

# Rates and pathways of methanogenesis in hypersaline environments as determined by $^{13}\text{C}$ -labeling

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**Abstract** Rates and pathways of methane production were determined from photosynthetic soft microbial mats and gypsum-encrusted endoevaporites collected in hypersaline environments from California, Mexico and Chile, as well as an organic-rich mud from a pond in the El Tatio volcanic fields, Chile. Samples (mud, soft mats and endoevaporites) were incubated anaerobically with deoxygenated site water, and the increase in methane concentration through time in the headspaces of the incubation vials was used to determine methane production rates. To ascertain the substrates used by the methanogens,  $^{13}\text{C}$ -labeled methylamines, methanol, dimethylsulfide, acetate or bicarbonate were added to the incubations (one substrate per vial) and the stable isotopic composition of the resulting methane was measured. The vials amended with  $^{13}\text{C}$ -labeled methylamines produced the most  $^{13}\text{C}$ -enriched methane, generally followed by the

$^{13}\text{C}$ -labeled methanol-amended vials. The stable isotope data and the methane production rates were used to determine first order rate constants for each of the substrates at each of the sites. Estimates of individual substrate use revealed that the methylamines produced 55–92 % of the methane generated, while methanol was responsible for another 8–40 %.

**Keywords**  $^{13}\text{C}$ -labeling · Hypersaline environments · Methane · Stable carbon isotopes

## Introduction

Atmospheric methane may be a good indicator of past or present biological activity in the search for life on other planets, especially Mars (e.g., Parnell et al. 2007). In addition, on Mars, chloride-containing deposits (Osterloo et al. 2008) and the possibility of liquid brines on the surface (McEwen et al. 2011) have also been reported. As these features may provide a setting for biogenic methane production in extraterrestrial environments, the characterization of the relative importance of substrates that support methane production in hypersaline terrestrial environments becomes important.

On Earth approximately 80–90 % of all methane in the atmosphere is of biogenic origin (Whiticar 1999). The two main pathways that methanogens, obligate anaerobic microorganisms, use to produce methane are acetate cleavage and  $\text{CO}_2$  reduction by  $\text{H}_2$ :

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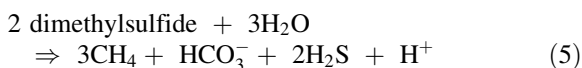
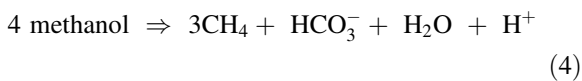
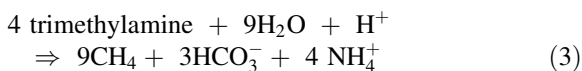
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However, in hypersaline environments, it is thought that non-competitive substrates fuel methane production (Bebout et al. 2004; Sørensen et al. 2009; Kelley et al. 2012, 2014; Tazaz et al. 2013), because of the well-known competition between sulfate reducers and methanogens for both acetate and  $\text{H}_2$  (Reeburgh 2007). These non-competitive substrates include the methylamines, methanol, and dimethylsulfide (e.g., Oremland and Polcin 1982; King 1984):



A powerful technique that has been used to infer substrate usage is stable isotopic analysis, since methanogens are known to fractionate carbon isotopes during methanogenesis producing methane that is  $^{13}\text{C}$ -depleted relative to the substrate (Whiticar 1999; Conrad 2005). Culture and environmental work suggests that methane produced from methanol should be the most  $^{13}\text{C}$ -depleted, followed by trimethylamine,  $\text{CO}_2/\text{H}_2$ , dimethylsulfide and acetate, in that order (Krzycki et al. 1987; Summons et al. 1998; Conrad 2005; Londry et al. 2008). Potter et al. (2009) used these reported differences in fractionation to predict substrate use along a salinity gradient in the salterns of Guerrero Negro, Mexico. However, in hypersaline environments, the full fractionation may not be expressed due to low substrate concentrations and near-quantitative substrate utilization (Kelley et al. 2012; Tazaz et al. 2013). Therefore, natural stable isotope abundance analyses may not be the best approach to unambiguously determine substrate usage.

Carbon isotope labeling experiments using radio-carbon ( $^{14}\text{C}$ ) have been used to determine substrates used in many metabolic pathways, including methane production (e.g., Crill and Martens 1986; Oremland et al. 1982; Lovley and Klug 1983). However, because of health and safety concerns over the use of radioactive substances for both workers and the environment,

more and more non-radioactive  $^{13}\text{C}$ -labeled substances are now available for use. The objective of this study was to use  $^{13}\text{C}$ -labeled substrate additions to determine the relative importance of methanogenic substrates in hypersaline environments.

## Methods

Methane production at three hypersaline field localities was investigated (Table 1). Two sites were managed salt ponds (the salterns of Exportadora de Sal, Guerrero Negro, Baja California Sur, Mexico and Don Edwards National Wildlife Refuge, California USA) and the third site, located within the Atacama Desert, Chile, contained salars. We also investigated a lower salinity pond (salinity was about a third that of seawater) associated with the El Tatio volcanic field, also located in the Atacama Desert in Chile.

The Guerrero Negro, Mexico site was sampled in March and October 2009 along a salinity gradient of 55 parts per thousand, ppt (Area 1) to 190 ppt (Area 9) (Kelley et al. 2012). Areas 1 and 4 (salinity of approximately 90 ppt) were both covered with thick, soft, laminated microbial mats. Area 9 had a thick gypsum crust with endoevaporitic microbial communities. In March 2012, the gypsum crust in Area 10 (salinity of 284 ppt) was also sampled. At the Don Edwards, California site, two ponds were sampled in January and August 2010 (Kelley et al. 2012): Pond 15, with a salinity of approximately 120 ppt, had a thin microbial mat veneer over black mud, and Pond 23, with a much higher salinity of approximately 300 ppt, was covered with both gypsum and halite crusts. In Chile, the Salar de Llamara (salinity of approximately 130 ppt) and the El Tatio (salinity of approximately 10 ppt) sites were sampled in May of both 2012 and 2013. Two areas within the Salar de Llamara (LL2 and LL3), containing gypsum-encrusted microbial communities, were sampled. These sites were on opposite sides of a large pool. Another small pool at Llamara (LL4), which contained a soft microbial mat overlying a gypsum sand subsurface, was also sampled. The pond sampled at El Tatio contained organic-rich sediments. During the 2013 field sampling period, an area within the Laguna Cejar of the Salar de Atacama (Cejar 3) was also sampled. Similar to the Salar de Llamara, this area also contained a gypsum-encrusted endoevaporitic microbial mat. At the time of sampling, the salinity

