

# Effect of methanogenic substrates on anaerobic oxidation of methane and sulfate reduction by an anaerobic methanotrophic enrichment

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Received: 16 February 2010 / Revised: 31 March 2010 / Accepted: 31 March 2010 / Published online: 6 May 2010  
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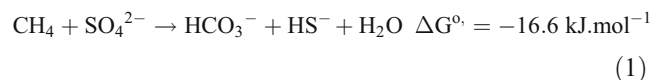
**Abstract** Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is assumed to be a syntrophic process, in which methanotrophic archaea produce an interspecies electron carrier (IEC), which is subsequently utilized by sulfate-reducing bacteria. In this paper, six methanogenic substrates are tested as candidate-IECs by assessing their effect on AOM and SR by an anaerobic methanotrophic enrichment. The presence of acetate, formate or hydrogen enhanced SR, but did not inhibit AOM, nor did these substrates trigger methanogenesis. Carbon monoxide also enhanced SR but slightly inhibited AOM. Methanol did not enhance SR nor did it inhibit AOM, and methanethiol inhibited both SR and AOM completely. Subsequently, it was calculated at which candidate-IEC concentrations no more Gibbs free energy can be conserved from their production from methane at the

applied conditions. These concentrations were at least 1,000 times lower than the final candidate-IEC concentration in the bulk liquid. Therefore, the tested candidate-IECs could not have been produced from methane during the incubations. Hence, acetate, formate, methanol, carbon monoxide, and hydrogen can be excluded as sole IEC in AOM coupled to SR. Methanethiol did inhibit AOM and can therefore not be excluded as IEC by this study.

**Keywords** Anaerobic oxidation of methane · Interspecies electron carrier · Methanogenic substrates

## Introduction

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) according to Eq. 1, is an important process in the global carbon cycle (Hinrichs and Boetius 2002). The process was discovered during geochemical studies in marine sediments (Martens and Berner 1974; Barnes and Goldberg 1976; Reeburgh 1976).



Phylogenetic analysis of AOM-SR sediments identified three novel groups of archaea putative called anaerobic methanotrophs (ANME); ANME-1, ANME-2, and ANME-3. These ANME are distantly related to cultivated methanogenic members from the orders *Methanosarcinales* and *Methanomicrobiales* (Hinrichs et al. 1999; Orphan et al. 2002; Knittel et al. 2005; Niemann et al. 2006). Orphan et al. (2001, 2002) showed that cells belonging to ANME-1 and ANME-2 assimilated carbon from methane (CH<sub>4</sub>) during AOM. ANME probably mediate a form of reversed methano-

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genesis since AOM, like methanogenesis, is inhibited by bromoethanesulfonate (Nauhaus et al. 2005); an analogue of the methyl-coenzyme M reductase was found to make up 7% of the extracted soluble proteins from an AOM-mediating microbial mat from the Black Sea (Krüger et al. 2003), and ANME-1 cells were found to contain most of the genes typically associated with CH<sub>4</sub> production (Hallam et al. 2003, 2004; Meyerdierks et al. 2010). Moreover, methanogens (Zehnder and Brock 1980; Harder 1997; Moran et al. 2007) and methanogenic sludge (Meulepas et al. 2010) also mediate CH<sub>4</sub> oxidation, however during net methanogenesis.

No gene analogues for enzymes involved in dissimilatory SR were found in archaea belonging to ANME groups (Thauer and Shima 2008; Meyerdierks et al. 2010) and FISH techniques showed that ANME occur in aggregates (Boetius et al. 2000; Michaelis et al. 2002; Knittel et al. 2005) with bacteria related to *Desulfosarcina-Desulfococcus* or *Desulfobulbus*. These findings suggest that AOM coupled to SR is a syntrophic process, in which ANME convert CH<sub>4</sub> to a metabolite which forms the electron donor for the sulfate-reducing bacterial (SRB) partner.

Given the evidence for reversed methanogenesis, methanogenic substrates have been proposed to act as interspecies electron carrier (IEC) (Hoehler et al. 1994; DeLong 2000). The standard Gibbs free energy change at pH 7 ( $\Delta G^{\circ}$ ) of the production of these IECs from CH<sub>4</sub> is positive (Table 1), but when the IEC concentration is kept low enough by the sulfate-reducing partner, the Gibbs free energy change will be negative. However, the SR activity of Hydrate Ridge sediment with hydrogen, formate, acetate, methanol, carbon monoxide, and methylamines was lower than SR activity on CH<sub>4</sub>, indicating that SRB involved in AOM, were not adapted to these substrates (Nauhaus et al. 2002, 2005). Wegener (2008) showed that the bacterial partners mainly assimilated inorganic carbon, which makes the uptake of an

