Factors affecting constancy of acetate concentration and correct determination of methanogenic activity in pH-stat experiments

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Abstract The determination of methanogenic activity with a pH-stat titration bioassay is evaluated utilising a mathematical model of this system. For given kinetic parameters and experimental conditions the model calculates the development of titrant flow and acetate concentration during experiments. Simulations of experiments under various conditions are compared. They show that the original method inherently causes a strong drift of acetate concentration during the experiments and a misestimation of methanogenic activity. As a solution to these disadvantages the addition of sodium hydroxide to the titrant and a careful control of pH during flushing the reactor with gas prior to the experiment are recommended. In this way a better constancy of acetate concentration and a more accurate determination of methanogenic activity should be achievable. The accuracy of this method is limited by the stability of pH-electrode calibration parameters.

Keywords Anaerobic digestion model; CO₂ supersaturation; methanogenic activity; pH; titration biosensor

Introduction
In a former issue of Water Science and Technology, Rozzi et al. (2001) presented a pH-stat titration bioassay for measurement of methanogenic activity. The basic principle of this assay is the following: after adding acetate to a sludge sample, the pH tends to increase (because of the consumption of acetic acid). However, in a pH-stat reactor the pH is kept constant by computer-controlled addition of acetic acid. The amount of acetic acid added corresponds to the amount of acetate consumed, and thus, to the methanogenic activity. By logging the data (i.e. flow of acetic acid) to the computer, a continuous on-line monitoring of activity is achieved.

This property makes the method particularly suited for the investigation of dynamic effects after the addition of toxicants. An eventual increase of decay rate after toxicant addition, as reported by Mösche and Meyer (2002), or the effect of inhibitors on the bacterial growth rate can be quantified, if a pH-stat experiment is run during a few days. A basic condition for such experiments is that the acetate concentration does not drift into the ranges of either substrate limitation or substrate inhibition.

Based on simplified mass balances Rozzi et al. (2001, 2002) postulated that the acetate concentration should remain constant during a pH-stat experiment of short duration, but a certain drift was expected in experiments lasting several days. In the present study a mathematical model of the pH-stat reactor is set up in order to find out the factors that might cause a drift of acetate concentration and to evaluate their impact. Finally, suggestions for an improved experimental procedure shall be given.

Mathematical model
Liquid phase
Mass balances of a Fed-Batch Reactor are applied. This implies that the liquid volume, \( V_{\text{liq}} \), is treated as a separate dynamic state variable (Eq. (1)). The differential equations for the concentrations, \( c \), (Eq. (2)) resemble those for a CSTR. The only difference is that the
output term in the mass balance of the CSTR has the meaning of a dilution term in the Fed-Batch Reactor (Bailey and Ollis, 1986).

$$\frac{d V_{\text{liq}}}{d t} = q_{in}$$  (1)

$$\frac{d c_{i,\text{liq}}}{d t} = \frac{q_{in}}{V_{\text{liq}}} \left( c_{i,\text{in}} - c_{i,\text{liq}} \right) + r_i$$  (2)

with: $q_{in} =$ inflow to the reactor = titrant flow

The state components, $i$, of the liquid phase are: acetate, $\text{Ace}$; inorganic carbon, $\text{IC}$; methane, $\text{CH}_4,\text{liq}$; ammonium, $\text{NH}_4^+$ and biomass, $X$. Further variables are dissolved carbon dioxide, $\text{CO}_2,\text{liq}$; and hydrogen carbonate, $\text{HCO}_3^-$. The Anaerobic Digestion Model No.1 (ADM1; Batstone et al., 2002) was employed to describe the corresponding reaction rates, $r_i$. Among the various processes included in the ADM1 only the processes “Uptake of Acetate”, “Decay of $X_{\text{ac}}$”, “Acid-Base Equilibrium of $\text{CO}_2$”, “Liquid-Gas Transfer of $\text{CO}_2$” and “Liquid-Gas Transfer of $\text{CH}_4$” were used.