**Table 1** The location of sites sampled, including the date, type of environment, temperature and salinity of the overlying water, as well as the rate and  $\delta^{13}\text{C}$  values of methane produced and DIC concentrations and  $\delta^{13}\text{C}$  values

Site	Date	Type	Temp (°C)	Salinity (ppt)	CH <sub>4</sub> prod (nmol g <sup>-1</sup> d <sup>-1</sup> )	CH <sub>4</sub> $\delta^{13}\text{C}$ (‰)	DIC conc (mM)	DIC $\delta^{13}\text{C}$ (‰)
Atacama Desert, Chile								
ET2	May 2012	Mud	14	13	6.0 (2.9)	-75.0 (4.1)	n.d.	n.d.
ET2	May 2013	Mud	3	10	290 (5.1)	-79.0 (0.3)	1.7 (0.3)	-5.5 (0.4)
LL2	May 2012	Gypsum crust	25	138	1.3 (0.4)	-31.1 (0.6)	n.d.	n.d.
LL2	May 2013	Gypsum crust	22	128	1.1 (0.4)	-20.2 (0.7)	1.1 (0.2)	4.4 (0.9)
LL3	May 2012	Gypsum crust	27	131	6.8 (1.4)	-32.7 (1.0)	n.d.	n.d.
LL3	May 2013	Gypsum crust	23	132	4.0 (2.5)	-43.9 (13.1)	1.1 (0.0)	4.8 (0.8)
LL4	May 2012	Soft mat over gypsum rubble	31	132	0.1 (0.0)	-44.5 (3.4)	n.d.	n.d.
LL4	May 2013	Soft mat over gypsum rubble	30	124	0.1 (0.0)	-45.7 (0.2)	1.2 (0.0)	2.6 (2.3)
Cejar 3	May 2013	Gypsum crust	13	77	1.0 (0.1)	-35.0 (4.0)	3.8 (0.5)	0.2 (0.1)
Don Edwards National Wildlife Refuge, California, USA								
Pond 15	Jan 2010	Soft mat/mud	12	126	0.7 (0.0)	-62.9 (0.2)	4.4 (0.4)	-12.7 (0.2)
Pond 15	Aug 2010	Soft mat/mud	24	115	0.1 (0.0)	-64.3	3.2 (1.1)	-11.9 (0.2)
Pond 23	Jan 2010	Gypsum/halite crust	24	320	0.4 (0.1)	-41.5 (0.7)	n.d.	n.d.
Pond 23	Aug 2010	Gypsum/halite crust	40	275	2.3 (0.2)	-44.5 (1.1)	4.6 (0.1)	-9.9 (0.1)
Guerrero Negro, Mexico <sup>a</sup>								
Area 1	Mar 2009	Soft mat	23	55	15.8 (4.5)	-48.7 (1.7)	2.2 (0.3)	-0.5 (0.6)
Area 1	Oct 2009	Soft mat	31	55	15.5 (5.7)	-48.1 (3.4)	2.2 (0.3)	-0.5 (0.6)
Area 4	Mar 2009	Soft mat	19	93	3.4 (0.4)	-75.4 (5.3)	2.0 (0.3)	-5.8 (1.1)
Area 4	Oct 2009	Soft mat	25	84	2.2 (1.4)	-47.6 (2.0)	2.0 (0.3)	-5.8 (1.1)
Area 9	Oct 2009	Gypsum rubble	25	192	5.1 (1.2)	-33.1 (0.6)	2.7 (0.3)	-6.2 (1.3)
Area 9	Oct 2009	Gypsum crust	25	192	19.6 (0.8)	-36.2 (2.8)	2.7 (0.3)	-6.2 (1.3)
Area 10	Mar 2012	Gypsum/halite crust	26	284	0.1 (0.0)	-60.9 (4.6)	3.5 (0.6)	-5.1 (2.0)

Numbers in parentheses represent the standard deviation of triplicate samples, or half the range of duplicate samples

*n.d.* not determined

<sup>a</sup> The DIC concentrations and isotopic composition for the Guerrero Negro sites are from Des Marais et al. (1989)

of the overlying water at Laguna Cejar was below gypsum saturation at 77 ppt, presumably due to the relatively wet 2013 fall season. See Table 1 for a list of sites, dates sampled, types of environment, temperatures and salinities of the overlying water.

At all sites, methane production was determined from incubations of slurries in serum vials. For the thick soft microbial mats (Areas 1, 4), the upper 1–3 cm of the mat was used, whereas with the predominantly sediment samples (Pond 15, Et Tatio), the upper approximately 10 cm of sediment was homogenized. With LL4, the soft mat with some gypsum sand subsurface was sampled. Surface

gypsum crusts (Area 9, Area 10, Pond 23, LL2, LL3, Cejar 3), as well as the underlying gypsum rubble at Area 9, were broken into small (sand/gravel sized) pieces and further mixed to homogenize the sample. Approximately 10–20 g of material was added to deoxygenated 10 mL of site water in 38 mL serum vials. The headspace was flushed with nitrogen gas and methane concentrations in the headspace were analyzed through time to determine methane production rate. In addition to control incubation vials, in which no substrates were added, vials containing <sup>13</sup>C-labeled (99 at.%) substrates were used to determine which substrate was preferentially used by the

methanogens. The substrates tested were monomethylamine (MMA), trimethylamine (TMA), dimethylsulfide (DMS), methanol (MeOH), acetate and bicarbonate. MMA was used only in March 2009, after which a commercial source for  $^{13}\text{C}$ -labeled TMA was found and used. TMA is the breakdown product of glycine betaine, a dominant osmolyte in hypersaline environments (Oren et al. 2013), so additions of TMA over MMA should better simulate the natural environment.  $^{13}\text{C}$ -labeled DMS was attained starting in 2012, however  $^2\text{H}$ -labeled DMS was used to determine usage in October 2009. The  $^{13}\text{C}$ -labeled non-competitive substrates, as well as acetate, were added to the incubation vials in concentrations of 0.1, 1.0 and 10  $\mu\text{M}$  (final concentrations). This range in the concentrations of added substrates was chosen to maximize the chances of detecting  $^{13}\text{C}$  label in the produced methane without stimulating methanogenesis (i.e., being added in trace quantities). Only  $^{13}\text{C}$ -labeled bicarbonate was added at a single concentration of 10  $\mu\text{M}$  final concentration; dissolved inorganic carbon (DIC) was expected to be in the low mM range (Des Marais et al. 1989; Demergasso et al. 2003; Dorador et al. 2009; Tassi et al. 2010), and so an addition of 10  $\mu\text{M}$  would not significantly change the bicarbonate pool. Each incubation vial received only one labeled substrate at one concentration (in triplicate). Since no stimulation of methane production from any of the 1.0  $\mu\text{M}$  additions was observed at any of the sites, in May 2013 all  $^{13}\text{C}$ -labeled additions were added at a single concentration (1.0  $\mu\text{M}$  for substrates other than bicarbonate and 10  $\mu\text{M}$  for bicarbonate).

