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# The effect of substrate and operational parameters on the abundance of sulphate-reducing bacteria in industrial anaerobic biogas digesters

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## HIGHLIGHTS

- ▶ Abundance of sulphate-reducing bacteria was determined by qPCR targeting dsrB in biogas digesters.
- ▶ Sulphate-reducing bacteria were tolerant to most operational strategies used in industrial biogas plants.
- ▶ High concentrations of ammonia and ammonium lead to decreased abundance of sulphate-reducing bacteria.

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## ABSTRACT

This study evaluated the effects of operational parameters and type of substrate on the abundance of sulphate-reducing bacteria in 25 industrial biogas digesters using qPCR targeting the functional dissimilatory sulphite reductase gene. The aim was to find clues for operational strategies minimizing the production of $H_2S$. The results showed that the operation, considering strategies evaluated, only had scarce effect on the abundance, varying between $10^5$ and $10^7$ gene copies per ml. However, high ammonia levels and increasing concentration of sulphate resulted in significantly lower and higher levels of sulphate-reducing bacteria, respectively.

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## 1. Introduction

Biogas is produced through biological degradation of organic material in the absence of oxygen. The process occurs in natural environments, but is also exploited in commercial plants for waste treatment and for production of biogas. In commercial biogas plants, commonly used waste streams include sludge from wastewater treatment plants (WWTP), slaughterhouse waste, food waste, manure or other industrial waste streams, crops and crop residues.

The anaerobic degradation of organic material and production of biogas proceeds through four sequential steps and requires the activity of different microbiological groups (Angelidaki et al., 2011). The last step, methanogenesis, is performed by the activity of two main groups of methanogens, the hydrogenotrophic and acetoclastic methanogens, or by syntrophic acetate-oxidising bacteria (SAOB) operating in cooperation with hydrogenotrophic methanogens (Angelidaki et al., 2011; Westerholm et al., 2011).

The biogas produced in industrial biogas digesters mainly consists of methane and carbon dioxide, but also small amounts of other gases such as hydrogen sulphide. The later compound has corrosive properties causing damage on equipment and thus during industrial scale production the hydrogen sulphide has to be removed (Appels et al., 2008). In Sweden this is accomplished by precipitation of sulphides with ferric or ferrous iron inside the digester (Ek et al., 2011). Alternative methods are aeration of the gas to obtain elemental sulphur or biological treatment with for example *Thiobacillus* strains etc. (Ramírez et al., 2011; van der Zee et al., 2007). Regardless of the choice of technique, removing sulphides requires either expensive, extensive use of chemicals or large investments in new equipment. In addition to the problem related to gas usage, hydrogen sulphide may also cause inhibition to the microbial community by direct toxic effects or by precipitation of trace metals needed for enzymatic activity (Chen et al., 2008;

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Lopes et al., 2010a; van der Veen et al., 2007). The production of sulphides is influenced by different factors. Two important regulatory parameters being: (i) the amount of sulphur-containing amino acids in the incoming material, (ii) the level of sulphate in the incoming material (Dewil et al., 2008; Rabus et al., 2006).

In the presence of sulphate in a biogas process, SRB and methanogens compete for the same substrate, i.e. acetate and hydrogen/carbon dioxide. SRB typically win this competition owing to several interacting factors: (i) anaerobic respiration with sulphate as the final electron acceptor yields more energy for growth compared with carbon dioxide; (ii) SRB possess higher affinity for both hydrogen and acetate, enabling them to consume substrates below levels possible for use by methanogens (Rabus et al., 2006); and (iii) SRB generally have a higher specific growth rate than methanogens (Oude Elferink et al., 1994). Several previous studies have sought to decrease the activity of SRB and hydrogen sulphide production in biogas processes. These studies have mainly focused on changes in hydrogen sulphide production or levels of fermentation products. Parameters that have been investigated include COD:sulphate ratio in the substrate (Hirasawa et al., 2008; Lopes et al., 2010b), addition of different SRB inhibitors (Isa and Anderson, 2005; Nemati et al., 2001), pH (Visser et al., 1996; Chaiprapat et al., 2011) and temperature (Pender et al., 2004). Overall, these studies provide no conclusive solution for optimising the management of a biogas process towards lower sulphide levels in the biogas.

