>kg COD m$^{-3}$</td>
<td>0.3</td>
<td>0.3</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{C4+}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.06</td>
<td>0.06</td>
<td>Biomass yield</td>
</tr>
<tr>
<td>$K_{\text{DET},\text{C4+}}$</td>
<td>kg COD m$^{-3}$</td>
<td>5.5$\times$10$^{-5}$</td>
<td>3$\times$10$^{-5}$</td>
<td>Inhibition constant for H$_3$</td>
</tr>
<tr>
<td>Propionate oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{PRO}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>12</td>
<td>20</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,PRO}}$</td>
<td>kg COD m$^{-3}$</td>
<td>0.1</td>
<td>0.1</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{PRO}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.04</td>
<td>0.04</td>
<td>Biomass yield</td>
</tr>
<tr>
<td>$K_{\text{DET},\text{PRO}}$</td>
<td>kg COD m$^{-3}$</td>
<td>1.08$\times$10$^{-7}$</td>
<td>1.0$\times$10$^{-8}$</td>
<td>Inhibition constant for H$_3$</td>
</tr>
<tr>
<td>Acetoclastic methanogenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{AC}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>18</td>
<td>16</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,AC}}$</td>
<td>kg COD m$^{-3}$</td>
<td>0.3</td>
<td>0.3</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{AC}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.05</td>
<td>0.05</td>
<td>Biomass yield</td>
</tr>
<tr>
<td>$pH_{\text{AC,MAXH}}$</td>
<td>-</td>
<td>8.5</td>
<td>8.5</td>
<td>Upper value for pH inhibition</td>
</tr>
<tr>
<td>$pH_{\text{AC,MINH}}$</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>Lower value for pH inhibition</td>
</tr>
<tr>
<td>$K_{\text{DET},\text{AC}}$</td>
<td>kmol m$^{-3}$</td>
<td>0.011</td>
<td>0.011</td>
<td>Inhibition constant for NH$_3$</td>
</tr>
<tr>
<td>Hydrogenotrophic methanogenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{\text{DET},\text{H2}}$</td>
<td>g COD g$^{-1}$ d$^{-1}$</td>
<td>35</td>
<td>35</td>
<td>Maximal uptake rate</td>
</tr>
<tr>
<td>$K_{\text{S,H2}}$</td>
<td>kg COD m$^{-3}$</td>
<td>1.16$\times$10$^{-4}$</td>
<td>5$\times$10$^{-5}$</td>
<td>Half saturation constant</td>
</tr>
<tr>
<td>$Y_{\text{H2}}$</td>
<td>g COD g$^{-1}$</td>
<td>0.6</td>
<td>0.6</td>
<td>Biomass yield</td>
</tr>
<tr>
<td>$pH_{\text{H2,MAXH}}$</td>
<td>-</td>
<td>8.5</td>
<td>8.5</td>
<td>Upper value for pH inhibition</td>
</tr>
<tr>
<td>$pH_{\text{H2,MINH}}$</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>Lower value for pH inhibition</td>
</tr>
</tbody>
</table>
B – Example for the $^{13}$C fractionation in the anaerobic acetate degradation

Figure I-B1 exemplifies the $^{13}$C variation through fractionation of acetate degradation processes in anaerobic conditions. The equations and parameters follow calculation framework in Conrad (2005) and the conversion equations of $^{13}$C abundances in atoms percentages (AP) are based on Fry (2006).

**Figure I-B1:** The flowchart exemplifies the $^{13}$C abundance variations and isotopic mass conservation for the degradation of 1.0 g carbon of acetate (or 2.5 g of acetate) through acetoclastic methanogens (70 %) and syntrophic acetate oxidation (30 %), adapted from Conrad et al. (2005). A degradation efficiency of 95 % was adopted for both acotrophic processes. a) $\delta$ and atom percentage (AP) values; b) absolute masses of $^{12}$C and $^{13}$C atoms in grams of carbon (gC). These calculations did not account for the difference in the molar masses of both carbon atoms (thus 1 gC = $^{12}$C and $^{13}$C moles) and for the dissolved inorganic carbon that should exist in the system.
A – Two-stage experimental plant: pictures and additional design data

*Pictures of the two-stage experimental plant*

Figure II-A1 shows the leach bed reactors (LBR) and their storage tank reactors (STR) in detail. Markers with different colors identify each reactor pair: red for the LBR-STR$_{01}$, blue for the LBR-STR$_{02}$ and purple for the LBR-STR$_{03}$. Figure II-A1e,d shows the custom-made structures of stainless steel and PVC for digestate removal from the LBRs.

*Figure II-A1:* Pictures of the LBRs. a) Overall view of the LBRs-STRs; b) detail view of a custom-made PVC cover of a LBR, with a temperature sensor (red cable), gas tubes (gray and black tubes), STR$_{UAF}$ recirculation inflow (transparent tube) and internal recirculation (black tube); c) overflow tube from a LBR-STR to the STR$_{UAF}$ (a small opening in the rubber balloon allowed for the outgassing of the CH$_4$ thermophilic produced CH$_4$); d,e) removable structure for substrate feeding and digestate removal in the LBRs.
Figure II-A2 presents the internal structure of the acetogenic/methanogenic reactors at start-up phase, i.e. the biofilm carrier holding system from the UAF and the stirring device from the CSTR. The overflow structures depicted in Figures II-A1c (T-connector with the rubber balloon) and II-A2c (open T-connector) had an important safety function, avoiding an overpressure build up in the reactors in case of any obstruction in the gas outflow tubing system. In the UAF the effluent outflow (upper reactor output in Figure II-A2) balanced any overpressure within the system. Further details of materials and other constructive aspects for the two-stage experimental plant are reported in Berzio (2013).

![Figure II-A2](image)

**Figure II-A2:** Pictures of the acetogenic/methanogenic reactors. a) View of the upflow anaerobic filter (UAF) internal structure; b) UAF at operation; c) control CSTR during a test run with tap water; d) CSTR at operation.

**Additional design data**

Table II-A1 gives all volumes for the reactors of the two-stage plant in addition to the other design parameters depicted in the Table 2-2.