The vials were incubated at room temperature in the dark from about 2 to 60 days, depending on the rate of methane production. Methane concentration was analyzed generally between 3 and 5 times during this time period. By 60 days, either enough methane had accumulated in the headspace to determine its isotopic composition or it was determined that the production rate was too low for further study. The production rate was calculated from the initial linear portion of the increase in methane concentration within the headspace of the incubation vials. At the end of the incubation, the vials were frozen to stop production. The methane in the headspace was then analyzed for its stable isotopic composition using a gas chromatograph in-line with a combustion interface connected to a Thermo-Finnigan Delta Plus XL isotope

ratio mass spectrometer (GC-IRMS). If the concentrations were too low to adequately detect by injecting the headspace gas directly onto the head of the analytical GC column (<approximately 1000 ppm), larger volumes of the headspace gas were first cryo-concentrated and then cryo-focused onto the column using a set-up similar to Rice et al. (2001). Repeated injections of methane standards, either directly (3 % v/v) or by cryo-focusing (1000 ppm v/v), yielded  $\delta^{13}\text{C}$  values of  $-36.7 \pm 0.2 \text{‰}$  ( $n = 131$ ) and  $-36.9 \pm 0.2 \text{‰}$  ( $n = 207$ ) for the 3 % and 1000 ppm methane standards, respectively. The nominal value for both standards is  $-36.8 \text{‰}$ . Methane  $\delta^{13}\text{C}$  values resulting from the labeled substrates were compared to controls using  $t$  tests at the  $\alpha = 0.05$  level.

Samples for DIC concentrations and stable isotopes were obtained in August 2010 for the Don Edwards sites, and in May 2013 for the Chile sites. Sampling methods for DIC followed the approach of Kelley et al. (2006). Known volumes of filtered site water were injected into evacuated serum vials, and frozen upside-down. In the lab, 30 % phosphoric acid was added to the vial to drive all the DIC into the headspace as  $\text{CO}_2$ . The pressure in the headspace was brought back to atmospheric pressure by adding high purity helium.  $\text{CO}_2$  concentrations and stable isotopic composition were then obtained by injecting known quantities of headspace into the GC-IRMS, and comparing to the  $\text{CO}_2$  reference gas. DIC data for the Guerrero Negro sites were taken from Des Marais et al. (1989).

Since the DIC concentrations and isotopic compositions were known, a simple mass balance could be employed to determine the isotopic composition (in at.%) of the DIC pool within vials containing the added  $^{13}\text{C}$ -label:

$$\begin{aligned} \text{at.\% of DIC in labeled vials} \\ = \frac{(DIC_{\text{conc}})(DIC_{\text{at.\%}}) + (DIC_{\text{label}})(DIC_{\text{label at.\%}})}{(DIC_{\text{conc}} + DIC_{\text{label}}} \end{aligned} \quad (6)$$

where  $\text{DIC}_{\text{conc}}$  is the concentration of DIC in the pond water,  $\text{DIC at.\%}$  is the stable isotopic composition of the DIC in the pond water expressed as at.%,  $\text{DIC}_{\text{label}}$  is the concentration of labeled DIC added (=10  $\mu\text{M}$ ),  $\text{DIC}_{\text{label at.\%}}$  is the stable isotopic composition of the labeled DIC expressed as at.% (=99 at.%).

Similarly, once the isotopic composition of the DIC pool within labeled vials was determined, another

mass balance could be used to determine the fraction ( $f_{\text{DIC}}$ ) of methane produced from the labeled DIC:

yielded the  $^{13}\text{C}$ -methane production rate (i.e., the methane production rate from the labeled substrate).

$$f_{\text{DIC}} = \frac{(\text{at.}\% \text{ of methane in labeled vial}) - (\text{at.}\% \text{ of methane in control vial})}{(\text{at.}\% \text{ of DIC in labeled vial}) - (\text{at.}\% \text{ of methane in control vial})} \quad (7)$$

This fraction ( $f_{\text{DIC}}$ ) was then multiplied by the measured methane production rate to calculate the rate of methane produced from DIC (i.e., the  $\text{CO}_2/\text{H}_2$  pathway).

For the above mass balances, the DIC concentrations and  $\delta^{13}\text{C}$  values used were those obtained from the various ponds at the time of sample collection. No attempt was made to correct for increases in DIC concentrations (and decreases in DIC  $\delta^{13}\text{C}$  values) due to respiration within the incubations vials, although this undoubtedly occurred (e.g., Beaudoin, 2015). The above fraction ( $f_{\text{DIC}}$ ) of methane produced from labeled DIC would therefore be a minimum, since the pool of DIC from respiration would dilute the  $^{13}\text{C}$ -labeled DIC within the vial causing the at.% of DIC in the labeled vial to be lower.

Unlike the DIC, the concentrations and in situ stable isotopic compositions were not known for the other methanogenic substrates. In order to assess their usage at these sites, the stable isotope values in the labeled incubation vials were used to obtain a first order rate constant ( $k$ ) for each of the pathways of methanogenesis, since the amount of  $^{13}\text{C}$  label added and the  $^{13}\text{C}$ -methane production rate was known or could be calculated. To obtain rate constants for the substrates at each site, a mass balance similar to Eq. (7) was used to obtain the fraction of methane ( $f$ ) produced from the labeled substrate. Instead of using the at.% of the substrate pool, the at.% of the labeled substrate (=99 at.%) was used:

The measured rate ( $\text{nmol g}^{-1} \text{d}^{-1}$ ) was corrected by the density of the sample at each of the sites, determined by water displacement, which ranged from 1.0 to  $2.3 \text{ g cm}^{-3}$ , to obtain a volumetric rate ( $\mu\text{M d}^{-1}$ ). This volumetric production rate was then plotted against the concentration of  $^{13}\text{C}$ -labeled substrate added to the vials (0.1, 1.0 and  $10 \mu\text{M}$ ); the slope of the line yielded the rate constant. An example of such a plot is shown in Fig. 1. The slopes for each substrate and site were tested for significance (greater than zero) at the  $\alpha = 0.05$  level. In addition, within a site, slopes were compared and tested, again at the  $\alpha = 0.05$  level. At all sites tested, TMA/MMA and MeOH had positive slopes ( $p < 0.05$ ) and were different from each other ( $p < 0.05$ ), whereas slopes for acetate usage were not different from zero ( $p > 0.05$ ).