organic IEC less likely. Sørensen et al. (2001) excluded hydrogen, acetate, and methanol as IEC because the maximum diffusion distances of those compounds at in situ concentrations and rates were smaller than the thickness of two prokaryotic cell walls. Orcutt and Meile (2008) showed with process-based modeling that hydrogen and formate could not be exchanged fast enough between syntrophic partners to sustain the sulfate reduction rates found by Nauhaus et al. (2007), but for acetate this might be possible. Using a spherical diffusion-reaction model Alperin and Hoehler (2009) concluded that hydrogen, formate, and acetate are thermodynamically and physically possible intermediates in AOM coupled to SR.

This study presents a novel approach to investigate whether a compound acts as IEC. Firstly, the effect of the presence of candidate-IECs, at relative high concentrations, on AOM and SR by an enrichment, of ANME-2a and bacteria mainly belonging to *Deltaproteobacteria* and *Flavobacteriales* (Meulepas et al. 2009; Jagersma et al. 2009), is assessed. And secondly, the concentration of each candidate-IEC is calculated at which no more energy can be obtained from their production from CH<sub>4</sub>. If AOM still occurs at IEC concentrations far above this theoretical maximum, the AOM does not proceed via the production of that particular IEC. Due to the high AOM and SR activity of the enrichment (0.2 mmol L<sup>-1</sup> day<sup>-1</sup>), the effect of these additives can be assessed within a period of 4 days.

## Material and methods

### Inoculate

The biomass used for this research was taken from a 1-L submersed-membrane bioreactor, inoculated with 10 g dry weight Eckernförde Bay sediment (Baltic Sea), operated at

**Table 1** The production and consumption of candidate interspecies electron carriers (IECs) in AOM coupled to SR

Candidate-IEC	Potential subconversions in AOM coupled to SR		
Acetate	CH <sub>4</sub> +HCO <sub>3</sub> <sup>-</sup> →CH <sub>3</sub> COO <sup>-</sup> +H <sub>2</sub> O	$\Delta G^{\circ}$ <sub>IEC production</sub>	+31 kJ mol <sup>-1</sup> CH <sub>4</sub>
	CH <sub>3</sub> COO <sup>-</sup> +SO <sub>4</sub> <sup>2-</sup> →HS <sup>-</sup> +2HCO <sub>3</sub> <sup>-</sup>	$\Delta G^{\circ}$ <sub>IEC consumption</sub>	-47 kJ mol <sup>-1</sup> SO <sub>4</sub> <sup>2-</sup>
Formate	CH <sub>4</sub> +3HCO <sub>3</sub> <sup>-</sup> →4HCO <sub>2</sub> <sup>-</sup> +H <sup>+</sup> +H <sub>2</sub> O	$\Delta G^{\circ}$ <sub>IEC production</sub>	+128 kJ mol <sup>-1</sup> CH <sub>4</sub>
	4HCO <sub>2</sub> <sup>-</sup> +SO <sub>4</sub> <sup>2-</sup> +H <sup>+</sup> →HS <sup>-</sup> +4HCO <sub>3</sub> <sup>-</sup>	$\Delta G^{\circ}$ <sub>IEC consumption</sub>	-144 kJ mol <sup>-1</sup> SO <sub>4</sub> <sup>2-</sup>
Methanol	CH <sub>4</sub> + <sup>1</sup> / <sub>3</sub> HCO <sub>3</sub> <sup>-</sup> + <sup>1</sup> / <sub>3</sub> H <sup>+</sup> + <sup>1</sup> / <sub>3</sub> H <sub>2</sub> O→ <sup>4</sup> / <sub>3</sub> CH <sub>3</sub> OH	$\Delta G^{\circ}$ <sub>IEC production</sub>	+104 kJ mol <sup>-1</sup> CH <sub>4</sub>
	<sup>4</sup> / <sub>3</sub> CH <sub>3</sub> OH+SO <sub>4</sub> <sup>2-</sup> →HS <sup>-</sup> + <sup>4</sup> / <sub>3</sub> HCO <sub>3</sub> <sup>-</sup> + <sup>1</sup> / <sub>3</sub> H <sup>+</sup> + <sup>4</sup> / <sub>3</sub> H <sub>2</sub> O	$\Delta G^{\circ}$ <sub>IEC consumption</sub>	-120 kJ mol <sup>-1</sup> SO <sub>4</sub> <sup>2-</sup>
Carbon monoxide	CH <sub>4</sub> +3HCO <sub>3</sub> <sup>-</sup> +3H <sup>+</sup> →4CO+5H <sub>2</sub> O	$\Delta G^{\circ}$ <sub>IEC production</sub>	+196 kJ mol <sup>-1</sup> CH <sub>4</sub>
	4CO+SO <sub>4</sub> <sup>2-</sup> +4H <sub>2</sub> O→HS <sup>-</sup> +4HCO <sub>3</sub> <sup>-</sup> +3H <sup>+</sup>	$\Delta G^{\circ}$ <sub>IEC consumption</sub>	-212 kJ mol <sup>-1</sup> SO <sub>4</sub> <sup>2-</sup>
Methanethiol	CH <sub>4</sub> + <sup>1</sup> / <sub>3</sub> HCO <sub>3</sub> <sup>-</sup> + <sup>5</sup> / <sub>3</sub> H <sup>+</sup> + <sup>4</sup> / <sub>3</sub> HS <sup>-</sup> → <sup>4</sup> / <sub>3</sub> H <sub>3</sub> CSH+H <sub>2</sub> O	$\Delta G^{\circ}$ <sub>IEC production</sub>	+55 kJ mol <sup>-1</sup> CH <sub>4</sub>
	<sup>4</sup> / <sub>3</sub> H <sub>3</sub> CSH+SO <sub>4</sub> <sup>2-</sup> → <sup>7</sup> / <sub>3</sub> HS <sup>-</sup> + <sup>4</sup> / <sub>3</sub> HCO <sub>3</sub> <sup>-</sup> + <sup>5</sup> / <sub>3</sub> H <sup>+</sup>	$\Delta G^{\circ}$ <sub>IEC consumption</sub>	-71 kJ mol <sup>-1</sup> SO <sub>4</sub> <sup>2-</sup>
Hydrogen	CH <sub>4</sub> +3H <sub>2</sub> O→4H <sub>2</sub> +HCO <sub>3</sub> <sup>-</sup> +H <sup>+</sup>	$\Delta G^{\circ}$ <sub>IEC production</sub>	+136 kJ mol <sup>-1</sup> CH <sub>4</sub>
	4H <sub>2</sub> +SO <sub>4</sub> <sup>2-</sup> +H <sup>+</sup> →HS <sup>-</sup> +4H <sub>2</sub> O	$\Delta G^{\circ}$ <sub>IEC consumption</sub>	-152 kJ mol <sup>-1</sup> SO <sub>4</sub> <sup>2-</sup>