<table>
<thead>
<tr>
<th>Reactors</th>
<th>Volumes (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>LBR01</td>
<td>14.0</td>
</tr>
<tr>
<td>STR01</td>
<td>14.0</td>
</tr>
<tr>
<td>LBR02</td>
<td>13.5</td>
</tr>
<tr>
<td>STR02</td>
<td>13.5</td>
</tr>
<tr>
<td>LBR03</td>
<td>13.5</td>
</tr>
<tr>
<td>STR03</td>
<td>14.0</td>
</tr>
<tr>
<td>STRUAF</td>
<td>12.0</td>
</tr>
<tr>
<td>UAF</td>
<td>14.0</td>
</tr>
<tr>
<td>CSTR</td>
<td>12.5</td>
</tr>
</tbody>
</table>


*Not included in the reactor total volume*
**Daily control sheet**

Figure II-A3 presents a copy of a daily control sheet for the operation of the two-stage experimental plant.

### 1) Heating water system / Temperatures

- Water reservoir is filled
- Water re-filling
- Heating water flow is o.k

<table>
<thead>
<tr>
<th>Temperatures [°C]</th>
<th>LBR01</th>
<th>°C</th>
<th>LBR02</th>
<th>°C</th>
<th>LBR03</th>
<th>°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR01</td>
<td>°C</td>
<td>STR02</td>
<td>°C</td>
<td>STR03</td>
<td>°C</td>
<td></td>
</tr>
<tr>
<td>Room</td>
<td>°C</td>
<td>UAF</td>
<td>°C</td>
<td>CSTR</td>
<td>°C</td>
<td></td>
</tr>
</tbody>
</table>

### 2) pH-Conductivity-Redox / Fill levels / Liquids inputs and outputs

<table>
<thead>
<tr>
<th>Reactor</th>
<th>pH [-]</th>
<th>Cond. [mS cm⁻¹]</th>
<th>Redox [mV]</th>
<th>Fill level [L]</th>
<th>Out [mL]</th>
<th>In [mL]</th>
<th>H₂O₂ [mL]</th>
<th>Sample [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STR2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STR3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAFSTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAFpit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAFpitp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBR0500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total:**

### 3) Milligascounter / Arite online gas measurements (control)

- all magnetic valves are on
- UAF overpressure is o.k
- CSTR overpressure is o.k

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Gas [L]</th>
<th>CH₄ [%]</th>
<th>O₂ [%]</th>
<th>CO₂ [%]</th>
<th>H₂ [ppm]</th>
<th>H₂S</th>
<th>Gas ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB01 - GS6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB02 - GS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB03 - GS3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FB - GS4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RK - GS5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- UAFSTR gas bag is not full
- New gas bag was placed
- UAFSTR overpressure is o.k

### 4) Pumps and tubes

- Water alarm is turned on
- Water sensor are turned on
- Water sensor are dry
- Tube Heidolph pump is o.k.
- Tubes Watson-Marlow pump are o.k.
- Overflow connection from the STRs to the UAF are all o.k
- LBR internal recirculation pumps (Eheim) are all o.k
- LBRs-STRs gas bag are all full
Figure II-A3: Daily control sheet for the operation of the two-stage experimental plant.

**B – Gas sample pipes for the $\delta^{13}$C analysis**

Figure II-B1 depicts the gas sample pipes (GSP) and a schematic illustration of how the pipes were connected to the UAF and control CSTR. The GSPs were fixed with a pipe clamp approximately 1 m above the acetogenic/methanogenic reactors. A valve system allowed for the removal of the gas sample tubes without affecting the reactor gas headspace. A by-pass guaranteed a continuous gas flow to the Milligacounters during the removal or connection of a new GSP. The GSPs were connected for at least 24 hours prior to a $^{13}$C analysis, which allowed for the complete removal of the nitrogen gas inside the tubes (nitrogen gas was used for the tubes flushing).

![Image of gas sample pipes](image)

**Figure II-B1:** a) Schematic view of the connection of the glass sample pipes (GSP) with the UAF and control CSTR; b) Picture of the 500 mL GSP used for sampling the UAF and CSTR reactors; c) pictures of the 50 mL GSP utilized for sampling the batch assays.

**Additional cited literature in Appendix II:**

Appendix II

C – Calculation routines implemented in the MATLAB® 2009a

Calculation routine for error propagations for the \( f_{mc} \) values

Calculation routine of the \( f_{mc} \) variations in dependence of the measurements uncertainties (standard errors) is depicted below:

```matlab
%Methanogenic Pathway Estimation through Stable Carbon Isotopes Fractionation
%Load measured data
M=load('Meas_d13C_OLR2b.txt');
t=M(:,1);  %Time
l=length(t);
%Measured Isotopic ratios [per mil values to the PDB standard]
d_ac=M(:,2);  % Acetate
d_co2=M(:,5)  % carbon dioxide
d_ch4=M(:,3)  % methane
%Substitution of the points without measurements for acetate
d_ac(find(isnan(d_ac)))=3.11;  
d_ac_rel=-10.0;  % Relative delta values between the C1 and C2 from acetate
% Fractionation factors
alfa_ma=1.010*ones(l,1);  % acetoclastic
alfa_mc=1.070*ones(l,1)  % hydrogenotrophic
% Acetate methyl
d_ac_m=d_ac + d_ac_rel
%Standard errors from the measured data
std_E_ac=0*ones(l,1);  % adopted as +/- 1.0
std_E_ch4=M(:,4);  % Errors for CH4
std_E_co2=M(:,6);  % Errors for CO2
vr_d_ac_rel=1;  %Variation in the internal acetate distribution
%Variation range for the fractionation parameters
vr_alfa_ma=0.00;  %No variation considered for alpha-ma
vr_alfa_mc=0.00;  %No variation considered for alpha-mc
%fmc calculation considering SE
n=10^4;  % number of calculations
fmc_max=0*ones(l,1);  %Start matrix maximal fmc values
fmc_min=1.0*ones(l,1);  %Start matrix minimal fmc values
for i=1:n  
  % Random number sampling
  X=rand(6,1)
  % Calculation of the variable SE
  d_ac_mr=d_ac_m+((X(1,1)-0.5)*2*vr_d_ac_rel).*ones(l,1);  
  d_ch4r=d_ch4+((X(3,1)-0.5)*2*std_E_ch4).*ones(l,1);  
  d_co2r=d_co2r+((X(4,1)-0.5)*2*std_E_co2).*ones(l,1);  
  % Calculation of the variable fractionation factors
  alfa_mar=alfa_ma+((X(5,1)-0.5)*2*vr_alfa_ma);  
  alfa_mcr=alfa_mc+((X(6,1)-0.5)*2*vr_alfa_mc);  
```
% Methane isotopic ratio resulting from acetoclastic methanogenesis
\[ d_{ma} = \left( \frac{1}{\alpha_{ma}} \right) \cdot \left( (d_{ac\_mr} + 1000) - (\alpha_{ma} \cdot 1000) \right) \cdot \text{ones}(1,1) \]

