Brief report

Toxicity of long chain fatty acids towards acetate conversion by Methanosaeta concilii and Methanosarcina mazei

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Summary
Long-chain fatty acids (LCFA) can inhibit methane production by methanogenic archaea. The effect of oleate and palmitate on pure cultures of Methanosaeta concilii and Methanosarcina mazei was assessed by comparing methane production rates from acetate before and after LCFA addition. For both methanogens, a sharp decrease in methane production (> 50%) was observed at 0.5 mmol L⁻¹ oleate, and no methane was formed at concentrations higher than 2 mmol L⁻¹ oleate. Palmitate was less inhibitory than oleate, and M. concilii was more tolerant to palmitate than M. mazei, with 2 mmol L⁻¹ palmitate causing 11% and 64% methanogenic inhibition respectively. This study indicates that M. concilii and M. mazei tolerate LCFA concentrations similar to those previously described for hydrogenotrophic methanogens. In particular, the robustness of M. concilii might contribute to the observed prevalence of Methanosaeta species in anaerobic bioreactors used to treat LCFA-rich wastewater.

Introduction
Long-chain fatty acids (LCFA) are released during lipid hydrolysis, and hold the majority of the energy potential of these biomolecules (Alves et al., 2009). LCFA are degraded by anaerobic bacteria through β-oxidation to form acetate and hydrogen, which are then used by acetoclastic and hydrogenotrophic methanogens to produce methane. In anaerobic bioreactors, approximately 70% of the methane produced from LCFA results from acetoclastic activity, whereas about 30% derives from hydrogenotrophic activity (Sousa et al., 2009). Low methane production has been reported during continuous bioreactor operation with LCFA, which was associated with inhibition and toxicity of these compounds towards the methanogenic communities (Chen et al., 2014; Dereli et al., 2014).

Studies on the toxic effect of LCFA on the acetoclastic and hydrogenotrophic activities of anaerobic sludge indicate that acetoclastic methanogens are the most sensitive to LCFA (Alves et al., 2009; Palatsi et al., 2010). Nevertheless, results obtained in our research group showed a significant increase in the relative abundance of methanogens in anaerobic sludge exposed to continuous feeding of oleate (C₁₈:₁) and palmitate (C₁₆:₀) followed by batch incubation (Sousa et al., 2007). Endurance of acetoclastic methanogens in a continuous bioreactor treating LCFA-rich effluent, at organic loading rates up to 21 kg m⁻³ day⁻¹, has also been reported (Salvador et al., 2013). Additionally, activity of acetoclastic methanogens in sludge incubated with LCFA in batch assays has been shown, as more than 80% of the proteins assigned to the archaeal community were from Methanosaeta concilii (Salvador, 2013). The prevalence of acetoclastic methanogens belonging to Methanosaeta and Methanosarcina genera in LCFA-degrading environments has been reported in other studies (Shigematsu et al., 2006; Palatsi et al., 2010; Baserba et al., 2012; Ma et al., 2015), suggesting some controversy in the reported sensitivity of acetoclastic methanogens to LCFA.

In lipid-containing wastewaters, oleate (C₁₈:₁) is generally the most abundant LCFA, and palmitate (C₁₆:₀) tends to accumulate in anaerobic bioreactors treating...
these effluents (Pereira et al., 2002; Dereli et al., 2014). The effect of LCFA on anaerobic sludge has been studied before (Sousa et al., 2007; Palatsi et al., 2010; Silva et al., 2014), and a few studies report the sensitivity of pure cultures of hydrogenotrophic methanogens (Sousa et al., 2013; Zhou et al., 2013). Information on the sensitivity of pure cultures of aceticlastic methanogens is lacking. Methanosaeta concilii and Methanosarcina mazei were the ones commonly found in mesophilic anaerobic bioreactors treating LCFA-based wastewaters (Table S1). In this work, the effect of saturated (C16:0, palmitate) and unsaturated (C18:1, oleate) LCFA on the aceticlastic methanogenesis of pure cultures of M. concilii and M. mazei was investigated.

Results and discussion

Methanosaeta concilii (DSM 3671T) and M. mazei (DSM 2053T) were grown on sodium acetate as substrate for methane production, which was quantified over time before and after LCFA addition (Figs S1 and S2). Differences in methane production rate before and after LCFA addition were used to determine the methanogenic inhibition at oleate or palmitate concentrations of 0.5, 1, 2 and 4 mmol L−1 (see Fig. 1 as example).