When only one concentration of labeled substrate was used (i.e., the additions in 2013), the rate constant was determined from the  $^{13}\text{C}$ -methane production rate divided by the concentration of added label:

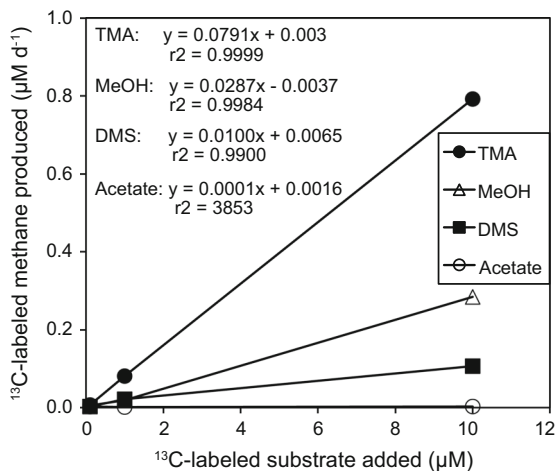
$$k = \frac{\text{rate of } ^{13}\text{C} - \text{methane produced}}{\text{concentration of } ^{13}\text{C} - \text{labeled substrate added}} \quad (9)$$

Since the relative amount of  $^{13}\text{C}$ -label measured in the produced methane pool may be dependent upon the incubation time as that affects the dilution of the label by the production of  $^{13}\text{C}$ -depleted methane from in situ substrates (e.g., Conrad and Claus 2005), the

$$f = \frac{(\text{at.}\% \text{ of methane in labeled vial}) - (\text{at.}\% \text{ of methane in control vial})}{(\text{at.}\% \text{ of labeled substrate}) - (\text{at.}\% \text{ of methane in control vial})} \quad (8)$$

Multiplying this fraction ( $f$ ) by the measured methane production rate for each substrate concentration then

fraction of methane produced from substrates in May 2013 are maximum values. However, the rate



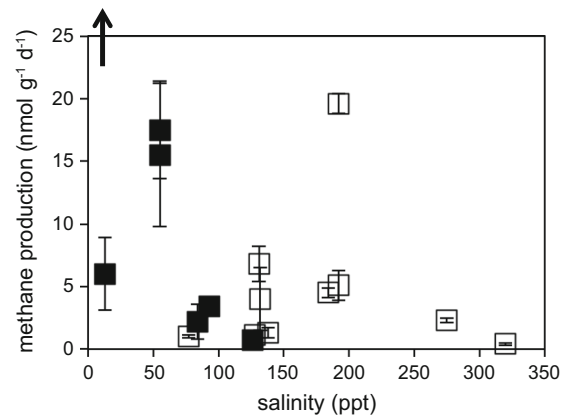
**Fig. 1**  $^{13}\text{C}$ -methane production rates as a function of  $^{13}\text{C}$ -labeled substrate additions for the El Tatio, Chile site in May 2012. The slopes of the linear regression lines are the first order rate constants for each of the substrates tested

constants calculated from the linear regressions using three substrate concentrations (e.g., Fig. 1) would be unaffected, assuming that dilution from  $^{13}\text{C}$ -depleted methane production in labeled vials was similar across each substrate concentration.

## Results

Both soft microbial mats/muds and gypsum-encrusted endoevaporites were sampled for methane production. A few sites of both types, including LL4, Pond 15 (sampled in August 2010), and Area 10, produced little to no methane ( $\leq 0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$ ) (Table 1). Data from these sites/times will not be discussed further. Of the sites that produced methane, production rates (Table 1) ranged from  $0.4 \pm 0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$  (Pond 23 sampled in January 2010) to  $290 \pm 5 \text{ nmol g}^{-1} \text{ d}^{-1}$  (El Tatio in May 2013). The production rates did not scale with salinity ( $p > 0.05$ ; Fig. 2), although the lowest salinity site (El Tatio) did have the highest production rate.

The isotopic composition of the methane produced in the control incubation vials (no  $^{13}\text{C}$ -labeled substrates added) also had a wide range of values. Of those samples that produced more than  $0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$ , the  $\delta^{13}\text{C}$  values ranged from  $-79.0$  to  $-20.2 \text{ ‰}$  (Table 1). Methane produced at sites dominated by soft microbial mats/muds was consistently more



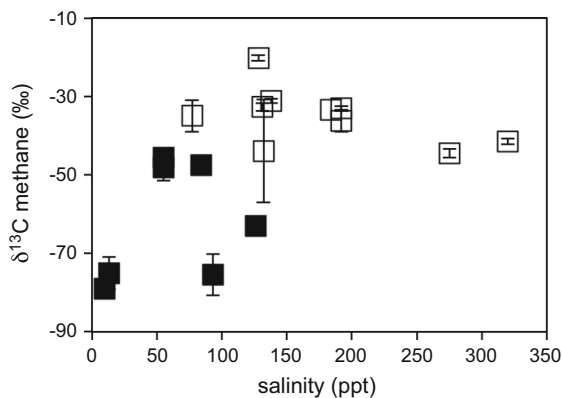
**Fig. 2** Methane production rates as a function of the salinity at the sites. In order to show the variations among the sites, the data point representing the pond at El Tatio in May 2013 ( $10 \text{ ppt}$ ,  $290 \text{ nmol g}^{-1} \text{ d}^{-1}$ ) is off-scale (indicated by the arrow). The sites are divided into soft mat/mud (filled squares) and gypsum-encrusted (open squares) sites. Error bars represent the standard deviation of triplicate samples. Only those samples/sites that produced more than  $0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$  of methane are shown

$^{13}\text{C}$ -depleted ( $p < 0.05$ ) than that produced in the gypsum (Fig. 3). The range of  $\delta^{13}\text{C}$  values for the soft microbial mats/mud was  $-79.0$  to  $-47.6 \text{ ‰}$ , whereas the range for the gypsum-encrusted sites was  $-44.5$  to  $-20.2 \text{ ‰}$ .