% Methane isotopic ratio resulting from hydrogenotrophic methanogenesis
\[ d_{mc} = \left( \frac{1}{\alpha_{mcr}} \right) \cdot \left( (d_{co2r} + 1000) - (\alpha_{mcr} \cdot 1000) \right) \cdot \text{ones}(1,1) \]

% Average calculation
\[ f_{mc} = \frac{d_{ch4r} - d_{ma}}{d_{mc} - d_{ma}}; \]

% Calculation routine for the f_{mc} sensitivity analysis

A second routine determined the f_{mc} sensitivity analysis in dependence of the methanogenic fractionation parameters:

% Calculation routine for the f_{mc} sensitivity analysis
% Load of the measured data matrix (MD)
% deltaCH4 with SE, deltaCO2 with SE and deltaAc for the UAF reactor:
MD = load('OLR_d13C.txt');
% Defined fractionation parameters
ama = 1.010; % alpha-ma
amc = 1.070; % alpha-ma
% Initial parameters
acm = -10.0; % 13C distribution for the acetate; value for the methyl group
% Measured 13C abundance data
dch4 = MD(:,3); % methane
dco2 = MD(:,5); % carbon dioxide
dac = MD(:,7); % acetate
% Substitution of the points without measurements for acetate
dac(find(isnan(dac))) = 3.1;
dacm = dac + acm; % acetate-methyl
% Defined variation values for alpha-ma
ama1 = 1.008;
ama2 = ama;
ama3 = 1.012;
ama4 = 1.014;
% Defined variation range for alpha-mc
amc_min = 1.060; % min
amc_max = 1.080; % max
n = 5; % number of values
% Data separation for the three OLR-stages in matrixes with same length:
nanfill = NaN.*ones(6,3);
d13FB = [dacm, dch4, dco2];
d13FB1 = d13FB(1:8, :);
Appendix II

d13FB1=[d13FB1; nanfill]
d13FB2=d13FB(9:16,:);
d13FB2=[d13FB2; nanfill]
d13FB3=d13FB(17:30,:);
for i=1:n;
amci=amc_min+(i-1)*(amc_max-amc_min)/(n-1);
\%
alpha-a1
dma11=1./ama1.*((d13FB1(:,1)+1000)-(ama1.*1000))
dmc11=1./amci.*((d13FB1(:,3)+1000)-(amci.*1000))
fmc11i=(d13FB1(:,2)-dma11)./(dmc11-dma11)
fmc_ama1(1,i)=mean(fmc11i(~isnan(fmc11i)));
fmc_bma1(:,(1+3*(i-1)))=fmc11i;
dma12=1./ama1.*((d13FB2(:,1)+1000)-(ama1.*1000))
dmc12=1./amci.*((d13FB2(:,3)+1000)-(amci.*1000))
fmc12i=(d13FB2(:,2)-dma12)./(dmc12-dma12)
fmc_ama1(2,i)=mean(fmc12i(~isnan(fmc12i)));
fmc_bma1(:,(2+3*(i-1)))=fmc12i;
dma13=1./ama1.*((d13FB3(:,1)+1000)-(ama1.*1000))
dmc13=1./amci.*((d13FB3(:,3)+1000)-(amci.*1000))
fmc13i=(d13FB3(:,2)-dma13)./(dmc13-dma13)
fmc_ama1(3,i)=mean(fmc13i);
fmc_bma1(:,(3+3*(i-1)))=fmc13i;
\%
alpha-a2
 dma21=1./ama2.*((d13FB1(:,1)+1000)-(ama2.*1000))
dmc21=1./amci.*((d13FB1(:,3)+1000)-(amci.*1000))
fmc21i=(d13FB1(:,2)-dma21)./(dmc21-dma21)
fmc_ama2(1,i)=mean(fmc21i(~isnan(fmc21i)));
fmc_bma2(:,(1+3*(i-1)))=fmc21i;
dma22=1./ama2.*((d13FB2(:,1)+1000)-(ama2.*1000))
dmc22=1./amci.*((d13FB2(:,3)+1000)-(amci.*1000))
fmc22i=(d13FB2(:,2)-dma22)./(dmc22-dma22)
fmc_ama2(2,i)=mean(fmc22i(~isnan(fmc22i)));
fmc_bma2(:,(2+3*(i-1)))=fmc22i;
dma23=1./ama2.*((d13FB3(:,1)+1000)-(ama2.*1000))
dmc23=1./amci.*((d13FB3(:,3)+1000)-(amci.*1000))
fmc23i=(d13FB3(:,2)-dma23)./(dmc23-dma23)
fmc_ama2(3,i)=mean(fmc23i);
fmc_bma2(:,(3+3*(i-1)))=fmc23i;
fmc_ama2(4,i)=amci;
\%
alpha-a3
 dma31=1./ama3.*((d13FB1(:,1)+1000)-(ama3.*1000))
dmc31=1./amci.*((d13FB1(:,3)+1000)-(amci.*1000))
fmc31i=(d13FB1(:,2)-dma31)./(dmc31-dma31)
fmc_ama3(1,i)=mean(fmc31i(~isnan(fmc31i)));
fmc_bma3(:,(1+3*(i-1)))=fmc31i;
\[
\begin{align*}
\text{dma32} &= 1./\text{ama3} \cdot ((d13FB2(:,1)+1000) - (\text{ama3} \cdot 1000)) \\
\text{dmc32} &= 1./\text{amci} \cdot ((d13FB2(:,3)+1000) - (\text{amci} \cdot 1000)) \\
\text{fmc}\text{ama}_3(2,i) &= \text{mean(fmc21i(~isnan(fmc21i))}); \\
\text{fmc}_\text{bma}_3(:,(2+3*(i-1))) &= \text{fmc32i}; \\
\text{dma33} &= 1./\text{ama3} \cdot ((d13FB3(:,1)+1000) - (\text{ama3} \cdot 1000)) \\
\text{dmc33} &= 1./\text{amci} \cdot ((d13FB3(:,3)+1000) - (\text{amci} \cdot 1000)) \\
\text{fmc}\text{33i} &= (d13FB3(:,2)-\text{dma33})/(\text{dmc33}-\text{dma33}) \\
\text{fmc}\text{ama}_3(3,i) &= \text{mean(fmc33i}); \\
\text{fmc}_\text{bma}_3(:,(3+3*(i-1))) &= \text{fmc33i}; \\
\text{fmc}_\text{ama}_3(4,i) &= \text{amci}; \\
\%\alpha - \text{ma}_4 \\
\text{dma41} &= 1./\text{ama4} \cdot ((d13FB1(:,1)+1000) - (\text{ama4} \cdot 1000)) \\
\text{dmc41} &= 1./\text{amci} \cdot ((d13FB1(:,3)+1000) - (\text{amci} \cdot 1000)) \\
\text{fmc}\text{ama}_4(1,i) &= \text{mean(fmc41i(~isnan(fmc41i))}); \\
\text{fmc}_\text{bma}_4(:,(1+3*(i-1))) &= \text{fmc41i}; \\
\text{dma42} &= 1./\text{ama4} \cdot ((d13FB2(:,1)+1000) - (\text{ama4} \cdot 1000)) \\
\text{dmc42} &= 1./\text{amci} \cdot ((d13FB2(:,3)+1000) - (\text{amci} \cdot 1000)) \\
\text{fmc}\text{ama}_4(2,i) &= \text{mean(fmc42i(~isnan(fmc42i))}); \\
\text{fmc}_\text{bma}_4(:,(2+3*(i-1))) &= \text{fmc42i}; \\
\text{dma43} &= 1./\text{ama4} \cdot ((d13FB3(:,1)+1000) - (\text{ama4} \cdot 1000)) \\
\text{dmc43} &= 1./\text{amci} \cdot ((d13FB3(:,3)+1000) - (\text{amci} \cdot 1000)) \\
\text{fmc}\text{ama}_4(3,i) &= \text{mean(fmc43i)}; \\
\text{fmc}_\text{bma}_4(:,(3+3*(i-1))) &= \text{fmc43i}; \\
\text{fmc}_\text{ama}_4(4,i) &= \text{amci}; \end{align*}
\]