**Gas phase**

It is assumed that the liquid-gas transfer occurs rather between the liquid and the gas bubbles dispersed therein than between the liquid and the headspace. Therefore, the gas volume, $V_{\text{gas}}$, is not set to the headspace volume, but to $0.005 \cdot V_{\text{liq}}$, an estimate of the total volume of all gas bubbles. Apart from this, the simulation of the $\text{CO}_2$ and $\text{CH}_4$ content in the gas bubbles is performed as described in Batstone et al. (2002).

**Model parameters**

The model parameters used for the simulations are given in Table 1. The values of the kinetic parameters $\mu_{\text{max}}$, $Y_{\text{XS}}$, $K_{S,\text{Ace}}$ and $k_{\text{dec}}$ were determined in our lab for an enrichment culture of acetoclastic methanogens.

In order to correctly simulate the liquid-gas mass transfer under dynamic conditions the mass transfer coefficient $k_{La}$ is assumed to be linearly correlated to the gas flow rate (approximation based on data of Merkel and Krauth, 1999). Since the gas flow rate is proportional to the acetate uptake rate, $r_{\text{ace}}$, and the liquid volume, $V_{\text{liq}}$, a simple formula (Eq. (3)) can be used to simulate dynamic changes of the mass transfer coefficient. Here, $r_{\text{ace}}^*$ is the reference acetate uptake rate given by the initial biomass concentration, $X_0$, the

### Table 1  Input parameters for the model

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Ace}_0$</td>
<td>500 mg Cod l$^{-1}$</td>
<td>$K_{\text{H,CO}_2}$</td>
<td>25 mmol l$^{-1}$ bar$^{-1}$</td>
<td>$p_{\text{H}_20}$</td>
<td>62 mbar</td>
</tr>
<tr>
<td>$\text{Ace}_{\text{in}}$</td>
<td>64 g COD l$^{-1}$</td>
<td>$k_{\text{a}}$</td>
<td>0.02 d$^{-1}$</td>
<td>$p_{\text{K}_{\text{a},\text{CO}_2}}$</td>
<td>6.2</td>
</tr>
<tr>
<td>$\text{CH}_4,\text{gas},_0$</td>
<td>50%</td>
<td>$K_{S,\text{ace}}$</td>
<td>10 mg COD l$^{-1}$</td>
<td>$V_{\text{g},0}$</td>
<td>1.8</td>
</tr>
<tr>
<td>$\text{CH}_4,\text{liq},_0$</td>
<td>36 mg COD l$^{-1}$</td>
<td>$K_{\text{H}_4}$</td>
<td>3 mmol l$^{-1}$</td>
<td>$V_{\text{reactor}}$</td>
<td>2.8</td>
</tr>
<tr>
<td>$\text{CO}_2,\text{gas,}_0$</td>
<td>50%</td>
<td>$N_{\text{H}_4}$</td>
<td>15 mmol l$^{-1}$</td>
<td>$X_0$</td>
<td>500 mg COD l$^{-1}$</td>
</tr>
<tr>
<td>$\text{IC},_0$</td>
<td>see Eq. (14)</td>
<td>$p_{\text{H}_20}$</td>
<td>7.00</td>
<td>$Y_{\text{XS}}$</td>
<td>0.03 g COD gCOD$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{dec}}$</td>
<td>0.02 d$^{-1}$</td>
<td>$p_{\text{am}}$</td>
<td>1.013 bar</td>
<td>$\mu_{\text{max}}$</td>
<td>0.14 d$^{-1}$</td>
</tr>
</tbody>
</table>

$k_{\text{dec}}$: decay rate, $K_{\text{H}}$: Henry’s law coefficient, $k_{\text{a}}$: see Eq. (3), $K_{S,\text{ace}}$: half saturation value, $p_{\text{K}_{\text{a},\text{CO}_2}}$: dissociation constant, $p_{\text{am}}$: atmospheric pressure, $p_{\text{H}_20}$: vapour pressure of H$_2$O, $T$: temperature, $Y_{\text{XS}}$: biomass yield, $\mu_{\text{max}}$: maximum growth rate. Indices: 0: initial concentration, in: influent concentration, gas: gas phase, liq: liquid phase.
maximum growth rate, $\mu_{\text{max}}$, and the biomass yield, $Y_{\text{XS}}$. $k_L a^*$ is the hypothetical value of $k_L a$ at the beginning of the experiment, assuming maximal activity of the biomass.