Slope ratio (Sratio) was calculated for each incubation condition according to equation $S_{ratio} = \frac{SlopeA}{SlopeB}$, where SlopeB and slopeA represent the cumulative methane production slopes (mmol L−1 day−1) before and after the headspace flushing, second acetate addition and LCFA (oleate or palmitate) addition. Methane production rate of M. concilii was affected by oleate, as shown by the sharp decrease in the $S_{ratio}$ with 0.5 mmol L−1 oleate, compared with the $S_{ratio}$ obtained in the control (Table 1). This corresponds to 67% methanogenic inhibition and thus the IC50 of oleate was below 0.5 mmol L−1. No methane was produced, nor was acetate consumed, after the addition of 2 and 4 mmol L−1 oleate to M. concilii cultures (Table 1 and Table S2), which suggests complete methanogenic inhibition. Palmitate also affected methane production of M. concilii, although not as much as oleate, i.e. palmitate concentrations up to 2 mmol L−1 resulted in a maximum inhibition of 11%, whereas in the presence of 4 mmol L−1 methanogenesis was inhibited by 94% (Table 1).

Similar results were obtained with M. mazei in the presence of oleate, which caused 64% inhibition at 0.5 mmol L−1 and complete inhibition at 2 and 4 mmol L−1 (Table 1 and Table S2). However, although Methanosarcina spp. are reported as highly tolerant to others toxicants, such as ammonia and salts (De Vrieze et al., 2012; Hao et al., 2015), M. mazei was more vulnerable to palmitate than M. concilii. Palmitate concentrations of 2 mmol L−1 caused a 64% decrease of methane production by M. mazei, while for the same concentration the methanogenic inhibition of M. concilii was only 11% (Table 1).

The predominance of Methanosaeta spp. in anaerobic reactors containing high concentrations of palmitate (Shigematsu et al., 2006; Salvador et al., 2013) also indicates that Methanosaeta spp. might be more tolerant than Methanosarcina spp. In methanogenic bioreactors treating LCFA-based wastewater, the presence of both Methanosaeta and Methanosarcina species has been reported. Methanosaeta spp. are usually the dominant aceticlastic methanogens when acetate concentrations are low, due to their higher affinity for acetate compared with Methanosarcina spp., while Methanosarcina spp are generally more abundant at high acetate concentrations (De Vrieze et al., 2012). Nevertheless, Methanoseta
by their different sensitivity to these compounds. LCFA-degrading environments might also be prevalent of these acetoclastic microorganisms in the presence of different oleate and palmitate concentrations.

### Table 1. Slopes ratio ($S_{ratio}$) calculated for Methanosaeta concilii and Methanosarcina mazei in the presence of different oleate and palmitate concentrations.

| LCFA/mmol L$^{-1}$ | M. concilii | M. mazei |
|--------------------|-------------|----------|
|                    | SlopeB      | SlopeA   | $S_{ratio}$ | Inhibition$S_{ratio}$ |
| Oleate             |             |          |             |                       |
| 0.5                | 1.1 ± 0.1   | 1.9 ± 0.1| 1.8 ± 0.2   | -                      |
| 1                  | 1.1 ± 0.1   | 0.6 ± 0.2| 0.6 ± 0.2   | 67 ± 13                |
| 2                  | 1.0 ± 0.1   | 0.0 ± 0.0| 0.0 ± 0.0   | 100 ± 16               |
| 4                  | 1.0 ± 0.1   | 0.0 ± 0.1| 0.0 ± 0.1   | 100 ± 16               |
| Palmitate          |             |          |             |                       |
| 0.5                | 1.1 ± 0.1   | 1.9 ± 0.1| 1.8 ± 0.2   | 0                      |
| 1                  | 1.1 ± 0.1   | 1.8 ± 0.3| 1.7 ± 0.3   | 6 ± 22                 |
| 2                  | 1.1 ± 0.1   | 1.8 ± 0.1| 1.6 ± 0.1   | 11 ± 11                |
| 4                  | 1.2 ± 0.1   | 0.1 ± 0.1| 0.1 ± 0.1   | 94 ± 15                |

- Differences in methane production rate before and after LCFA addition were expressed as a slope ratio ($S_{ratio}$) that was calculated for each condition using the cumulative methane production slope, according to the equation ($S_{ratio} = SlopeA/SlopeB$). For control assays, in which no LCFA was added, $S_{ratio}$ were equally calculated and SlopeA determined after the headspace flushing and second acetate addition (Figs S1 and S2).
- The inhibitory effect of the different LCFA concentrations on methane production was expressed in percentage, by comparing the $S_{ratio}$ obtained from the LCFA supplemented assays ($S_{ratio,c}$) with the slopes ratio obtained from the control assays ($S_{ratio,c}$), according to equation (Inhibition $= (|S_{ratio,c} - S_{ratio} |/S_{ratio,c})\times100$).
- Average ± standard deviation of duplicate assays.