end

\% Plot data
D – Constants and initial concentrations calculations for the $^{12}$C$^{13}$C Model

Table II-D1 presents the defined physicochemical coefficients utilized for the simulations with the $^{12}$C$^{13}$C-Model corrected for 37°C. The definition of the initial conditions of the batch assays are a fundamental aspect for the simulations, especially considering the definition of the isotopic compositions. Therefore a standardized calculation for the initial conditions was used for all assay simulations. Only the initial biomass fractions, fX$_{0,Xma}$ (acetoclastic methanogens), fX$_{0,Xma}$ (acetoclastic methanogens) and fX$_{0,Xma}$ (acetate oxidizers) varied as described in Sections 3.4.1 and 3.4.5. Table II-D2 lists the parameters necessary for the calculations of the state variables initial conditions. All the equations for definition of the initial state variables are depicted in the Table II-D3. This calculation framework allows for further simulations of carbon stable isotopes dynamics in acetate activity assays.

Table II-D1: Physicochemical constants, values obtained from Batstone et al. (2002) and corrected for 37°C.

<table>
<thead>
<tr>
<th>Constant</th>
<th>Unit</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{a_{ac}}$</td>
<td>M</td>
<td>$1.74 \cdot 10^{-5}$</td>
<td>Acetate acidity coefficient</td>
</tr>
<tr>
<td>$K_{a_{co2}}$</td>
<td>M</td>
<td>$5.03 \cdot 10^{-7}$</td>
<td>Carbon dioxide acidity coefficient</td>
</tr>
<tr>
<td>$K_{a_{h2o}}$</td>
<td>M</td>
<td>$2.40 \cdot 10^{-14}$</td>
<td>Water acidity coefficient</td>
</tr>
<tr>
<td>$K_{a_{nh4}}$</td>
<td>M</td>
<td>$1.27 \cdot 10^{-9}$</td>
<td>Ammonium nitrogen acidity coefficient</td>
</tr>
<tr>
<td>$K_{H_{CH4}}$</td>
<td>$M_{liq} \cdot L^{-1} \cdot Bar^{-1}$</td>
<td>$0.0011^a$</td>
<td>Henry coefficient for methane</td>
</tr>
<tr>
<td>$K_{H_{CO2}}$</td>
<td>$M_{liq} \cdot L^{-1} \cdot Bar^{-1}$</td>
<td>$0.0258^a$</td>
<td>Henry coefficient for carbon dioxide</td>
</tr>
<tr>
<td>$K_{H_{H2}}$</td>
<td>$M_{liq} \cdot L^{-1} \cdot Bar^{-1}$</td>
<td>$0.0007^a$</td>
<td>Henry coefficient for hydrogen</td>
</tr>
</tbody>
</table>