\[
k_L a = k_L a^* \cdot \frac{r_{\text{ace}}}{r_{\text{ace},0}} \cdot \frac{V_{\text{liq}}}{V_{\text{liq},0}} = k_L a^* \cdot \frac{r_{\text{ace}} Y_{\text{XS}}}{X_0 \cdot \mu_{\text{max}}} \cdot \frac{V_{\text{liq}}}{V_{\text{liq},0}}
\]

(3)

**Calculation of titrant flow**

The idea of pH-stat experiments is that the titrant flow, $q_{\text{in}}$, is not an independent variable, but the result of the experiment representing the biological activity. Thus, the titrant flow has to be calculated as an output of the model. This is achieved by setting up the proton balance:

\[
\frac{d H^+}{dt} = \text{Inflow} - \text{Dilution} + \text{Biochemical Reaction} + \text{CO}_2 \text{ Transfer Effect} = 0
\]

(4)

The inflow of protons results from the acetic acid in the titrant, $HA^e_{\text{in}}$, and is eventually reduced by sodium hydroxide added to the titrant, $NaOH_{\text{in}}$. The dilution is proportional to the actual $H^+$ concentration in the liquid phase, $H^+_{\text{liq}}$.

\[
\text{Inflow} - \text{Dilution} = \frac{q_{\text{in}}}{V_{\text{liq}}} \cdot (H^+_{\text{in}} - H^+_{\text{liq}}) = \frac{q_{\text{in}}}{V_{\text{liq}}} \cdot (HA^e_{\text{in}} \cdot C_{\text{Ace}} - NaOH_{\text{in}} - H^+_{\text{liq}})
\]

(5)

The factor $C_{\text{Ace}}$ (carbon content of acetate = 1/64 mole g COD$^{-1}$) is needed for the conversion between the concentrations of organics and inorganics (g COD$^{-1}$ to mol$^{-1}$).

For the biochemical reaction the stoichiometric equation (6) is assumed:

\[
\text{CH}_3\text{COO}^- + 0.4 \cdot Y_{\text{XS}} \text{NH}_4^+ + (0.6 \cdot Y_{\text{XS}} + \alpha (1 - Y_{\text{XS}})) H^+ + (1 - 2.2 \cdot Y_{\text{XS}} - \alpha (1 - Y_{\text{XS}})) \text{H}_2\text{O} \rightarrow
\]

\[
(1 - Y_{\text{XS}}) \text{CH}_4 + \alpha (1 - Y_{\text{XS}}) \text{CO}_2 + (1 - \alpha) (1 - Y_{\text{XS}}) \text{HCO}_3^- + 0.4 \cdot Y_{\text{XS}} \text{C}_5\text{H}_7\text{O}_2\text{N}
\]

(6)

where $\alpha$ is the fraction of $\text{CO}_2$ among the total inorganic carbon:

\[
\alpha = \frac{CO_{2,\text{liq}}}{CO_{2,\text{liq}} + HCO_{3,\text{liq}}} = \frac{H^+_{\text{liq}}}{H^+_{\text{liq}} + K_{\alpha,\text{CO}_2}}
\]

(7)

Consequently, the contribution of the biochemical reaction to the proton balance is:

\[
\text{Reaction} = r_{\text{Ace}} \cdot C_{\text{Ace}} \cdot \left(0.6 \cdot Y_{\text{XS}} + \alpha \cdot (1 - Y_{\text{XS}})\right)
\]

(8)

The mass transfer of $\text{CO}_2$ from the liquid to the gas phase consumes protons according to:

\[
\alpha \ CO_{2,\text{liq}} + (1 - \alpha) \ HCO_3^- + (1 - \alpha) \ H^+ \leftrightarrow \ CO_{2,\text{gas}} + (1 - \alpha) \ H_2\text{O}
\]

(9)

The corresponding rate of proton consumption is:

\[
\text{CO}_2 \text{ Transfer Effect} = -(1 - \alpha) \cdot k_L a \cdot \left(CO_{2,\text{liq}} - CO_{2,\text{eq}}\right)
\]