### Table 2. Concentration (mmol L$^{-1}$) of oleate and palmitate necessary to inhibit 50% methanogenesis of pure cultures of acetoclastic and hydrogenotrophic methanogens.

| LCFA   | Acetoclastic methanogens | Hydrogenotrophic methanogens$^a$ |
|--------|--------------------------|----------------------------------|
|        | Methanosaeta concilii    | Methanosarcina mazei              |
|        | Methanospirillum hungatei| Methanobacterium formicicum       |
| Oleate | <0.5                     | <0.5                             |
| Palmitate | 2–4$^c$               | 1–2$^c$                          |

- a. Sousa et al. (2013).

was found to persist and dominate over Methanosarcina in unstable anaerobic bioreactors with acetate concentrations up to 44 mmol L$^{-1}$ (Chen and He, 2015). The prevalence of these acetoclastic microorganisms in LCFA-degrading environments might also be influenced by their different sensitivity to these compounds.

A comparison between IC$_{50}$ values obtained for oleate and palmitate towards acetoclastic methanogens in this study, and towards hydrogenotrophic methanogens (Sousa et al., 2013) is presented in Table 2. Our results show that M. concilii and M. mazei are similarly affected by the presence of oleate as the hydrogenotroph Methanospirillum hungatei, and M. concilii seems to be even more tolerant to the presence of palmitate than M. hungatei. Previous studies on the toxicity of LCFA towards anaerobic sludge highlighted the higher sensitivity of acetoclasts compared with hydrogenotrophs. Since LCFA can absorb to the cells at variable amounts, its toxicity might be explained by a physical inhibition phenomenon rather than by direct metabolic inhibition (Pereira et al., 2005). Mass transfer limitations exerted by LCFA are likely more pronounced for acetate than for hydrogen transport, since hydrogen is a smaller molecule (Pereira et al., 2005).

Differences in cell envelopes composition might also influence the sensitivity of microorganisms. For example, the cell wall of Methanosarcina contains mannochondroitin and the one of Methanosaeta contains a sheath surrounding the S-layer and the cytoplasmic membranes. The sheath might have a protective effect since

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it is reported to be resistant to detergents (Claus and König, 2010).

Although the studies with mixed communities degrading LCFA are important, information about the sensitivity of individual species growing in pure cultures show the unequivocal metabolic behaviour of each tested species in the presence of LCFA.

The tolerance to LCFA can be higher when methanogens are growing in complex microbial communities than in pure cultures, due to a structural protection provided by aggregation of different microbial species. IC50 values between 0.1 and 1 mmol L⁻¹ and approximately 3 mmol L⁻¹ were reported for suspended and granular sludge respectively (Table S3). These values are, however, close to the ones obtained in this study for acetoclastic methanogens, and by Sousa et al. (2013) for pure cultures of hydrogenotrophic methanogens, and are indicative of LCFA concentrations that might cause operational problems due to direct inhibition of methanogens. These studies, all together, allow to discriminate between mass transport-related physical inhibition and metabolic inhibition, which impacts practical applications of anaerobic processes for treatment of LCFA-containing wastewater.

Because in continuous bioreactors oleate is for a large part converted to palmitate (Pereira et al., 2002), the potential toxicity of palmitate is, in this context, most relevant. Palmitate concentrations between 1 and 2 mmol L⁻¹ can be tolerated by methanogenic communities, allowing to feed higher oleate concentrations than the IC50 for oleate (<0.5 mmol L⁻¹). The higher IC50 exhibited by the acetoclastic M. concilii and the hydrogenotrophic Methanobacterium formicicum (Table 2), particularly for palmitate, may explain why these species are commonly found in bioreactors and point to their importance in the conversion of LCFA to methane in anaerobic wastewater treatment systems.

Conclusions
In this work, two acetoclastic methanogens revealed different tolerance to LCFA. Methanoseta concilii demonstrated a tolerance to palmitate similar to hydrogenotrophic methanogens, which generally are considered to be more resistant. These results are relevant in the context of lipid-rich wastewater treatment, where the presence and prevalence of Methanoseta species could be a good indicator of the system potential to efficiently convert LCFA to methane.

Conflict of Interest
The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Cumulative methane production from acetate consumption by Methanoseta concilii during exposure to oleate or palmitate.

Fig. S2. Cumulative methane production from acetate consumption by Methanosarcina mazei during exposure to oleate or palmitate.

Table S1. Main acetoclastic methanogens detected in anaerobic sludges from LCFA-fed reactors.

Table S2. Acetate concentration (mmol L⁻¹) determined before LCFA was added (Acinit) and at the end of the assays (Acend).

Table S3. Inhibition of acetoclastic methanogenic activity by LCFA, in several sludges exposed to different wastewater compositions.