The standard Gibbs free energy changes were obtained from Thauer et al. (1977)

15 °C, sparged with 4.8 L L<sup>-1</sup> day<sup>-1</sup> pure CH<sub>4</sub> gas and fed with 0.14 L L<sup>-1</sup> day<sup>-1</sup> marine medium. The basal medium consisted of: NaCl (19.8 g L<sup>-1</sup>), KCl (0.45 g L<sup>-1</sup>), MgCl<sub>2</sub>·6H<sub>2</sub>O (4.25 g L<sup>-1</sup>), NH<sub>4</sub>Cl (0.25 g L<sup>-1</sup>), CaCl<sub>2</sub>·2H<sub>2</sub>O (1.19 g L<sup>-1</sup>), MgSO<sub>4</sub>·7H<sub>2</sub>O (5.10 g L<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (0.34 g L<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O (1.25 g L<sup>-1</sup>), a trace element solution (1 mL L<sup>-1</sup>), a vitamin solution (1 mL L<sup>-1</sup>), a 0.5 g L<sup>-1</sup> resazurin solution (1 mL L<sup>-1</sup>), a 0.1 M Na<sub>2</sub>S solution (1 mL L<sup>-1</sup>), and demineralized water. The trace elements and vitamin solutions were made according to Widdel and Bak (1992). The first 330 days, 70 μmol L<sup>-1</sup> day<sup>-1</sup> acetate was supplied as cosubstrate, after that CH<sub>4</sub> was the sole supplied electron donor and carbon source. During 884 days, the volumetric AOM rate increased exponentially from 0.002 to 0.6 mmol L<sup>-1</sup> day<sup>-1</sup> (Meulepas et al. 2009). The biomass used for this research was sampled at day 584. To ensure homogeneous sampling, liquid recirculation (0.5 L min<sup>-1</sup>) and gas sparging (2 L min<sup>-1</sup>) were applied prior to and during sampling. Microbial analyses of the enrichment (clone library and fluorescent in situ hybridization) showed that the archaeal community was dominated by ANME-2a and that the bacterial community mainly consists of members of the *Deltaproteobacteria* and Bacteroidetes (Jagersma et al. 2009). During AOM, carbon derived from CH<sub>4</sub> was incorporated in both archaeal and bacterial lipids (Jagersma et al. 2009).