(10)

Thus, the complete proton balance is:

\[
\frac{q_{\text{in}}}{V_{\text{liq}}} \cdot \left(HAce_{\text{in}} \cdot C_{\text{Ace}} - NaOH_{\text{in}} - H^+_{\text{liq}}\right) - \left(0.6 \cdot Y_{\text{Ace}} + \alpha \cdot (1 - Y_{\text{Ace}})\right) \cdot r_{\text{ace}} \cdot C_{\text{Ace}}
\]

\[
-(1 - \alpha) \cdot k_L a \cdot \left(CO_{2,\text{liq}} - CO_{2,\text{eq}}\right) = 0
\]

(11)

This can be resolved to the titrant flow, $q_{\text{in}}$:
Implementation

The simulations were performed with a MATLAB program, using the subroutine “ode15s.m” a variable order solver for stiff sets of ordinary differential equations.

Results and discussion

Simulation of the original procedure

An experiment according to the original procedure of Rozzi et al. (2001) was simulated: the titrant was assumed to consist of 1 mol l\(^{-1}\) acetic acid, the pH was set to 7.00, the initial concentration of acetate was 500 mg COD l\(^{-1}\), and the initial concentration of inorganic carbon was assumed to be in equilibrium with 50% CO\(_2\) in the gas phase. The simulation (Figure 1) shows that the acetate concentration in the reactor drops to zero within 1.5 days. Following the model of Rozzi et al. (2001) the titrant flow should be directly proportional to the acetate degradation rate, \(r_{\text{Ace}}\), according to:

\[
q_{\text{in}} = V_{\text{liq}} \cdot \frac{(0.6 \cdot Y_{\text{Ace}} + \alpha \cdot (1 - Y_{\text{Ace}})) \cdot r_{\text{Ace}} \cdot C_{\text{Ace}} + (1 - \alpha) \cdot k_{\text{L}} \cdot \alpha \cdot (C_{O2,\text{liq}} - C_{O2,\text{eq}})}{H_{\text{Ace}} \cdot C_{\text{Ace}} - NaOH_{\text{in}} - H_{\text{liq}}^+} \tag{12}
\]

Liquid-gas equilibrium of CO\(_2\)

According to the procedure of Rozzi et al. (2002), the liquid phase is flushed with an equimolar mixture of CO\(_2\)/N\(_2\) before the experiment. Because of the CO\(_2\) stripping or dissolution, the pH changes during flushing until an equilibrium is reached. The resulting concentration of inorganic carbon in the liquid phase depends strongly on the final pH after flushing (\(pH_{\text{flush}}\)):

\[
IC_0 = CO_{2,\text{gas,flush}} \cdot (P_{\text{am}} - P_{\text{H2O}}) \cdot K_{H,CO2} \cdot \left(1 + 10^{(pH_{\text{flush}} - pK_a,CO2)}\right) \tag{14}
\]

Only if \(pH_{\text{flush}}\) is equal to the pH during the titration experiment, \(pH_{\text{titr}}\), are gas and liquid phases in equilibrium at the beginning of the experiment. However, in practice there will be a certain deviation, so that the pH has to be adjusted by addition of HCl or NaOH. In case of \(pH_{\text{flush}} > pH_{\text{titr}}\) the result is a supersaturation of the liquid phase with CO\(_2\) at the beginning.
of the experiment, and when $pH_{\text{flush}} < pH_{\text{titr}}$ the liquid phase is not fully saturated. The release of CO$_2$ from a supersaturated liquid phase would consume protons (cf. Eq. (9)), which, in turn, would simulate a consumption of acetic acid. In this case, more acetic acid would be added than consumed and consequently the acetate concentration would increase.