DIC concentrations in the water columns of the ponds ranged from  $3.2$  to  $4.6 \text{ mM}$  at Ponds 15 and 23 of Don Edwards, and from  $1.1$  to  $3.8 \text{ mM}$  at the Chilean sites (Table 1). Isotopically, the  $\delta^{13}\text{C}$  values of the DIC ranged from  $-11.9$  to  $-9.9 \text{ ‰}$  at the Don Edwards sites. The  $\delta^{13}\text{C}$  values of the DIC were much higher at the Chilean sites, ranging from  $-5.5$  to  $+4.6 \text{ ‰}$  (Table 1). The highest DIC  $\delta^{13}\text{C}$  values came from the gypsum sites of the Salar de Llamara. The DIC concentrations and stable isotopic composition used for the Guerrero Negro sites came from Des Marais et al. (1989) and are also listed in Table 1. These values ranged from  $2.2$  to  $3.5 \text{ mM}$  and  $-0.5$  to  $-6.2 \text{ ‰}$ , respectively (Des Marais et al. 1989).

The addition of  $^{13}\text{C}$ -labeled substrates to the incubation vials did not stimulate methane production relative to the overall rates in the control vials, similar to what had been seen previously (Kelley et al. 2012). The vials at all sites receiving the  $^{13}\text{C}$ -labeled MMA or TMA produced methane with the most  $^{13}\text{C}$ -enriched  $\delta^{13}\text{C}$  values ( $p < 0.05$ ; Table 2; Figs. 4, 5).





**Fig. 3** The isotopic composition of methane produced as a function of salinity. The sites are divided into soft mat/mud (filled squares) and gypsum-encrusted (open squares) sites. Error bars represent the standard deviation of triplicate samples. Only those samples/sites that produced more than  $0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$  of methane are shown

Based on  $^{13}\text{C}$ -enrichments in the produced methane, TMA/MMA followed by MeOH were the main substrates used at the Guerrero Negro and Don Edwards sites (Fig. 4). The vials receiving the

$^{13}\text{C}$ -labeled competitive substrates (bicarbonate and acetate) yielded methane  $\delta^{13}\text{C}$  values that were not different from the controls ( $p > 0.05$ ), except on three occasions for the  $^{13}\text{C}$ -labeled bicarbonate-amended vials. For both Don Edwards sites in January 2010 and the Area 9 crust in October 2009, the  $\delta^{13}\text{C}$  values of the methane produced in the  $^{13}\text{C}$ -labeled bicarbonate-amended vials were statistically higher ( $p < 0.05$ ) than the  $\delta^{13}\text{C}$  values of the control vials, however overall enrichments were only on the order of, at most, 6 ‰ (Table 2). The Chilean sites also exhibited greatest methane  $^{13}\text{C}$  enrichments in TMA-amended ( $p < 0.05$ ) followed by MeOH-amended ( $p < 0.05$ ) vials (Fig. 5), indicating the use of these substrates by methanogens at the sites. In addition, the methanogens in the gypsum crusts of Salar de Llamara and at El Tatio also used bicarbonate ( $p < 0.05$ ), with greater methane  $^{13}\text{C}$ -enrichments observed in 2012 than in 2013 (Table 2). In addition to using bicarbonate, methanogens within the sediments at the volcanic fields of El Tatio also used DMS, producing  $^{13}\text{C}$ -enriched methane in  $^{13}\text{C}$ -labeled DMS-amended vials ( $p < 0.05$ ). In fact, the organic-rich sediments in the

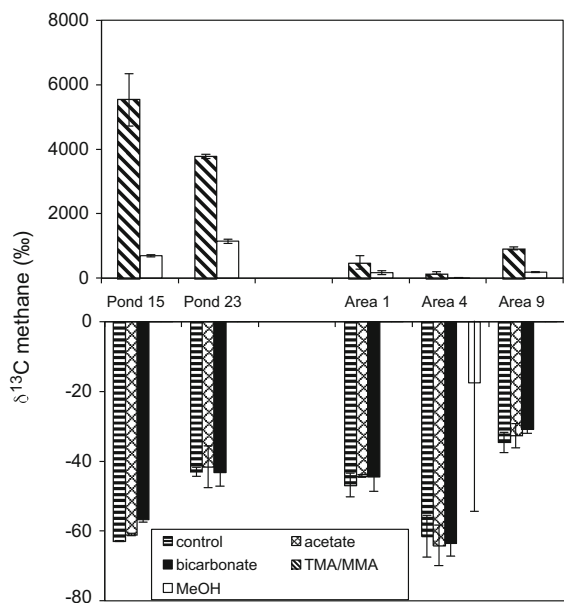
**Table 2** Stable carbon isotopic composition of methane produced in control (no  $^{13}\text{C}$ -label added) vials, and in vials receiving  $^{13}\text{C}$ -labeled substrates

Site	Date	Control	Acetate	Bicarb	DMS	TMA/MMA	MeOH
Atacama Desert, Chile							
ET2	May 2012	-75.0 (4.1)	-61.4 (13.7)	5.4 (20.4)	221 (190)	899 (597)	156 (235)
ET2	May 2013	-79.0 (0.3)	-75.1 (0.6)	-64.5 (1.1)	-45.7 (3.4)	67.1 (12.2)	-23.6 (8.0)
LL2	May 2012	-31.1 (0.6)	-30.9 (0.7)	240 (28.3)	-22.6 (1.2)	2090 (560)	58.8 (29.1)
LL3	May 2013	-43.9 (13.1)	-44.1 (10.6)	-28.6 (2.5)	-49.7 (1.3)	448 (67.6)	-29.0 (24.6)
Cejar 3	May 2013	-35.0 (4.0)	-42.3 (5.4)	-40.4 (2.0)	-41.9 (0.8)	243 (3.2)	-19.8 (6.8)
Don Edwards National Wildlife Refuge, California, USA							
Pond 15	Jan 2010	-62.9 (0.2)	-61.0 (0.4)	-56.7 (0.8)	n.d.	5530 (814)	694 (34.9)
Pond 23	Jan 2010	-41.5 (0.7)	-35.6 (5.0)	-37.9 (0.2)	n.d.	6380 (60.1)	1850 (33.9)
Pond 23	Aug 2010	-44.5 (1.1)	-47.5 (3.4)	-48.5 (3.9)	n.d.	1170 (25.1)	444 (60.1)
Guerrero Negro, Mexico							
Area 1	Mar 2009	-45.6 (0.3)	-44.2 (0.4)	-44.5 (4.1)	n.d.	382 (30.4) <sup>a</sup>	254 (58.2)
Area 1	Oct 2009	-48.1 (34)	n.d.	n.d.	n.d.	601 (209)	86.5 (17.6)
Area 4	Mar 2009	-75.4 (5.3)	-70.8 (0.3)	-69.1 (0.9)	n.d.	-68.3 (0.7) <sup>a</sup>	-70.2 (1.1)
Area 4	Oct 2009	-47.6 (2.8)	-57.3 (5.8)	-58.0 (3.5)	n.d.	394 (42.2)	35.2 (36.8)
Area 9 rubble	Oct 2009	-33.1 (0.6)	-32.8 (1.2)	-31.8 (1.0)	n.d.	1310 (48.8)	276 (14.8)
Area 9 crust	Oct 2009	-36.2 (2.8)	-32.6 (3.3)	-29.9 (0.5)	n.d.	526 (1.4)	99.4 (1.2)