The overall aim of the present study was to obtain further information concerning SRB in biogas processes and by doing so find clues on how to decrease $H_2S$ production. The specific objective was to determine SRB abundance in biogas digesters and to evaluate possible correlations to substrate composition and operational parameters. To our knowledge, no previous study has examined the effects of different management strategies on the level of SRB. In total, 25 large-scale biogas digesters in Sweden were analysed with quantitative PCR targeting the dissimilatory sulphite reductase gene (*drs*B). In addition, SRB abundance was investigated over time in one digester subjected to increasing sulphate concentrations in the incoming substrate.

## 2. Methods

### 2.1. Samples and operational parameters

Representative samples were taken from 25 industrial biogas digesters at 17 different biogas plants in Sweden (Table 1). In total, six thermophilic digesters (H1, H2, K, M, N and P) and 19 mesophilic digesters (A1, A2, B1, B2, C, D, E, F, G, I1, I2, L1, L2, O1, O2, Q, R and S) were sampled, including two second stage digesters (D and F). At six of the biogas plants, two parallel digesters with slightly differing operational parameters were sampled. These digesters are denoted 1 and 2 accordingly for each plant (A1–A2, B1–B2, H1–H2, I1–I2, L1–L2 and O1–O2). The selected production plants included WWTP (L1, L2, O1, O2, P, Q and R) and co-digestion plants treating slaughterhouse waste or food waste as the main substrate (B1, B2, H1, H2, I1, I2, J, L1, L2, M, N and S). Digesters treating either brewery waste or crops (A1, A2, C, D, E and F) and digesters treating mainly manure (G and K) were also included. The samples taken from the digesters were frozen on-site (−20 °C) and sent to the laboratory for analysis. Information on substrate composition, iron addition and operational parameters for the different plants is presented in Table 1. Data on volatile fatty acids (VFA), pH, ammonium–nitrogen, hydrogen sulphide in raw gas (if available) were obtained from the different biogas plants. The fraction of ammonium–nitrogen that was present as ammonia was calculated according to Hansen et al. (1998) using pH and temperature. The ammonium–nitrogen concentration was also adjusted according to this calculation to only show nitrogen in the form of ammonium.

In addition, consecutive sampling of one industrial plant (B) was performed during a period when the sulphate level in the substrate involuntarily increased on average by 870 mg/l. In total, eight samples were taken over a period of 70 days.

### 2.2. Sulphate analysis

Sulphate concentrations in typical substrates common for the biogas plants included in the study were analysed using a sulphate cell test manufactured by WTW PhotoLab Spektral, Weilheim, Germany. Samples were diluted to the desired concentration, centrifuged (20 min, 11,000*g*) and filtered (0.45 μm). Barium in excess was added to the samples and turbidity was measured using a WTW turbidity meter (WTW PhotoLab Spektral). Analysis of thin stillage was performed according to ISO 22743 using a Skalar San++ Continuous Flow Analyzer (Skalar, Breda, Netherlands).

The increased amount of sulphate added to digester B in the time study was calculated by analysis of sulphate concentration in the substrate (EN ISO 10304-1:2009, Eurofins Environment Sweden AB) and by multiplying this by the total amount of substrate added to the digester.

### 2.3. DNA extraction

Frozen samples were thawed and DNA was extracted from small aliquots (300 μl) with the FastDNA® SPIN kit for soil (MP Biomedicals, Solon, OH, USA), according to the protocol given by the manufacturer, with small adjustments to increase yield (SEWS-M washing was performed twice). For monitoring of DNA yield, Qubit® (Invitrogen, Carlsbad, CA, USA), fluorometric quantification was used.