#### Incubations with candidate-IECs

Experiments were done in 35-mL serum bottles closed with butyl rubber stoppers and caps. After determining the exact weight and volume, the bottles were eluted with nitrogen gas. Subsequently, 30 mL undiluted reactor suspension was transferred from the bioreactor to the bottles by syringe. The reactor suspension contained 0.59 g volatile suspended solids per liter (a measure for the biomass content). The headspace of each bottle was evacuated again and replaced by 0.16 (±0.01) MPa <sup>13</sup>C-labeled CH<sub>4</sub> (<sup>13</sup>CH<sub>4</sub>) with a purity of 99% from Campro (Veenendaal, The Netherlands). Subsequently, 1.0 mM acetate, 1.0 mM formate, 1.0 mM methanol, 7.2 kPa carbon monoxide, 1.0 mM methanethiol, or 7.2 kPa hydrogen were added as candidate-IECs from stock bottles. In addition, control incubations without IEC were done. Incubations were done in duplicate. However, one incubation with methanethiol and one with hydrogen had to be terminated before day 4, due to leaking. These duplicates could not be repeated due to a limited biomass stock. The bottles were incubated at 15 °C and shaken in an orbital shaker at 100 rpm. The gas composition, pH, and pressure were determined once or twice a day. The carbon monoxide and hydrogen fraction in the headspace, the sulfide and formate concentration, the dissolved sulfide concentration, and the concentration of fatty acids and

alcohols were analyzed immediately after inoculation and after 4 days. Sampling was done at the incubation temperature (15 °C).

#### Analysis

The headspace composition was measured on a gas chromatograph–mass spectrometer (GC-MS) from Inter-science (Breda, The Netherlands). The system was composed of a Trace GC equipped with a GS-GasPro column (30 m by 0.32 mm; J&W Scientific, Folsom, CA, USA), and a Ion-Trap MS. Helium was the carrier gas at a flow rate of 1.7 mL min<sup>-1</sup>. The column temperature was 30 °C. The fractions of CH<sub>4</sub> and CO<sub>2</sub> in the headspace were derived from the peak areas in the gas chromatograph. The fractions of <sup>13</sup>C-labeled CH<sub>4</sub> (<sup>13</sup>CH<sub>4</sub>) and <sup>13</sup>C-labeled CO<sub>2</sub> (<sup>13</sup>CO<sub>2</sub>) were derived from the mass spectra as done by Shigematsu et al. (2004). The headspace pressure, sulfide concentration, and pH were analyzed as described by Meulepas et al. (2009). The sulfate concentration was analyzed according to Sipma et al. (2004), and the acetate and methanol concentration were analyzed according to Weijma et al. (2000). Formate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA) equipped with IonPac AG17 and AS17 4 mm columns, operated at a temperature of 30 °C, and a flow rate of 1.5 mL min<sup>-1</sup>. The injection volume was 25 μL. The eluent was made on-line using the EG40 Eluent Generator (Dionex) equipped with a KOH cartridge (Dionex P/N 053921) and deionized water as the carrier. Prior to analysis, samples were centrifuged and diluted 20 times. Hydrogen and carbon monoxide were measured on a gas chromatograph HP 5890 (Hewlett Packard, Palo Alto, USA) as described previously (Sipma et al. 2004). Methanethiol was measured on a HP 6890 gas chromatograph equipped with a Supelco sulfur SPB-1 column (Bellefonte, PA, USA) according to van den Bosch et al. (2009).