Data shown are for vials receiving  $1 \mu\text{M}$  (final concentration)  $^{13}\text{C}$ -labeled substrates except bicarbonate, which was added to a final concentration of  $10 \mu\text{M}$ ; the standard deviation of triplicate samples or half the range of duplicate samples is shown in parentheses

n.d. not determined

<sup>a</sup> For the March 2009 samples, MMA was used instead of TMA



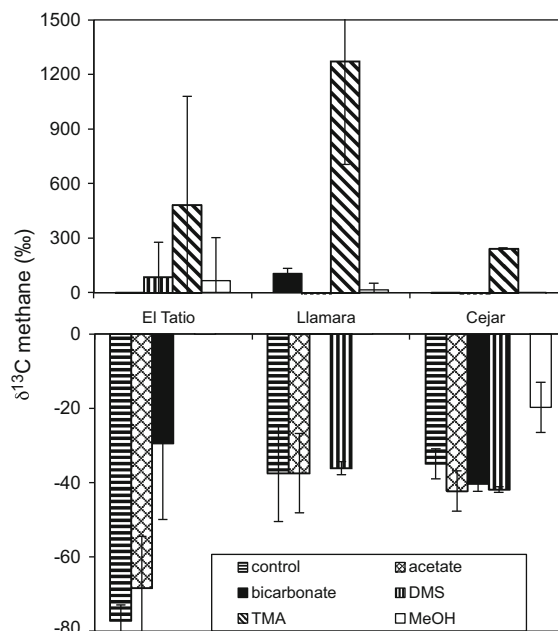
**Fig. 4** The stable isotopic composition of methane produced at the Guerrero Negro and Don Edwards sites. The data shown are the controls plus 1  $\mu\text{M}$   $^{13}\text{C}$ -labeled substrate additions, except for  $^{13}\text{C}$ -labeled bicarbonate, which was added to a final concentration of 10  $\mu\text{M}$ . Guerrero Negro data (Areas 1, 4, 9) are averages from March and October 2009; the Don Edwards data (Ponds 15 and 23) are averages from January and August 2010. *Top panel* those samples that yielded positive  $\delta^{13}\text{C}$  values; *bottom panel* controls and those samples that yielded negative  $\delta^{13}\text{C}$  values, despite the addition of  $^{13}\text{C}$ -labeled substrate

brackish pool at El Tatio appeared to use, to some extent, all substrates tested (Fig. 5), although the use of acetate was only significant, as shown by  $^{13}\text{C}$ -enrichment in the produced methane, in 2013 ( $p < 0.05$ ).

Not surprisingly, the calculated rate constants (Table 3) mirror the stable isotope results. The highest rate constants for each of the sites were for TMA/MMA and MeOH use. Interestingly, although the production rates were very different at the El Tatio site (ET2) between May 2012 and May 2013 (Table 1), the rate constants for TMA were about threefold higher than the rate constants for MeOH (Table 3) indicating the relative utilization of these two substrates were similar in both years.

## Discussion

This paper reports on the use of  $^{13}\text{C}$ -labeled substrates (both competitive and non-competitive) to determine



**Fig. 5** The stable isotopic composition of methane produced at the Chilean sites. The data shown are the controls plus 1  $\mu\text{M}$   $^{13}\text{C}$ -labeled substrate additions, except for  $^{13}\text{C}$ -labeled bicarbonate, which was added to a final concentration of 10  $\mu\text{M}$ . The El Tatio and the Salar de Llamara data were averaged over 2012 and 2013. Laguna Cejar was only sampled in 2013. *Top panel* those samples that yielded positive  $\delta^{13}\text{C}$  values; *bottom panel* controls and those samples that yielded negative  $\delta^{13}\text{C}$  values, despite the addition of  $^{13}\text{C}$ -labeled substrate

the relative importance of the substrates that support methane production in hypersaline sites in both North and South America. From these data it's evident that TMA/MMA, as well as MeOH, are significant sources of methane produced in these systems. Although all sites used TMA/MMA and MeOH, there were differences among the sites with regards to substrate usage. The methanogens at the sites in Guerrero Negro and those at the Don Edwards National Wildlife Refuge all relied heavily on TMA/MMA and MeOH as substrates for methane. Vials with either added  $^{13}\text{C}$ -labeled TMA/MMA or MeOH all produced methane that was very  $^{13}\text{C}$ -enriched ( $\delta^{13}\text{C}$  values generally  $\gg 0$  ‰), with TMA/MMA producing more  $^{13}\text{C}$ -enriched methane than the MeOH. TMA was also a preferred substrate at the Chilean sites, however, MeOH was used to a lesser extent there, and it appeared that  $\text{CO}_2/\text{H}_2$  was also used. In the low salinity El Tatio sediments, DMS was also used as a substrate for methane production, consistent with the high reduced