### 2.4. Quantitative PCR

The functional gene dissimilatory sulphite reductase (*dsr*B) was amplified with primer pair *DSR*p2060F-GC (5′ CAA CAT CGT YCA YAC CCA GGG 3′) and *DSR*4R (5′ GTG TAG CAG TTA CCG CA 3′) according to Geets et al. (2006). For the analysis, the iQ™ SYBR® Green Supermix (Bio Rad, Hercules, CA, USA) was detected with a C1000™ Thermal Cycler, CFX96™ Real-Time System (Bio Rad). PCR was performed according to the protocol described by Dar et al. (2007), with adjusted initial touchdown protocol using 10 cycles instead of 20 for step-wise decrease of annealing temperature from 65 to 55 °C. PCR amplification was followed by a melt curve analysis. Each reaction was loaded with 10 μl Supermix, 1 μl forward primer (1 μM), 1 μl reverse primer (1 μM), 5 μl sterile water and 3 μl template DNA. The samples were analysed at different dilutions; 1:500, 1:100, 1:50 and 1:10 in order to find the optimal dilution and to ensure that no PCR-inhibiting substance affected the results. Most samples had optimal PCR performance at the 1:100 dilution. However, DNA samples from digesters D, O1 and O2 were diluted 1:50 and samples from digester E were diluted 1:500. The melt curve analysis of the real-time PCR showed a lower melt temperature for some samples (digesters C, D, E, G and J) of 83 °C, which was 7 °C lower than the standard curve, motivating sequence analysis of the targeted sequence (Uppsala Genome Center). The sequences obtained was aligned against known sequences with BLASTN and the result showed that the amplified sequence was of correct length and closely related to the *dsr*B sequence (data not shown).

**Table 1**
Process and operational parameters of the 25 digesters, including the main substrates. Iron added indicates that the plant precipitates sulphides with iron. $H_2S$ is measured in raw gas, before upgrading and cleaning. Slaughterhouse waste (SW), Food waste (FW) and Industry waste (IW).

| Digester | A1 | A2 | B1 | B2 | C | D | E | F | G | H1 | H2 | I1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Main substrate | Stillage | Stillage | SW & IW | SW & IW | Crops | Crops | Brewery & crops | Brewery & crops | Manure & crops | FW & SW | FW & SW | SW & IW |
| Temperature (°C) | 37 | 37 | 37 | 37 | 38 | 37 | 38 | 37 | 40 | 52 | 52 | 37 |
| HRT (days) | 53 | 53 | 57 | 60 | 80 | 50 | 55 | 30 | 40 | 50 | 35 | 27,5 |
| Iron added | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| VFA (mg/l) | 396 | 583 | 718 | 276 | 602 | 822 | 4120 | 6440 | 790 | 0 | 0 | 2720 |
| pH | 7.6 | 7.6 | 8.0 | 8.0 | 7.6 | 7.6 | 7.5 | 7.5 | 8.0 | 8.0 | 7.9 | 7.6 |
| $NH_4^+$–N (mg/l) | 4634 | 4722 | 5168 | 5008 | 3509 | 3140 | 3355 | 3171 | 3591 | 1937 | 1861 | 2569 |
| $H_2S$ (ppmv) | <30 | <30 | <30 | <30 | 250 | 450 | 150 | 150 | 175 | 100 | 100 | 25 |
| $NH_3$–N (mg/l) | 237 | 241 | 662 | 642 | 191 | 160 | 145 | 129 | 559 | 632 | 483 | 131 |

| Digester | I2 | J | K | L1 | L2 | M | N | O1 | O2 | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Main substrate | SW & IW | FW & IW | Manure | Sludge | Sludge | FW & SW | SW | Sludge & stillage | Sludge & stillage | Sludge | Sludge | Sludge | FW & crops |
| Temperature (°C) | 37 | 37 | 52 | 37 | 37 | 53.3 | 53 | 37 | 37 | 53 | 37 | 37.5 | 37 |
| HRT (days) | 27.5 | 27.5 | 100 | 22 | 22 | 22.5 | 22 | 26 | 26 | 11.5 | 22 | 19 | 20 |
| Iron added | Yes | Yes | No | No | No | Yes | Yes | No | No | Yes | No | Yes | No |
| VFA (mg/l) | 1800 | 600 | 810 | 270 | 237 | 1730 | 4500 | 170 | 120 | 275 | 0 | 100 | 2700 |
| pH | 7.8 | 7.5 | 7.9 | 7.4 | 7.5 | 7.8 | 8.0 | 7.3 | 7.4 | 7.5 | 7.5 | 7.6 | 7.6 |
| $NH_4^+$–N (mg/l) | 2868 | 1634 | 1322 | 796 | 948 | 2406 | 2751 | 1463 | 1647 | 565 | 1233 | 1995 | 2569 |
| $H_2S$ (ppmv) | 25 | 125 | 680 | 3.5 | 3.5 | 90 | ND | ND | ND | ND | ND | ND | ND |
| $NH_3$–N (mg/l) | 232 | 66 | 343 | 26 | 38 | 536 | 849 | 37 | 53 | 55 | 50 | 105 | 131 |