$^a$ Dividing the parameter through the number of mols for 1 liter gas at 1 bar and 37 °C (0.0388) gives the non-dimensional Henry coefficient (KH' in $M_{liq} M_{gas}^{-1}$).
**Table II-D2**: Measured variables, calculations and general assumptions for the definition of the initial state variables.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Description / Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f_{\text{inoc}} )</td>
<td>-</td>
<td>Volume fraction of the inoculum relative to the total liquid volume</td>
</tr>
<tr>
<td>( f_{\text{Sac,added}} )</td>
<td>-</td>
<td>Volume fraction of the acetate solution relative to the total liquid vol.</td>
</tr>
<tr>
<td>( S_{\text{ac,added}} )</td>
<td>mmol L(^{-1})</td>
<td>Concentration of acetate in the acetate solution</td>
</tr>
<tr>
<td>( S_{\text{ac,inoc}} )</td>
<td>mmol L(^{-1})</td>
<td>Measured acetate concentration in the inoculum</td>
</tr>
<tr>
<td>( f_{\text{Sac,C2}} )</td>
<td>-</td>
<td>Fraction of [2(-^{13})C]acetate in the acetate solution</td>
</tr>
<tr>
<td>( S_{\text{in,inoc}} )</td>
<td>mmol L(^{-1})</td>
<td>Measured NH(_4)-N in the inoculum</td>
</tr>
<tr>
<td>( X_{\text{BM,inoc}} )</td>
<td>mmol L(^{-1})</td>
<td>Calculated concentration of biomass in the inoculum (Equation 2-6)(^a)</td>
</tr>
<tr>
<td>( X_{\text{C,inoc}} )</td>
<td>mmol L(^{-1})</td>
<td>Concentration of the slowly degradable organic matter; ( X_{\text{C,inoc}}=C_{\text{COD}}-X_{\text{BM,inoc}} )</td>
</tr>
<tr>
<td>( T_{\text{start}} )</td>
<td>K</td>
<td>Measured temperature during the batch assays preparation</td>
</tr>
<tr>
<td>( P_{\text{atm,start}} )</td>
<td>Bar</td>
<td>Measured atmospheric pressure during the batch assays preparation</td>
</tr>
<tr>
<td>( \text{pH}_{\text{start}} )</td>
<td>-</td>
<td>Measured pH at the assay start</td>
</tr>
<tr>
<td>( \text{pH}_{2\text{O},\text{start}} )</td>
<td>Bar</td>
<td>Water partial pressure; ( \text{pH}<em>{2\text{O}} = 0.0313 \cdot \exp(5290 \cdot (1/298-1/T</em>{\text{start}})) )</td>
</tr>
<tr>
<td>( \text{pCH}_4,\text{parent} )</td>
<td>Bar</td>
<td>Measured CH(_4) partial pressure at the parent reactor</td>
</tr>
<tr>
<td>( \text{pCO}_2,\text{parent} )</td>
<td>Bar</td>
<td>Measured CO(_2) partial pressure at the parent reactor</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{CH}_4,\text{parent}} )</td>
<td>‰ PDB</td>
<td>Measured (^{13})C abundance at the parent reactor for CH(_4)</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{CO}_2,\text{parent}} )</td>
<td>‰ PDB</td>
<td>Measured (^{13})C abundance at the parent reactor for CO(_2)</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{inoc}} )</td>
<td>‰ PDB</td>
<td>Estimated (^{13})C abundance at the parent reactor for the organic matter</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{ac,inoc,met}} )</td>
<td>‰ PDB</td>
<td>Adopted distribution; ( \delta^{13}C_{\text{ac,inoc,met}} = \delta^{13}C_{\text{ac,inoc}} - 10 ) (see Section 3.2.1)</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{ac,NL}} )</td>
<td>‰ PDB</td>
<td>Measured (^{13})C abundance for the not labeled acetate</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{XBM}} )</td>
<td>‰ PDB</td>
<td>Estimated internal distribution for the not labeled acetate</td>
</tr>
<tr>
<td>( \delta^{13}C_{\text{ac,NL}} )</td>
<td>‰ PDB</td>
<td>Assumed (^{13})C abundance for the biomass; ( \delta^{13}C_{\text{XBM}} = \delta^{13}C_{\text{inoculum}}+3.9 )</td>
</tr>
</tbody>
</table>

Calculation of the \(^{13}\)C-fraction \((^{13}C/^{12+13}C)\) of a substrate with an abundance of \(^{13}\)C\(^b\):

\[
f_{13,j} = \frac{(\delta^{13}C+1000)}{(\delta^{13}C+1000+(1000/R_{\text{PDB}}))}
\]

Calculation of the \(^{13}\)C-distribution for acetate with an abundance of \(^{13}\)C\(_\text{ac}\) and \(^{13}\)C\(_\text{ac,met}\)\(^b\):

\[
f_{\text{Sac,1212},j} = 1-(\delta^{13}C_{\text{Sac,i}}+1000)/((\delta^{13}C_{\text{Sac,i}}+1000+(1000/R_{\text{PDB}}))-2
\]
\[
f_{\text{Sac,1213},j} = 2*(\delta^{13}C_{\text{Sac,i}}+1000)/((\delta^{13}C_{\text{Sac,i}}+1000+(1000/R_{\text{PDB}})))-f_{\text{Sac,1312},j}
\]
\[
f_{\text{Sac,1312},j} = (\delta^{13}C_{\text{Sac,met,i}}+1000)/((\delta^{13}C_{\text{Sac,met,i}}+1000+(1000/R_{\text{PDB}}))
\]

\(^a\)Calculated first as COD and later converted to molar units. \( C_{\text{COD}} \) stands for the measured COD in the inoculum;