**Table 3** Calculated first order rate constants (k) for methane production at the sites

Site	Date	MMA	TMA	MeOH	DMS	Acetate	Bicarb	TMA/MeOH <sup>a</sup>
Atacama Desert, Chile								
ET2	May 2012		79.1	28.7	10.0	0.1	0.7	2.8
ET2	May 2013		434.9	150.2	94.5	11.4	4.3	2.9
LL2	May 2012		41.3	7.7	0.2	<0 <sup>b</sup>	0.3	5.4
LL3	May 2013		33.4	1.3	<0	<0	0.2	25.2
Cejar 3	May 2013		5.2	0.4	<0	<0	<0	14.5
Don Edwards National Wildlife Refuge, California, USA								
Pond 15	Jan 2010		50.3	5.9	n.d.	0.0	0.0	8.5
Pond 23	Jan 2010		45.1	9.5	n.d.	0.0	0.0	4.7
Pond 23	Aug 2010		39.9	16.6	n.d.	<0	<0	2.4
Guerrero Negro, Mexico								
Area 1	Mar 2009	121.2	n.d.	66.2	n.d.	<0	0.0	1.8 <sup>c</sup>
Area 1	Oct 2009		122.7	43.0	<0 <sup>d</sup>	n.d.	n.d.	2.9
Area 4	Mar 2009	0.1	n.d.	0.0	n.d.	0.0	0.0	5.0 <sup>e</sup>
Area 4	Oct 2009		16.4	1.4	0.0	<0	<0	11.7
Area 9 rubble	Oct 2009		119.2	33.7	0.6 <sup>d</sup>	0.0	0.0	3.5
Area 9 crust	Oct 2009		95.0	39.1	0.1 <sup>d</sup>	<0	0.2	2.4

The rate constants are in units of  $\text{d}^{-1}$ , however they have been multiplied by  $10^3$  for ease of view

<sup>a</sup> TMA/MeOH is the ratio between the rate constants of TMA and MeOH

<sup>b</sup> For samples showing <0, the isotopic composition of methane produced in vials containing the  $^{13}\text{C}$ -labeled substrate was more  $^{13}\text{C}$ -depleted than the control

<sup>c</sup> For March 2009 samples, MMA was used for the TMA/MeOH ratio

<sup>d</sup> For October 2009 samples,  $^2\text{H}$ -labeled DMS (99 at.%) was used to determine DMS usage

<sup>e</sup> The rate constants for MMA and MeOH were 0.00010 and 0.00002  $\text{d}^{-1}$ , respectively

sulfur content at this volcanic site (Tassi et al. 2010). In fact, all substrates, including acetate (which was not taken up at any other site), were used to some extent at El Tatio, probably due to the low sulfate concentration ( $\leq 2$  mM; unpublished data). At all other sites, sulfate concentrations greatly exceeded seawater concentrations ( $\sim 29$  mM), and so, presumably, was never depleted within the incubation vials.

It was interesting that the gypsum sites of the Atacama Desert in Chile had lower rate constants for MeOH than the gypsum site in either Guerrero Negro or Don Edwards (Table 3). This difference may be related to the environmental conditions of the sites. It is known that MeOH is derived from the degradation of pectin (Schink and Zeikus, 1982) found in vascular plants. Freshwater sites, or those sites with relatively higher allochthonous input, may have greater MeOH utilization. In fact, Zhang et al. (2008) and Jiang et al. (2010) found that methanogenesis occurring in

wetlands in the Tibetan plateau favored MeOH use. The Atacama Desert of Chile is known as the driest place on Earth (McKay et al. 2003), and as such, there was much less vegetation surrounding the sites at Salar de Llamara and Laguna Cejar than the sites in Guerrero Negro and Don Edwards. Although recent work has shown that MeOH can also be produced by phytoplankton (Mincer and Aicher 2013; Aicher and Mincer 2014), including cyanobacteria and diatoms, the relative amounts of MeOH released are presumably less than that released by vascular plants.

In addition to the rate constants, ideally one would like to determine the relative amounts of methane that were produced from each of the substrates. However, in order to do this, the substrate concentrations need to be known. Of all the substrates, only DIC concentrations were measured (Chilean and Don Edwards sites) or could be obtained from literature values (Guerrero Negro sites) for these sites (Table 1). DIC

concentrations for all sites were in the mM range, ranging from about 1 mM (Salar de Llamara) to about 5.5 mM (Don Edwards Pond 23). Using the measured or literature values for DIC concentrations for the sites, rates of methane production from CO<sub>2</sub>/H<sub>2</sub> usage ranged from 0 (not used at all) to 6.4 nmol g<sup>-1</sup> d<sup>-1</sup> (at El Tatio in May 2013). The total methane production for the El Tatio site at this time was 290 nmol g<sup>-1</sup> d<sup>-1</sup>, so CO<sub>2</sub>/H<sub>2</sub> accounted for 2.2 % of the total methane produced. In 2012, much less methane was produced at El Tatio (~6 nmol g<sup>-1</sup> d<sup>-1</sup>) with about 1 nmol g<sup>-1</sup> d<sup>-1</sup> coming from CO<sub>2</sub>/H<sub>2</sub>, accounting for about 15 % of the methane produced. At the Salar de Llamara, CO<sub>2</sub>/H<sub>2</sub> accounted for 7 % of the total methane produced in 2012 and 2013. At other sites that showed some CO<sub>2</sub>/H<sub>2</sub> use (Area 9 crust October 2009, Pond 15 January 2010, and Pond 23 January 2010), it accounted for less than 2 % of the total methane produced. As stated above, for the substrates other than DIC, the pool sizes at our sites are not known. In the few studies in which these substrates have been measured, the concentrations have been low, with the non-competitive substrates (TMA, MeOH, DMS) generally in the single μM range (Lovley and Klug 1983; King et al. 1983; Sørensen and Glob 1987; Lomans et al. 1999); acetate concentrations tend to be somewhat higher, ranging up into the low hundreds of μM (King 1991; Blair and Carter 1992; Stets et al. 2004; Waldron et al. 2007). Unfortunately, the above studies were not from hypersaline environments.