### 2.5. Standard curve

A DNA standard curve was prepared with a pure culture of *Desulfovibrio desulfuricans* (DSM 642). DNA was extracted with DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany) and PCR amplification was performed with ReadyToGo beads (GE Healthcare, Buckinghamshire, UK) according to the protocol described above. The product was visualised on agarose gel 1% wt/v, cut out from the gel and extracted with MinElute® Gel Extraction Kit (Qiagen). The extraction was followed by a ligation/cloning reaction with TOPO TA Cloning® Kit (Invitrogen), a plasmid purification with QIAprep® Spin Miniprep kit (Qiagen) and a second PCR amplification. The product obtained was quantified with Nanodrop ND-100 (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted to appropriate standard levels.

### 2.6. Data analysis

SRB abundance was evaluated as response variable for single biogas digesters. The digesters were clustered in groups of high and low when above or below the respective threshold value of each factor, but also by type of substrate. Factor levels used in the analysis were HRT (threshold 26 days), temperature (threshold 45 °C), pH (threshold 7.6), VFA (threshold 750 mg/l), ammonium (threshold 1800 mg/l), ammonia (threshold 200 mg/l), iron dosing, sulphate-rich substrate or not (sulphate-rich substrates included thin stillage, slaughterhouse waste and swine manure) and type of substrate. The threshold values were chosen to yield as high a difference as possible in SRB abundance. Confidence intervals were calculated with $\alpha = 0.05$.