#### Calculations

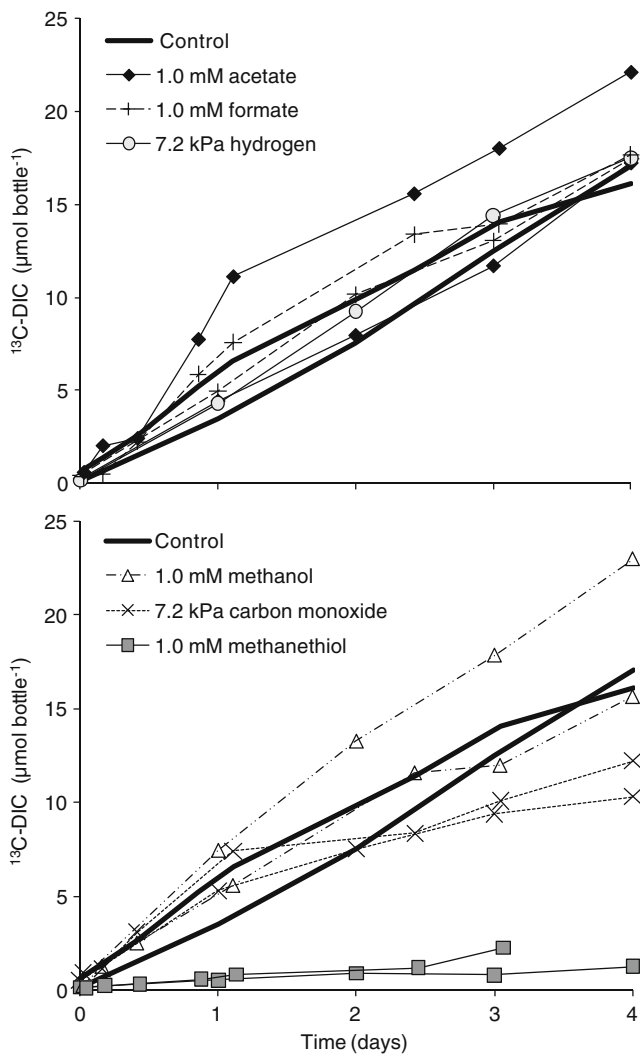
The <sup>13</sup>C-dissolved inorganic carbon (<sup>13</sup>C-DIC, <sup>13</sup>C-labeled CO<sub>2</sub>, and <sup>13</sup>C-labeled bicarbonate) and <sup>12</sup>C-DIC per bottle were calculated according to the equation given by Meulepas et al. (2009).

The concentration of each candidate-IEC at which no more energy can be obtained ( $\Delta G_{\text{IEC production}}=0$ ) from their production from CH<sub>4</sub> (Table 1) was calculated. This is done according to Eq. 2.

$$\Delta G_{\text{IEC-production}} = \Delta G_{\text{IEC-Production}}^{\circ} + R T \ln \frac{\prod [\text{products}]}{\prod [\text{substrates}]} \quad (2)$$

$R$  gas constant=8.314 J mol<sup>-1</sup> K<sup>-1</sup>

$T$  temperature in  $K=288.15$  K



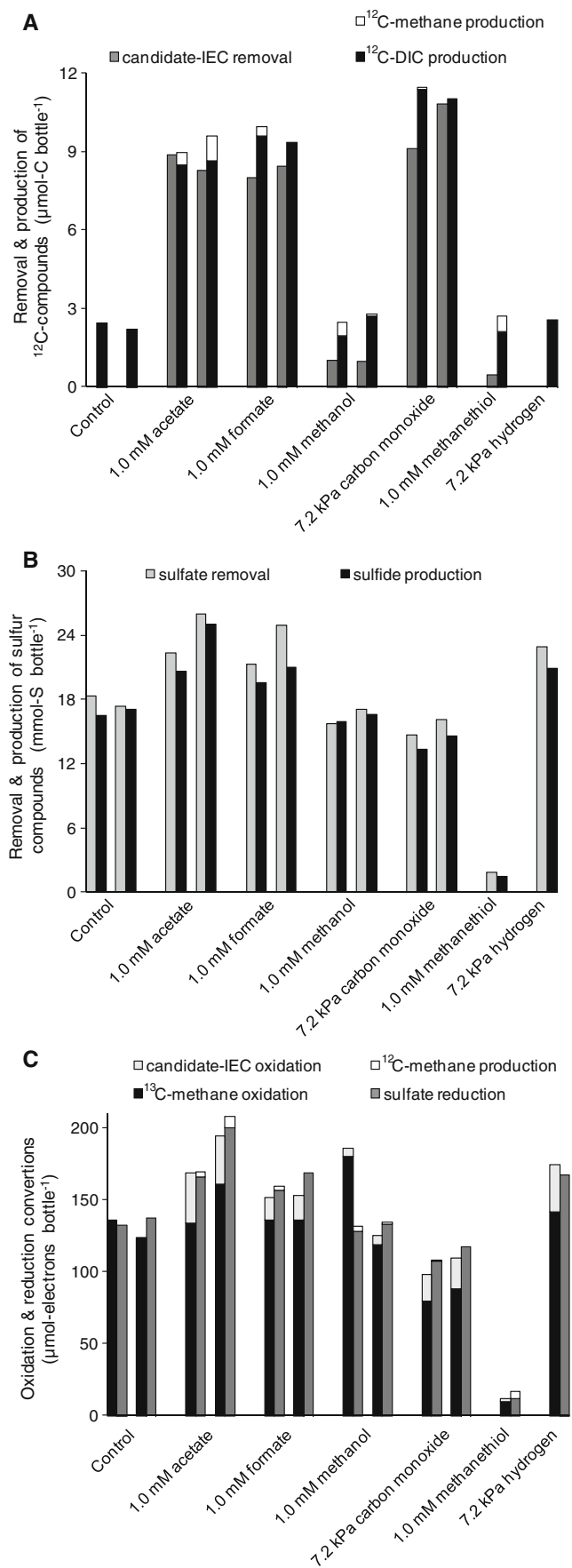
**Fig. 1**  $^{13}\text{C}$ -DIC production in time, during 4-day batch incubations, in the absence (*control*) or in the presence of one candidate-IEC. The bottles contained 30 mL biomass suspension from the enrichment bioreactor and initially  $0.16 (\pm 0.01)$  MPa  $^{13}\text{CH}_4$ , 15 ( $\pm 1$ ) mM sulfate and 0.2 ( $\pm 0.1$ ) mM sulfide