Figure 2 shows the effect of varying $pH_{\text{flush}}$ on the development of acetate concentration during the experiment ($pH_{\text{titr}} = 7.00$, $Ace_{in} = 1$ mol l$^{-1}$). Yet a few hundredth of pH have a strong impact. As expected from the reasoning above, high values of $pH_{\text{flush}}$ (supersaturation of CO$_2$,liq) cause the acetate concentration to increase during the first day. However, this effect lasts only for about 2 days. Then CO$_2$,liq reaches equilibrium with the gas phase. Subsequently, all curves show the same decreasing trend. Lower values of $pH_{\text{flush}}$ are compensated by this trend, and thus, no increase of acetate concentration is found. When $pH_{\text{flush}} < pH_{\text{titr}} = 7.0$, the decrease of acetate concentration is particularly fast. Figure 3 shows the effect of varying $pH_{\text{flush}}$ on the titrant flow. After the initial lag-phase, the titrant flow stabilises at clearly different levels depending on $pH_{\text{flush}}$, demonstrating the strong interference of the pH during previous gas flushing with the calculation of methanogenic activity from the titrant flow. This applies for both short and long term experiments.

**Reaching constant acetate concentration**

The previous section illustrates the adverse effects of too high or too low CO$_2$ saturation at the beginning of the experiment. In order to reach a steady-state of CO$_2$ concentration, in fact, a certain supersaturation of CO$_2$ in the liquid phase is needed, because a concentration gradient is necessary for liquid-gas mass transfer. According to the simulations, such a steady-state can be reached very fast, if $pH_{\text{flush}}$ is 7.04. Nevertheless, the acetate concentration decreases during an experiment under such conditions (Figure 2, curve for $pH_{\text{flush}} = 7.04$). The reason for this behaviour is the following: the titrant added to the reactor does not contain any inorganic carbon. In the reactor, this volume is saturated with the CO$_2$ produced. Because of the dissociation there is a constant production of protons. This causes the titration algorithm to dose less acetic acid than necessary, which, in turn, leads to the decrease of acetate concentration.

$$CO_2,\text{liq} + H_2O \rightarrow HCO_3^- + H^+ \quad (15)$$

This effect can be prevented, if a certain amount of sodium hydroxide is added to the titrant (with the concentration $NaOH_{in}$) in order to neutralise the protons produced according to Eq. (15). Figure 4 shows the simulation of an experiment with $pH_{\text{flush}} = 7.04$ and an addition of 90 mmol l$^{-1}$ NaOH to the titrant. It can be seen that after a “lag phase” the simulated titrant flow reaches the level of the expected titrant flow according to Eq. (13). This means that the methanogenic activity would be calculated without systematic error. The acetate concentration remains constant. This is not affected by the circumstance that the bacteria are growing in the reactor. The “lag phase” of the titration rate is due to dynamic changes of
the CO₂ content of the gas phase after the onset of the biochemical reaction. This can only be avoided, if the reaction is already running before the start of the experiment.

While it is no great effort to add 90 mmol l⁻¹ of NaOH to the titrant, the precise adjustment of pH 7.04 during gas flushing is rather cumbersome (because the flushing itself influences the pH). An alternative would be to operate the titration reactor for about 3 days until the CO₂ equilibrium is established. After correcting the acetate concentration to the desired initial value, the actual titration experiment could then be started.

Factors influencing the optimal values for pH_flush and NaOH_in

As shown above, constancy of acetate concentration and a correct determination of methanogenic activity can be achieved with pH_flush = 7.04 and NaOH_in = 90 mmol l⁻¹. However, these values cannot be regarded as constants. They are dependent on various other factors. Probably, the most important among these is the mass transfer coefficient k_La. This is, in turn, dependent on gas production, reactor geometry, mixing conditions, and physical properties of the liquid phase. For the simulation a value of k_La* = 20 d⁻¹ was chosen. This value fitted best the experimental data obtained in our lab with a 2-1 titration reactor (glass vessel with a magnetic stirrer, 500 rpm). Moreover, the simulated supersaturation of CO₂ in the liquid phase in the steady state is about 10%, which agrees very well with the observations of Merkel and Krauth (1999).

The ratio of k_La to the maximum acetate degradation rate, r_ace, is the relevant variable influencing the result of the simulations rather than k_La itself. The influence of this ratio on the optimal values for pH_flush and NaOH_in is plotted in Figure 5. It can be seen that lower values of k_La (corresponding to slow stirrer speed) have a strong influence on both parameters. In this case, it would be important to have exactly the same stirrer speed in various experiments to achieve reproducibility. At higher k_La values, this is less important.