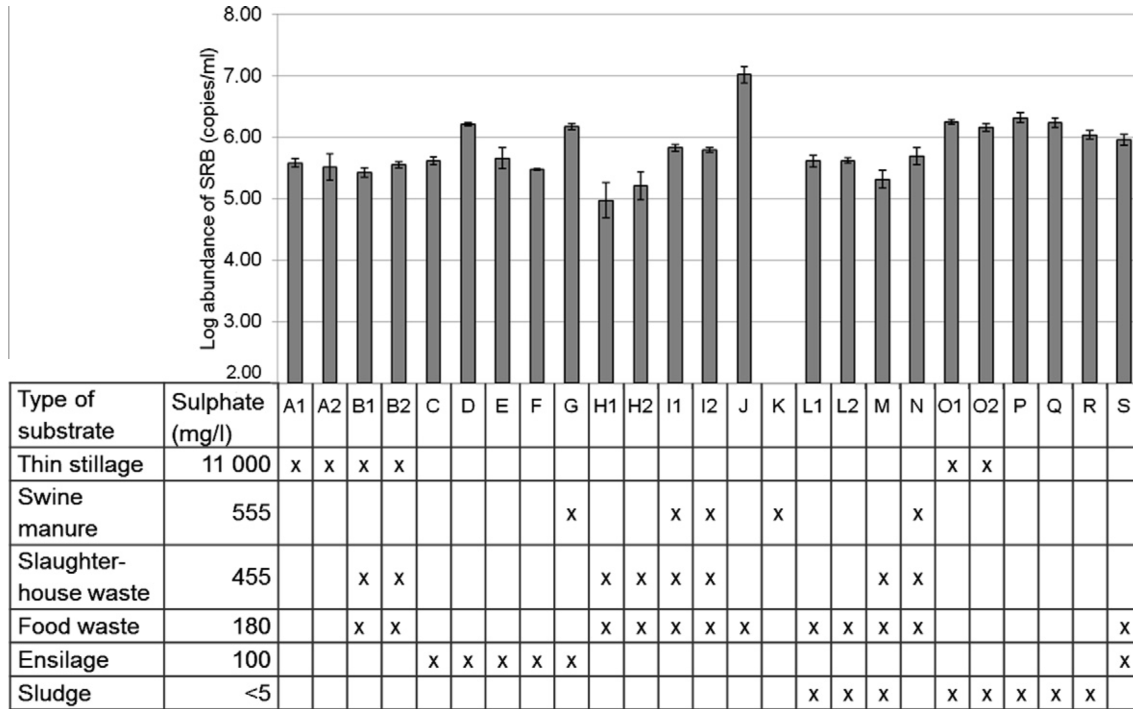
## 3. Results and discussion

### 3.1. Determination of SRB abundance by qPCR

SRB abundance was successfully analysed through amplification of the *drs*B gene for all digesters except one (digester K), which had SRB levels below the detection limit of the method (Fig. 1). The detected levels in the digester varied between $10^5$ and $10^7$ target gene copies per ml (Fig. 1). Furthermore, analysis with different DNA dilutions gave identical results, showing that the amplification was not affected by inhibitory substances, and that SRB abundance can be successfully determined in biogas digesters by qPCR. Analysis of SRB by targeting the *dsr* gene was first performed by Wagner et al. (1998), who showed that primers directed towards this gene targeted a range of different SRB and that none of the nitrite-reducing or sulphate-assimilating bacteria evaluated was amplified during the PCR reaction. The specific primer pair used in this study was designed and evaluated by Geets et al. (2006) and showed successful for amplification of 24 out of 25 different pure culture SRB strains. This primer pair has also been used in complex environments, such as soda lakes, sulphidogenic bioreactors, contaminated water samples from steel plants and harbours, with successful results (Dar et al., 2007; Foti et al., 2007; Geets et al., 2006). Although the prevailing sulphate concentration in the investigated biogas digesters likely was low compared with that in some natural environments, e.g. marine sediments, the results clearly showed that SRB can thrive in this environment. Furthermore, comparisons of the abundance of SRB in the biogas processes with the abundance in other environments, all determined with the same detection method (qPCR targeting the *dsr*B gene), revealed that the levels are lower than in estuarine sediments ($10^7$–$10^9$) and paddy soils ($10^7$–$10^9$), but similar or higher than those in marine sediments ($10^5$–$10^8$), soda lakes ($10^4$–$10^8$) and oil reservoirs ($10^2$–$10^6$) (Foti et al., 2007; Gittel et al., 2009; He et al., 2010; Kondo et al., 2004; Leloup et al., 2007).

### 3.2. Effects by choice of operational parameters

Correlating the qPCR results with operational parameters showed that two parameters had a significant impact on SRB levels (Fig. 2). The abundance was significant lower at high ammonia levels (>200 mg/l) and high ammonium levels (>1800 mg/l). Trends for lower abundance were also observed for high temperatures (>45 °C), pH (>7.6) and sulphate levels in the different substrate categories (Fig. 2). However, these differences were not significant (Figs. 1 and 2). SRB activity has in previous studies been shown to be optimal at neutral pH while decreasing at both acid and alkaline pH levels (Chaiprapat et al., 2011; Gutierrez et al., 2009). In line with our results, increased temperature and sulphate concentration has




| Type of substrate | Sulphate (mg/l) | A1 | A2 | B1 | B2 | C | D | E | F | G | H1 | H2 | I1 | I2 | J | K | L1 | L2 | M | N | O1 | O2 | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Thin stillage | 11 000 | x | x | x | x | | | | | | | | | | | | | | | | x | x | | | | |
| Swine manure | 555 | | | | | | | | | x | | | x | x | | x | | | | x | | | | | | |
| Slaughter-house waste | 455 | | | x | x | | | | | | x | x | x | x | | | | | x | x | | | | | | |
| Food waste | 180 | | | x | x | | | | | | x | x | x | x | x | | x | x | x | x | | | | | | x |
| Ensilage | 100 | | | | | x | x | x | x | x | | | | | | | | | | | | | | | | x |
| Sludge | <5 | | | | | | | | | | | | | | | | x | x | x | | x | x | x | x | x | |

**Fig. 1.** Logarithmic abundance of sulphate-reducing bacteria (gene copies per ml) in 25 large-scale biogas digesters. The table shows sulphate levels in substrates typical for the selected biogas plants. Digesters using certain substrates are indicated with (×). The error bars indicate standard deviation.