## Results

### Incubations

The  $^{13}\text{C}$ -DIC production is taken as a measure for  $^{13}\text{CH}_4$  oxidation because the percentage  $^{13}\text{C}$  in DIC produced from other (not  $^{13}\text{C}$ -enriched) sources is only around 1.1%, while the  $^{12}\text{C}$ -DIC production (Fig. 2a) did not even exceed the  $^{13}\text{C}$ -DIC production (Fig. 1) in any of the incubations. The sulfide production was taken as a measure for SR, which was in all incubations coupled to the sulfate removal (Fig. 2b).

**Fig. 2** a  $^{12}\text{C}$  carbon, b sulfur, and c reduction equivalent balances over 4-day batch incubations in the absence (*control*) or in the presence of one candidate-IEC. The 35-mL bottles contained 30 mL biomass suspension from the enrichment bioreactor and initially  $0.16 (\pm 0.01)$  MPa  $^{13}\text{CH}_4$ , 15 ( $\pm 1$ ) mM sulfate, and 0.2 ( $\pm 0.1$ ) mM sulfide



In the absence of candidate-IECs (controls), there was a linear accumulation of  $^{13}\text{C}$ -DIC during the 4-day incubation (Fig. 1); this  $^{13}\text{CH}_4$  oxidation was coupled to SR (Fig. 2c), according to the stoichiometry of Eq. 1.

In the presence of acetate, formate, methanol, or hydrogen, there was also a linear accumulation of  $^{13}\text{C}$ -DIC (Fig. 1), the rates ( $3.9$ – $5.7 \mu\text{mol bottle}^{-1} \text{ day}^{-1}$ ) were comparable with the rates without a candidate-IEC ( $3.9$  and  $4.2 \mu\text{mol bottle}^{-1} \text{ day}^{-1}$ ). In the incubations with carbon monoxide, the  $^{13}\text{C}$ -DIC production rate was slightly lower ( $2.7$  and  $3.0 \mu\text{mol bottle}^{-1} \text{ day}^{-1}$ ) and methanethiol completely inhibited  $^{13}\text{C}$ -DIC production.

Some acetate ( $8.8$  and  $8.3 \mu\text{mol-C}$ ), formate ( $8.0$  and  $8.4 \mu\text{mol-C}$ ), and carbon monoxide ( $9.1$  and  $10.8 \mu\text{mol-C}$ ) were being removed during the 4-day incubation. Figure 2a shows that the removal of these candidate-IECs was coupled to  $^{12}\text{C}$ -DIC production and not  $^{12}\text{CH}_4$  production, indicating complete oxidation. Methanol ( $1.0$  and  $1.0 \mu\text{mol-C bottle}^{-1}$ ) and methanethiol ( $0.4 \mu\text{mol-C bottle}^{-1}$ ) were hardly removed. All incubations showed some (up to  $2.6 \mu\text{mol bottle}^{-1}$ ) background  $^{12}\text{C}$ -DIC production, likely released from the inoculate.

Figure 2c compares oxidation conversions with reduction conversions. In the presence of acetate, formate, carbon monoxide, and hydrogen more sulfate was reduced than  $^{13}\text{CH}_4$  oxidized. This additional SR was coupled to the oxidation of candidate-IECs. Therefore,  $\text{CH}_4$ , acetate, formate, carbon monoxide, and hydrogen were all used as electron donor for SR by the enrichment, although the oxidation of  $^{13}\text{CH}_4$  was, in all incubations, dominant over the oxidation of candidate-IECs. Both SR and  $^{13}\text{CH}_4$  oxidation were inhibited by the presence of methanethiol.