Since the concentrations of these methane substrates were not measured at our sites, the fraction of total substrate of interest used over the incubation period relative to the initial amount was calculated:

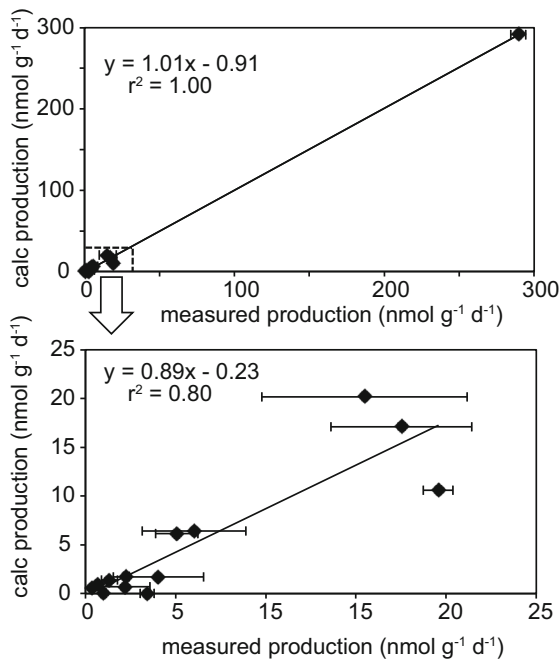
$$\text{Fraction of total substrate of interest used} = e^{-(\text{incubation time})/t_{1/2}} \quad (10)$$

where  $t_{1/2}$  is the half-life of the substrate of interest and is dependent on the first order rate constant ( $t_{1/2} = \ln 2/k$ ). Depending on the site and the substrate, from 0 (acetate at most sites) to 96 % (TMA at Don Edwards Pond 15) of the substrate of interest was used. This fraction of substrate used was then divided by  $f$ , the fraction of the total methane produced from the <sup>13</sup>C-labeled substrate (from Eq. 8), to yield a dilution factor representing the total amount of substrate of interest (both unlabeled and <sup>13</sup>C-labeled) used relative to the <sup>13</sup>C-labeled substrate used. This dilution factor

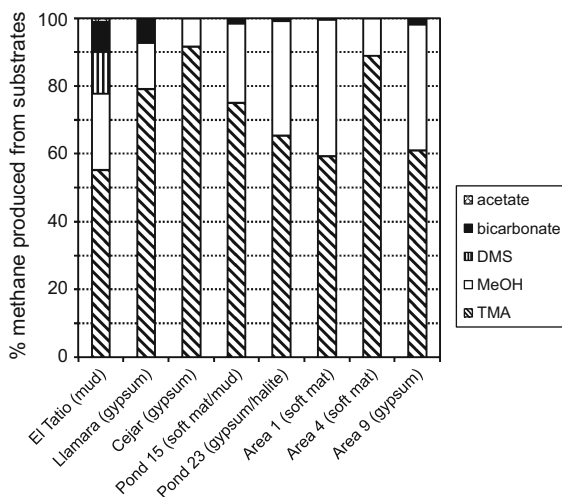
was then multiplied by the <sup>13</sup>C-methane production rate to obtain the total methane produced from any particular substrate. Because of the fact that there was methane produced from non-labeled substrates other than the substrate of interest (included in  $f$  above), implicit in this dilution factor is the assumption that the substrate of interest concentration is equal to the total amount of methane produced. We recognize that there is nothing a priori which would lead one to think that the concentrations of the substrates would be equal to the amount of methane produced (or to each other). However, this reasoning does imply that the substrate concentrations would scale with the amount of methane produced (i.e., more methane produced would require more substrate), which seems likely given that these environments are substrate-limited. Also, given the above literature search, it seems likely that the substrate concentrations would be of approximately the same order of magnitude. Because of the assumptions involved, the sum of these calculated rates (as determined from above and using the previously calculated methane produced from DIC) at each of the sites are plotted against the measured rates determined from the increase in headspace methane concentrations in the incubation vials (Fig. 6). The very good correlation between the calculated and measured production rates lends credence to our approach.

Using the above approach, the % methane produced from each of the various substrates at the different sites is plotted in Fig. 7. At all sites, TMA/MMA is the leading substrate used, producing 55–92 % of the methane generated, with MeOH contributing most of the rest (from 8 to 40 %). Taken together, TMA/MMA and MeOH were the substrates used to produce more than 98 % of all the methane generated at the sites in Guerrero Negro and Don Edwards National Wildlife Refuge, with the rest from CO<sub>2</sub>/H<sub>2</sub>. At the Chilean sites, TMA and MeOH together produced from 78 % (El Tatio) to 93 % (Salar de Llamara) to 100 % (Laguna Cejar) of the methane, with more than 90 % of the methane at Laguna Cejar being produced from TMA alone. At Salar de Llamara, use of CO<sub>2</sub>/H<sub>2</sub> made up the remaining 7 %, whereas at the low salinity El Tatio site, the remaining 22 % was divided between DMS (12 %), CO<sub>2</sub>/H<sub>2</sub> (9 %) and acetate use (1 %).

In conclusion, we have shown that methanogenesis occurring in hypersaline environments, representing a



**Fig. 6** Calculated versus measured methane production rates. The calculated rates were determined by summing the methane produced from each of the individual substrates at each site, as calculated from  $k$  values (for TMA, MeOH, DMS, and acetate) and DIC concentrations and  $\delta^{13}\text{C}$  values (for bicarbonate). The *bottom panel* is the data contained within the *dashed inset* in the *top panel* ( $<25 \text{ nmol g}^{-1} \text{ d}^{-1}$  data), with the regression curve omitting the  $290 \text{ nmol g}^{-1} \text{ d}^{-1}$  data point shown (and included in the regression) in the *upper panel*



**Fig. 7** The % methane produced from each of the substrates for the sites

wide range of salinities, depend on non-competitive substrates, with TMA being the dominant substrate used at all sites examined. This prevalence of TMA use is consistent with the important role of glycine betaine, a precursor to TMA, as a dominant osmotic solute used by organisms in hypersaline environments (Oren et al. 2013). From phylogenetic work, it has also been shown that methylotrophic Archaea, those methanogens who use the non-competitive methylated substrates, are the main methanogens in hypersaline environments (e.g., García-Maldonado et al. 2012; Lazar et al. 2011; Sørensen et al. 2009). However, the high usage of TMA and MeOH should produce quite  $^{13}\text{C}$ -depleted  $\delta^{13}\text{C}$  values of the methane since the fractionation of carbon isotopes is large with the use of these substrates (Krzycki et al. 1987; Summons et al. 1998; Londry et al. 2008). Although El Tatio and Area 4 had methane values  $<-70 \text{ ‰}$ , most of the sites, and all of the gypsum-encrusted sites, had methane  $\delta^{13}\text{C}$  values  $>-50 \text{ ‰}$  (Table 1; Fig. 3), supporting the generality of substrate limitation at hypersaline sites (e.g., Kelley et al. 2012, 2014). If methane  $\delta^{13}\text{C}$  values alone are used for the determination of biogenicity on other planets, especially those with evidence of current or past brines, such as Mars, false negatives may result. In light of this, the use of methane  $\delta^2\text{H}$  values or ratios of methane to higher hydrocarbons (e.g., Bernard et al. 1976) along with  $\delta^{13}\text{C}$  values could help to alleviate this problem.

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