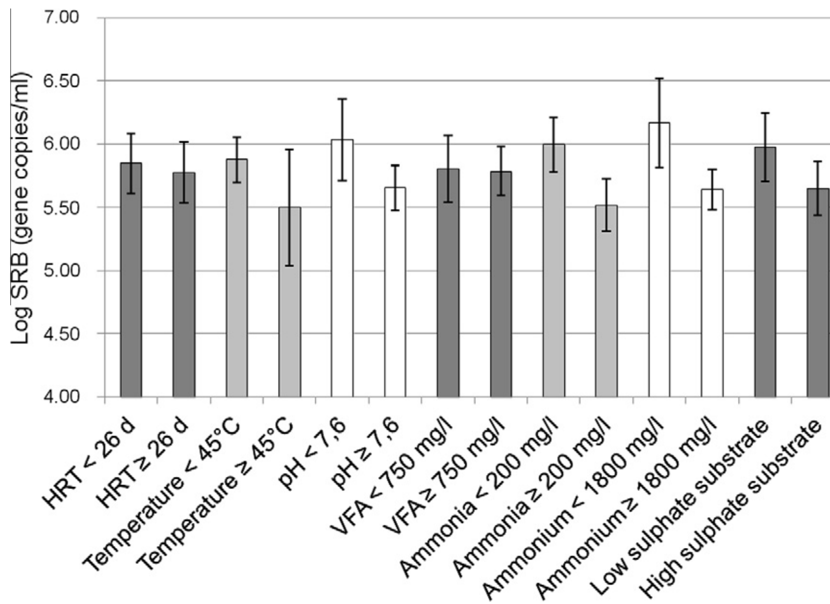



**Fig. 2.** Logarithmic abundance of SRB in biogas production plants grouped according to high and low HRT, temperature, pH, VFA, ammonia, ammonium and sulphate level in substrate. Error bars are confidence intervals, $\alpha = 0.05$, equal variance not assumed.

in a previous study been shown to give decreased SRB activity (Pender et al., 2004). The significantly lower SRB abundance in biogas digesters operating at high ammonia levels (above 200 mg/l) and high ammonium (above 1800 mg/l), suggest that SRB are sensitive to high nitrogen levels. Ammonia is the toxic fraction of nitrogen for various types of organisms, as it is uncharged and can pass through cell membranes. The concentration of ammonia is primarily determined by the ammonium concentration, but at increasing pH and temperature the fraction of nitrogen in the form of ammonia increases (Hansen et al., 1998). An alternative explanation for the lower SRB abundance in the high ammonia digesters is that SRB are outcompeted by hydrogenotrophic methanogens under sulphate-limited conditions, which can be assumed in biogas processes. Different groups of SRB compete for sulphate at limited conditions, leading to dominance of hydrogenotrophic SRB (Laanbroek et al., 1984). Furthermore, high ammonia concentrations have been shown to select for methane production by syntrophic acetate oxidation instead of acetoclastic methanogenesis (Schnürer et al., 1999; Schnürer and Nordberg, 2008; Westerholm et al., 2011). Under these conditions methane production mainly precedes through

the activity of ammonia-tolerant hydrogenotrophic methanogens (Schnürer et al., 1999; Westerholm et al., 2011). These methanogens may have a competitive advantage over SRB as hydrogen scavengers, as indicated by Pender et al. (2004).

### 3.3. Effect of substrates

Besides the choice of operational parameters, an alternative strategy for the industrial biogas production plants to reduce the level of hydrogen sulphide could be by selection of substrates with lower sulphur content. The substrates used by the digesters in this study were quite different, ranging from sulphate-rich thin stillage (11,000 mg/l sulphate; digesters A1, A2, O1, O2), protein-rich slaughterhouse waste with moderate sulphate levels (455 mg/l sulphate; digesters B1, B2, H1, H2, I1, I2, M and N), food waste (180 mg/l sulphate; digesters H1, H2, I1, I2, J, L1, L2, M, N and S), silage (100 mg/l sulphate; digesters C, D, E, F, G and S) and municipal WWTP (<5 mg/l sulphate; digesters L1, L2, M, O1, O2, P, Q and R) (Fig. 1). Furthermore, five plants treated sulphate-rich swine manure (555 mg/l sulphate; digesters G, I1, I2, K and N) (Fig. 1). Even so, no correlation between SRB abundance and sulphate content in the substrate could be seen in the digester screening (Fig. 2). Digesters treating sulphate-rich thin stillage and slaughterhouse waste even had a low SRB abundance compared to WWTP treating low sulphate sewage sludge. The function of SRB at low sulphate levels has been described elsewhere and it is reported that SRB are metabolically flexible and certain species can grow fermentative on, for example, lactate, fumarate or ethanol to produce propionate, acetate, $CO_2$ or $H_2$ (Oude Elferink et al., 1994; Plugge et al., 2011). Furthermore, SRB may grow as acetogens by syntrophic degradation of propionate, lactate or ethanol in association with hydrogenotrophic methanogens (Stams and Plugge, 2009). This can explain how SRB persist in biogas processes at low sulphate levels, as indicated by the relatively high SRB abundance in digesters with sulphate-depleted substrates included in this study (Fig. 1). The significantly lower SRB abundance in digesters treating slaughterhouse could have been caused by ammonia inhibition. The mean ammonia level in digesters treating slaughterhouse waste was 521 mg/l $NH_3$, which was 10-fold higher than that in WWTP (52 mg/l $NH_3$, Table 1).