\(^b\)See Fry (2006) for further details in the conversion of \(^{13}\)C abundances.
### Table II-D3: Definition of the initial values for the state variables in the $^{12}$C$^{13}$C-Model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Equation / Commentaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{ac,1212}$</td>
<td>$f_{inoc} \cdot S_{ac,inoc} \cdot (1-f_{S_{ac,C2}}) \cdot S_{ac,added} \cdot f_{Sac1212,NL}$</td>
</tr>
<tr>
<td>$S_{ac,1213}$</td>
<td>$f_{inoc} \cdot S_{ac,inoc} \cdot (1-f_{S_{ac,C2}}) \cdot S_{ac,added} \cdot f_{Sac1213,NL}$</td>
</tr>
<tr>
<td>$S_{ac,1312}$</td>
<td>$f_{inoc} \cdot S_{ac,inoc} \cdot (1-f_{S_{ac,C2}}) \cdot S_{ac,added} \cdot f_{Sac1312,NL} \cdot f_{Sac,C2} \cdot f_{Sac,added}$</td>
</tr>
<tr>
<td>$S_{ch4,12}$</td>
<td>$(1-f_{13,CH4,parent}) \cdot 1000 \cdot (p_{CH4, parent} \cdot KH_{CH4}) \cdot f_{inoc}$</td>
</tr>
<tr>
<td>$S_{ch4,13}$</td>
<td>$f_{13,CH4,parent} \cdot 1000 \cdot (p_{CH4, parent} \cdot KH_{CH4}) \cdot f_{inoc}$</td>
</tr>
<tr>
<td>$S_{in}$</td>
<td>$f_{inoc} \cdot S_{in,inoc}$</td>
</tr>
<tr>
<td>$S_{co2,12}$</td>
<td>$(1-f_{13,CO2,parent}) \cdot 1000 \cdot (p_{CO2, parent} \cdot KH_{CO2})$</td>
</tr>
<tr>
<td>$S_{co2,13}$</td>
<td>$f_{13,CO2,parent} \cdot 1000 \cdot (p_{CO2, parent} \cdot KH_{CO2})$</td>
</tr>
<tr>
<td>$X_{ma,12}$</td>
<td>$(1-f_{13,XBM}) \cdot 1000 \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot f_{X_{0,ma}})$</td>
</tr>
<tr>
<td>$X_{ma,13}$</td>
<td>$f_{13,XBM} \cdot 1000 \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot f_{X_{0,ma}})$</td>
</tr>
<tr>
<td>$X_{ac,12}$</td>
<td>$(1-f_{13,XBM}) \cdot 1000 \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot f_{X_{0,ac}})$</td>
</tr>
<tr>
<td>$X_{ac,13}$</td>
<td>$f_{13,XBM} \cdot 1000 \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot f_{X_{0,ac}})$</td>
</tr>
<tr>
<td>$X_{mc,12}$</td>
<td>$(1-f_{13,XBM}) \cdot 1000 \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot f_{X_{0,mc}})$</td>
</tr>
<tr>
<td>$X_{mc,13}$</td>
<td>$f_{13,XBM} \cdot 1000 \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot f_{X_{0,mc}})$</td>
</tr>
<tr>
<td>$X_{C,12}$</td>
<td>$(1-f_{13,inoc}) \cdot 1000 \cdot (f_{inoc} \cdot X_{C,inoc})$</td>
</tr>
<tr>
<td>$X_{C,13}$</td>
<td>$f_{13,inoc} \cdot (f_{inoc} \cdot X_{C,inoc})$</td>
</tr>
<tr>
<td>$X_{P,12}$</td>
<td>$(1-f_{13,XBM}) \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot (1-f_{X_{0,mc}} \cdot f_{X_{0,ma}} \cdot f_{X_{0,ca}}))$ (is the amount of not active biomass)</td>
</tr>
<tr>
<td>$X_{P,13}$</td>
<td>$f_{13,XBM} \cdot (f_{inoc} \cdot X_{BM,inoc} \cdot (1-f_{X_{0,mc}} \cdot f_{X_{0,ma}} \cdot f_{X_{0,ca}}))$ (is the amount of not active biomass)</td>
</tr>
<tr>
<td>$S_{ac,1212,ion}$</td>
<td>$S_{ac,1212} \cdot Ka_{ac}/(Ka_{ac}+S_{h+})$</td>
</tr>
<tr>
<td>$S_{ac,1213,ion}$</td>
<td>$S_{ac,1213} \cdot Ka_{ac}/(Ka_{ac}+S_{h+})$</td>
</tr>
<tr>
<td>$S_{ac,1312,ion}$</td>
<td>$S_{ac,1312} \cdot Ka_{ac}/(Ka_{ac}+S_{h+})$</td>
</tr>
<tr>
<td>$S_{nh4+}$</td>
<td>$S_{in} \cdot (S_{h+}/(S_{h+}+Ka_{nh4}))$</td>
</tr>
<tr>
<td>$S_{nh3}$</td>
<td>$S_{in} \cdot S_{nh4+}$</td>
</tr>
<tr>
<td>$S_{hco3,-12}$</td>
<td>$(1-f_{13,HCO3,parent}) \cdot S_{nh4+}$ (idealized bicarbonate-ammonium nitrogen buffer system)</td>
</tr>
<tr>
<td>$S_{hco3,-13}$</td>
<td>$f_{13,HCO3,parent} \cdot S_{nh4+}$ (idealized bicarbonate-ammonium nitrogen buffer system)</td>
</tr>
<tr>
<td>$S_{an}$</td>
<td>50 (fixed initial concentration; $S_{cat+}$ closes the ionic balance for the measured pH value)</td>
</tr>
<tr>
<td>$S_{cat+}$</td>
<td>$-S_{h+} \cdot 1000 + S_{an} \cdot 1000 + (S_{hco3,-12} + S_{hco3,-13}) \cdot S_{nh4+} + (S_{ac1212} + S_{ac1213} + S_{ac1312})$</td>
</tr>
<tr>
<td>$S_{h+}$</td>
<td>$10^4 \cdot (pH_{start})$</td>
</tr>
<tr>
<td>$S_{oh}$</td>
<td>$Ka_{h2o}/S_{h+}$</td>
</tr>
<tr>
<td>$S_{i2}$</td>
<td>$P_{air,start} + P_{H2O,start}$; for gas phase only (further gases with initial partial pressures of $1 \cdot 10^{-4}$ bar)</td>
</tr>
</tbody>
</table>
A – Operation of the leach bed reactors

Organic loading rates

Figure III-A1 depicts the complete overview of the organic loading rates (OLR) for the leach bed reactor (LBR) during the investigation period.