Another factor of influence is the atmospheric pressure, since it determines the liquid-gas equilibrium of CO₂. For the simulations, p_atm was assumed 1,013 mbar, corresponding to sea level. For experiments performed at higher altitude, the reduced atmospheric pressure has to be considered, using the following equations:

\[
p_{\text{atm}} \times \frac{k_{L,a}}{r_{\text{ace,max}}} = \frac{p_{\text{H}_2\text{O}}}{p_{\text{CO}_2}}
\]

Figure 4 Simulation of acetate concentration and titrant flow during an experiment with NaOH_in = 90 mM and pH_flush = 7.04

Figure 5 Influence of k_La and r_ace,max on the optimal values for pH_flush and NaOH_in. The vertical line indicates the values used for the simulations

Figure 6 Simulation of acetate concentration during an experiment with varying atmospheric pressure

Figure 7 Simulation of acetate concentration and titrant flow during an experiment with a drift of electrode calibration parameters
This dependency also causes a certain susceptibility of the pH-stat to changes of the weather situation. Figure 6 shows a simulation of an experiment considering atmospheric pressure data as input for the simulation. The resulting drift of acetate concentration is rather small (60 mg COD l⁻¹ in 2 days). The simulated titrant flow was on average 1.5% higher than the expected flow. This would lead to a slight error in the estimation of methanogenic activity.

Another potential source of errors is the instability of pH-electrodes. For high-precision measurements, pH-electrodes have to be calibrated immediately before the measurement. However, during a titration experiment this is not possible. It is rather unlikely that an electrode will keep the same calibration parameters during several days in a medium containing sulphide, proteins and particles (bacteria), all of which interfere with the diaphragm of a pH-electrode. Investigations in our lab have shown that a drift of the zero potential (electrode signal in a pH 7 buffer) can be up to 1 mV d⁻¹. This case was simulated, assuming that the zero potential of the electrode changes from 0 mV at the beginning to +3 mV at the end of the experiment. Although the pH-meter would display 7.00 throughout the experiment, the actual pH drifts from 7.00 to 7.05. As shown in Figure 7 the consequence is a significant drift of acetate concentration. After the “lag-phase”, the simulated titrant flow reaches almost the expected value, but then it deviates from the expected curve. At the end of the experiment, there is a difference of 5% between the two curves. The growth rate estimated from the increase of the simulated titrant flow is only 0.08 d⁻¹, compared to the theoretical value of μ_max – k_dec = 0.12 d⁻¹.

Conclusions
The pH-stat system appears to be much more complex than it seems at first sight. The original method of Rozzi et al. (2001; 2002) inevitably leads to a drift of acetate concentration during the experiments. A possible solution to this problem would be to start the experiments with very high acetate concentrations. However, such conditions are not representative for the normal environment of methanogenic sludges, and thus, unfavourable for an activity assay. A second disadvantage of the original method is the erroneous estimation of methanogenic activity.

As an effective solution for both problems, we suggest the addition of sodium hydroxide to the titrant and the careful control of pH during flushing the reactor with gas. The necessary concentration of NaOH in the titrant and the pH during gas flushing are slightly dependent on mass transfer characteristics of the reactor and the local atmospheric pressure. The exact values can be assessed from Figure 5 and Eqs (16) and (17).

Nevertheless, a perfect constancy of acetate concentration cannot be obtained due to the insufficient stability of pH-electrode calibration parameters. However, compared to the original procedure, the error in the determination of methanogenic activity is greatly reduced. At least for short-term determination of methanogenic activity, the modified procedure appears well applicable, whereas long-term experiments like the determination of bacterial growth rate are prone to errors. Although no experimental data are shown in this paper, the general trends of the model predictions have been verified in experiments (unpublished data).

\[
pH_{\text{flush}} = 7.036 + 9.22 \cdot 10^{-5} \cdot (1013 \, \text{mbar} - p_{\text{atm}}) \tag{16}
\]

\[
NaOH_{\text{in}} = 90.0 - 0.0715 \cdot (1013 \, \text{mbar} - p_{\text{atm}}) \tag{17}
\]
References