### Thermodynamic calculations

Table 2 presents the concentrations of candidate-IECs at which their production, under the applied experimental conditions, is no longer thermodynamically possible. To obtain maximum concentrations, the highest measured  $\text{CH}_4$  partial pressure ( $0.16 \text{ MPa}$ ),  $\text{HS}^-$  concentration ( $1 \text{ mM}$ ) and  $\text{HCO}_3^-$  concentrations ( $1 \text{ mM}$ ) were used for the calculations. Only for the calculation of the maximum  $\text{H}_2$  partial pressure, the lowest  $\text{HCO}_3^-$  concentration ( $0.2 \text{ mM}$ ) was used. The theoretical

maximum concentration for the production of each candidate-IEC was always at least 1,000 times lower than the actual concentration measured at the end of the experiment.

## Discussion

### Exclusion of candidate-IECs

This research shows that acetate, formate, methanol, carbon monoxide, and hydrogen can be excluded as sole IEC in AOM coupled to SR by an enrichment composed of ANME-2a and bacteria mainly belonging to *Deltaproteobacteria* and *Flavobacteriales*. The  $^{13}\text{CH}_4$  oxidation rates in the presence of these compounds were not or hardly lower than in the controls (Fig. 1). Moreover, during the 4-day incubations, the concentrations of these candidate-IECs were at least  $1,000\times$  higher than the candidate-IEC concentrations at which no more Gibbs free energy can be conserved from their production from  $\text{CH}_4$  at the applied conditions (Table 2). Nauhaus et al. (2002, 2005) already showed that acetate, formate, methanol, carbon monoxide, methylamines, and hydrogen are unlikely IECs in AOM coupled to SR by the ANME-2/bacteria community in Hydrate Ridge sediment because the SR activity on those compounds was lower than on  $\text{CH}_4$ . In Black Sea microbial mats, the SR activity with acetate was also lower than with methane, but with hydrogen and formate this was not the case, which was likely due to the rapid enrichment of SRB not involved in AOM (Nauhaus et al. 2005). Possibly the carbon monoxide concentration ( $10 \text{ kPa CO}$ ) used by Nauhaus et al. (2005) was inhibitory for sulfate reduction or the candidate-IECs were consumed by methanogens or homoacetogens. By also assessing the  $^{13}\text{CH}_4$  oxidation rate (Fig. 1), the  $^{12}\text{CH}_4$  production (Fig. 2a),  $^{12}\text{C}$ -DIC production (Fig. 2a), and the candidate-IEC consumption (Fig. 2a and c), those possibilities can be excluded in this study for acetate, formate, methanol, carbon monoxide, and hydrogen.

### Inhibition by carbon monoxide and methanethiol

$^{13}\text{CH}_4$  oxidation was slightly hampered by carbon monoxide and completely inhibited by methanethiol (Fig. 1), both

**Table 2** The concentration of candidate interspecies electron carriers (IECs) at which their production from  $\text{CH}_4$  is no longer thermodynamically possible ( $\Delta G^\circ=0$ ) at  $0.16 \text{ MPa}$   $\text{CH}_4$ ,  $0.2 \text{ mM HCO}_3^-$  (for  $\text{H}_2$ ) or  $1 \text{ mM HCO}_3^-$  (of the other potential-IECs),  $1 \text{ mM HS}^-$ ,  $30 \text{ }^\circ\text{C}$ , and a pH of 7

IEC	IEC concentration at which $\Delta G_{\text{IEC production}}^\circ=0$	IEC concentration on day 4
Acetate	$3.8 \times 10^{-9} \text{ M}$	$8.5 \times 10^{-4}$ , $8.6 \times 10^{-4} \text{ M}$
Formate	$1.0 \times 10^{-8} \text{ M}$	$7.3 \times 10^{-4}$ , $7.2 \times 10^{-4} \text{ M}$
Methanol	$1.8 \times 10^{-15} \text{ M}$	$9.3 \times 10^{-4}$ , $9.3 \times 10^{-4} \text{ M}$
Carbon monoxide	$8.4 \times 10^{-10} \text{ kPa}$	4.9, 4.4 kPa
Methanethiol	$8.4 \times 10^{-12} \text{ M}$	$9.9 \times 10^{-4} \text{ M}$
Hydrogen	$6.6 \times 10^{-4} \text{ kPa}$	3.2 kPa