Figure III-A1: OLRs for the LBR$_{01}$ (white circles), LBR$_{02}$ (gray squares) and LBR$_{03}$ (black triangles) and the average for the three LBRs (black line). The gray line represents a corrected average of the OLRs for the real hydraulic retention times considering the weeks without feedings (days 197 and 204).
Substrate distribution and digestate stratification

In Figure III-A2a it is possible to observe how the substrate stacked vertically within the LBRs. This occurred especially at the OLR-S3. This substrate stacking might lead to a heterogeneous substrate degradation, as illustrated by the digestate stratification in Figure III-A2b.

Figure III-A2: a) Leach bed reactor (LBR) fed with 520 g\textsubscript{VS} maize silage (1.7 kg fresh mass); b) Digestate removed from a LBR fed with 400 g\textsubscript{VS} maize silage (1.3 kg fresh maize and additionally 0.3 kg digestate from the previous cycle) after 21 day retention time.
B – Additional results from the two-stage experimental plant operation

*Leach bed reactor biogas production*

Figures III-B1 and III-B2 gives the daily gas production and the contents of methane, carbon dioxide and hydrogen. The similarity in the gas flow and gas composition dynamics between the three LBRs is evident. The simultaneous drop of both CO$_2$ and CH$_4$ contents after feeding resulted from atmospheric oxygen and nitrogen gases that entered the LBR through the reactor opening.

![Graph of daily biogas production and composition for LBRs OLR-S1, OLR-S2, and OLR-S3.](image)

**Figure III-B1:** Daily data for biogas production and composition for the LBR$_{01}$ (white circles and dashed black line), LBR$_{02}$ (gray squares and lines) and LBR$_{03}$ (black triangles and lines). a) Biogas production; b) methane contents. For a summary of this data see Figure 3-2 and Table 3-4.
Figure III-B2: Daily data for the biogas composition for the LBR-01 (white circles and dashed black line), LBR-02 (gray squares and lines) and LBR-03 (black triangles and lines). a) Carbon dioxide contents; (b) hydrogen contents. For a summary of this data see Table 3-4.
**Upflow anaerobic filter biogas production**

Figure III-B3 shows the gas production and composition in the UAF. It is possible to recognize that the problems in the overflow from the LBRs to STR\textsubscript{UAF} affected the UAF gas output (Figure III-B3a). The calculated average methane production curves from Figure 3-2 does not include the weeks in which disturbances were identified. Besides the acidification period (between days 176 and 196), the gas composition in the UAF stayed mostly invariant (Figures III-B3b and III-B4).

![Figure III-B3: Daily data for the biogas production and composition in the UAF. a) Biogas production, arrows identify operational problems which resulted in a reduced recirculation between the LBRs and the STR\textsubscript{UAF}; b) methane (white circles) and carbon dioxide (gray squares) contents. For a summary of this data see Figure 3-2 and 3-4 and Table 3-4.](image)

**Figure III-B3:** Daily data for the biogas production and composition in the UAF. a) Biogas production, arrows identify operational problems which resulted in a reduced recirculation between the LBRs and the STR\textsubscript{UAF}; b) methane (white circles) and carbon dioxide (gray squares) contents. For a summary of this data see Figure 3-2 and 3-4 and Table 3-4.
Figure III-B4: Daily data for the biogas composition in the UAF. a) Hydrogen gas (H\(_2\)); b) Hydrogen sulphide gas (H\(_2\)S). For a summary of this data see Figure 3-2 and 3-4 and Table 3-4.
pH and conductivity data

Figure III-B5 shows the pH values for the UAF and from the mixed solution from the three LBRs (i.e. the inflow to the UAF) as well as the correspondent conductivity values. Chapter 3 includes in detail a discussion of the pH values, while the conductivity values are not mentioned. Nevertheless, the continually increase in the conductivities corroborates with the accumulation of ammonium nitrogen and COD observed along this period (see Figure 3-4).

**Figure III-B5:** Daily data for pH and conductivity in the UAF (white circles) and in the leachate mixture from the three LBRs (gray triangles). a) pH; b) conductivity. For a summary of the pH data see Figure 3-3.
**Control continuously stirred tank reactor methane production**

Figure III-B5 shows the average weekly CH₄ production for the control CSTR at the OLR-S1, OLR-S2 and OLR-S3.

![Graph showing average weekly CH₄ production for control CSTR](image)

**Figure III-B6:** Weekly dynamics for the CSTR during OLR-S1 (white bars and circles), OLR-S2 (gray bars and squares) and OLR-S3 (black bars and triangles). Error bars indicate the 95% confidence intervals.

**C – UAF pictures: tracer experiment and batch assay preparation**

**Tracer experiment**

Figure III-C1 summarizes the results of a qualitative tracer experiment with fluorescein. In Figure III-C1a it is possible to visualize the fluorescein distribution over a period of 20 min. A flow plume accompanies the hot water flow from the heating jacket. In Figure III-C1b some detailed views are given to illustrate this preferential flow near the external walls. In addition, the outflow tube of the reactor is located asymmetrically, hence enhancing the non-uniformity of the inflow distribution within the UAF. Nevertheless, the sludge bed formation is likely to increase the mixture of the fluid substrate. A spatial distribution correlation for biofilm biomass properties was not identified in these investigations.
Figure III-C1: Visualization from tracer experiments with fluorescein in the UAF without biofilm carriers. a) Formation of a tracer plume (upper left of the UAF in fourth picture); b) detail views of the preferential tracer flow near the reactor external walls.

Pictures of the batch assays with biofilm carriers

Figure III-C2 shows the preparation of batch assays with a biofilm carrier and a finished assays vessel with a biofilm carrier. Figure III-C2c shows the biofilm carrier holding system during the UAF operation.

Figure III-C2: Preparation of batch assays with biofilm carriers. a) Cut of the biofilm carrier inside a batch assay vessel; b) closed batch assay vessel with a biofilm carrier; c) reposition of the removed biofilm carriers with new PVC strips (4 strips in total).