### 3.4. Time study in an industrial biogas plant

The analysis of SRB in the digesters was complemented by a time-study in digester B during a 70-day period of industrial biogas production. The sulphate concentration in the substrate, mainly consisting of slaughterhouse waste and diverse industrial waste, increased during this period from moderate sulphate concentration to, in this context, extraordinarily high concentrations (Fig. 3). The extra sulphate resulted in increasing hydrogen sulphide production and higher SRB abundance, with an increase from $10^5$ to $10^6$ gene copies per ml (Fig. 3). Even though the screening of the large-scale biogas plants showed no significant correlation between sulphate content in the substrate and SRB levels the results from the industrial time study clearly illustrated an increase in SRB levels, and consequently an increase in $H_2S$ production, in response to increased levels of sulphate of the incoming substrate (Fig. 3). The explanation for the somewhat contradictory results could be that the SRB abundance in the different biogas digesters included in the screening was influenced not only by sulphate, but also by other parameters. For example, the high sulphate substrates were all protein-rich materials, resulting in high ammonia levels. Thus, an inhibitory effect of ammonia might explain the trend towards lower levels of SRB associated with the high-sulphate substrates. In any case, the observed increase in the time study coincided with the observed differences in SRB levels in the 25 different biogas plants. These results suggest that an increase in SRB abundance of one log unit is sufficiently high to cause a significant increase in $H_2S$ levels in the biogas. The response to sulphate addition in the time study generated a hydrogen sulphide level in the raw gas corresponding to 3600 ppmv. In Sweden, the hydrogen sulphide content is limited to 23 ppmv if the gas is to be distributed as vehicle fuel. During the period of the time study, the iron dosage had to be increased by a factor of 3 in order to reduce the hydrogen sulphide levels to below 23 ppmv. Any remaining hydrogen sulphide was reduced by using activated coal and water scrubbers during the upgrading process. Thus reduction of the added sulphate by SRB resulted in increased production costs in the investigated industrial biogas plant.

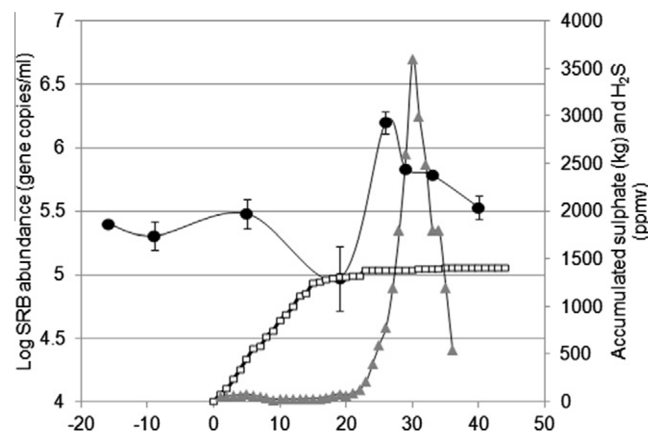



**Fig. 3.** Effect of sulphate addition to a industrial digester. Day 0 was the first day of increased sulphate addition. The lines illustrate SRB abundance (black circles), hydrogen sulphide (grey triangles) and accumulated amount of extra sulphate added to the process (white squares).

## 4. Conclusions

Abundance of SRB was for the first time successfully determined by qPCR targeting *dsr*B in biogas digesters. The abundance was only marginally influenced by the choice of the incoming material and process parameters, why operating strategies for biogas plants has little effect on SRB level. In total, only two parameters were shown to have a significant effect on SRB abundance. High levels of nitrogen resulted in lower levels of SRB and addition of excess sulphate to a running process resulted in increased growth of SRB.

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