compounds have been reported to be toxic. Carbon monoxide hampered SR by sulfate-reducing sludge at a concentration of 5% or higher (van Houten et al. 1995), and sulfate reducers used only methyl sulfides as substrate at low concentrations (<10  $\mu\text{M}$ ; Kiene et al. 1986). If these compounds would be produced in situ, the concentrations would remain much lower due to simultaneous consumption, therefore toxic effects would be less profound. Because methanethiol inhibited AOM, it can therefore not be excluded as IEC in AOM coupled to SR. Moran et al. (2007) also reported an inhibition of AOM by methanethiol. If electrons would be transferred via methanethiol, sulfate reducers would be able to utilize these compounds; however that did not occur (Fig. 2b). Possibly, the sulfate reducers were intoxicated by methanethiol.

#### Local concentration of candidate-IECs

Many of the candidate-IECs tested were consumed (Fig. 2a, c), which can result in a concentration gradient within the biomass flocks. Therefore, the concentration near the responsible organism can be lower than in the bulk liquid. A big difference between the concentration in the bulk liquid at the concentration near the organism mediating AOM is not expected though because the reactor suspension was well mixed (orbital shaker at 100 rpm), the biomass flocks were small (0.1 mm; Meulepas et al. 2009), and the candidate-IEC consumption rates were low (<4  $\mu\text{mol bottle}^{-1} \text{ day}^{-1}$ ).

#### Syntrophy between ANME and SRB

The reason that the addition of a candidate-IEC does not affect AOM and SR might be the involvement of more than one intermediate in AOM coupled to SR (Valentine and Reeburgh 2000; Stams and Plugge 2009). Other theories for the shuttling of electrons between ANME and SRB are that reduction equivalents are transferred via extracellular redox shuttles (Widdel and Rabus 2001; Wegener et al. 2008) or via membrane-bound redox shuttles or so-called “nanowires” (Reguera et al. 2005; Stams et al. 2006; Stams and Plugge 2009; Thauer and Shima 2008; Wegener et al. 2008). The extracellular redox shuttle theory requires the shuttle to be transported back to the ANME after donating the electrons to the SRB, giving rise to an additional loss in Gibbs free energy change available for the microorganisms, due to the concentration gradients between the syntrophic partners. The membrane-bound redox shuttles require the ANME and SRB to make direct physical contact, which is not always the case (Michaelis et al. 2002; Knittel et al. 2005; Orphan et al. 2002; Treude et al. 2005; Jagersma et al. 2009). However, Nielsen et al. (2010) showed that electrical currents in marine sediments coupled spatially separated biogeochemical processes, presumably through nanowires. Such mechanism

might also be responsible for interspecies electron transfer in AOM coupled to SR.

At in situ conditions there is only  $-22.35 \text{ kJ mol}^{-1}$  available for AOM coupled to SR (Harder 1997). Methanogenic archaea and sulfate-reducing bacteria have been shown to require a free energy change under physiological conditions of at least  $-10$  and  $-19 \text{ kJ mol}^{-1}$ , respectively, to support their metabolism in situ (Hoehler et al. 2001; Dale et al. 2006). Therefore, the in situ free energy change of AOM coupled to SR is probably not sufficiently large to fuel the energy metabolism of two microorganisms in tandem (Schink 1997; Thauer and Shima 2008). Further research should also consider the possibility that one microorganism is responsible for AOM coupled to SR.

#### Alternative electron donors

The enrichment was able to utilize acetate, formate, methanol, carbon monoxide, and hydrogen as electron donor for SR (Fig. 2c), although the enrichment was not fed with any other electron donor and carbon source than  $\text{CH}_4$  for 512 days (Meulepas et al. 2009). Prior to this, the enrichment was additionally fed with small amounts of acetate ( $70 \mu\text{mol L}^{-1} \text{ day}^{-1}$ ) for a period of 330 days. Possibly, the sulfate reducers involved in AOM coupled to SR are capable of utilizing acetate, formate, methanol, carbon monoxide, and hydrogen as alternative electron donors for the IEC or  $\text{CH}_4$ . If this would be the case, those microorganisms could be enriched on those alternative substrates instead of on  $\text{CH}_4$ . Another explanation is that other SRB, not involved in AOM coupled to SR, survived the enrichment period. This hypothesis would require inactive SRB to become active within the 4-day duration of the experiment, which is a rather short time span.

**Acknowledgments** This work was part the Anaerobic Methane Oxidation for Sulfate Reduction project supported by the Dutch ministries of Economical affairs, Education, culture and science, and Environment and special planning as part their EET program and was cofunded by King Abdullah University of Science and Technology through the SOWACOR project.

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