Figure III-C3 shows pictures of four biofilm carries prior to their utilization for batch assay preparation used for documentation. Figure III-C2a shows the damages on the biofilm caused through an inadequate biofilm carrier removal, resulting in the lowest biofilm densities, $24 \text{ g}_{\text{XMB,COD}} \text{ m}^{-2}$, of the assays with 3 % labeled acetate.
Figure III-C3: Example of biofilm carrier’s (BC) previous to their utilization for the batch assay preparation. a) BC D-II-2; b) BC D-II-3; c) BC A-III-2; d) BC D-III-3.
**D – Additional results for the $^{12}\text{C}^{13}\text{C}$ Model**

**$^{13}\text{C}$ mass balance**

Figure III-D1 presents the dynamic distribution of the $^{13}\text{C}$ mass for assays with labeled and unlabeled acetate. The total $^{13}\text{C}$ masses varies according to the total liquid volume of the assays (e.g., 160 and 100 mL, for diagrams a and b, respectively) and the amounts of labeled acetate added. These calculations are especially relevant for the design of experiments to support the dosage of labeled substrates. Moreover, this data confirms the mass conservation within the $^{12}\text{C}^{13}\text{C}$-Modell simulations, i.e. the total $^{13}\text{C}$ mass is constant.

**Figure III-D1:** Mass balance for the $^{13}\text{C}$ isotopes including the total (dotted black lines), the liquid phase (continuous black line) and the gas phase (continuous gray line) masses. The amounts of $^{13}\text{C}$ added as [2-$^{13}\text{C}$]-acetate are indicated by the dashed black lines. a) Sludge assays with low acetate concentrations; b) sludge assays with high acetate concentrations; c) biofilm assays with high acetate concentrations and with a gas sampling after 0.92 ($^{13}\text{C}$ outflow indicated by the gray dashed line); d) Sludge assays with high acetate concentrations and without labeled acetate dosage.
**Gas exchange coefficient**

Figure III-D2 depicts the difference from the utilization of a dynamic and constant gas exchange coefficients ($k_{L,a}$), upper and lower diagrams, respectively. Although the initial pressure increase can be adequately described through constant $k_{L,a}$ values, it overestimates the partial pressures when the gas production rates are very low (around day 3 in the Figure III-D2). A calibration with a constant $k_{L,a}$ for the low and high rates gas production periods was not possible, as exemplified for the $k_{L,a}$ values ranging from 200 to 800 $d^{-1}$ (Figure III-D2c,d). A dynamic $k_{L,a}$ based on the upflow gas flow velocity according to Merkel and Krauth (1999) allowed for a better fitting of the measured pressures in the batch assays vessels.

**Figure III-D2:** Measured (white circles) and simulated partial pressures with different gas exchange coefficients ($k_{L,a}$): dynamic $k_{L,a}$ according to Merkel and Krauth (1999; black continuous lines), $k_{L,a} = 400$ $d^{-1}$ (gray continuous lines), $k_{L,a} = 600$ $d^{-1}$ (gray dashed lines) and $k_{L,a} = 800$ $d^{-1}$ (gray continuous lines). a,c) Batch assays with a BC at low acetate concentrations; b,d) Batch assays with UAF sludge at low acetate concentrations.
Calculation of thermodynamic constraints

The $^{12}$C$^{13}$C-Modell also accounts for the calculation of Gibbs energy change ($\Delta G_R$) values (Equations 2-17 and 2-18). Nonetheless, as discussed in Section 3.2.1 and 3.2.2, the determination of the hydrogen (H$_2$) availability to the microorganism on basis of (H$_2$) partial pressure ($p_{H2}$) data is challenging. Thus, a high uncertainty on the calculated $\Delta G$ values exits. Still, the identification of the thermodynamics constraints is an important feature of the $^{12}$C$^{13}$C-Model, especially for further investigations in systems with H$_2$ accumulation. Figure III-D3 shows $\Delta G$ values together with the simulated $p_{H2}$ for assays with low and high acetate concentrations, left and right diagrams respectively. $\Delta G$ were directly calculated with the simulated $p_{H2}$ (Figure III-D3c,d) and with the same oversaturation factor of 20 (Equation 3-3) from Pauss and Guiot (1993) utilized for the UAF reactor (Figure III-D3e,f). The syntrophic acetate oxidation (SAO) $\Delta G$ values derived from both syntrophic processes, acetate oxidation and hydrogenotrophic methanogenesis, i.e. $\Delta G_{SAO}=\Delta G_{ca}^{+}\Delta G_{mc}$.

The $p_{H2}$ values were in the same range of the measured data for the UAF reactor. A gas sampling for the high acetate concentration assay (Figure III-D3b) at the time point 0.92 d did not significantly affect the $p_{H2}$ values. The hydrogenotrophic methanogenesis was found under feasible thermodynamically constraints, values lower than -20 kJ reaction$^{-1}$ Schink (1997), for calculations only with the oversaturation factor. On the contrary, the acetate oxidation alone becomes rapidly thermodynamically unfeasible when using the $p_{H2}$ oversaturation factor. Nevertheless, the $\Delta G$ for the SAO is not affected by this $p_{H2}$ variations. In fact, the SAO and the acetoclastic methanogenesis have virtually the same $\Delta G$ values.
Figure III-D3: Simulated hydrogen partial pressures ($p_{H_2}$) and Gibbs energy change ($\Delta G_R$) values for the acetoclastic methanogenesis (black continuous lines), hydrogenotrophic methanogenesis (black dashed lines), acetate oxidation (gray dashed lines) and for the syntrophic acetate oxidation (gray continuous lines). a,c,e) Simulation results for a biofilm carrier assay with low acetate concentrations; b,d,f) simulation results for a biofilm carrier assay with high acetate concentrations. Analogously to calculations for Figure 3-10 an oversaturation factor of 20 was utilized to determine the $\Delta G_R$ values for e and f.
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PEER-REVIEW PUBLICATIONS

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2013
Weitergehende Spurenstoffelimination mittels dynamischer Rezirkulation auf der Kläranlage Schwerte - GWF-Wasser Abwasser (Keysers, C., Grünebaum, T., Thölke, D., Lübken, M., Türk, J., Gehring, T., Pinnekamp, J., Wichern, M.)

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