C-1 utilization in marine estuary sediments: using energetics to predict microbial ecology and physiology

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Abstract

The utilization of C-1 carbon compounds as an energy source in marine environments are assumed to be the sole domain of methanogenic archaea, even though thermodynamics predicts that sulfate reducing bacteria (SRB) are able to generate more energy from the oxidation of such substrates. However if the use of the methyl-reduction pathway is invoked it is possible that methanogenesis should not only out-compete SRB for C-1 compounds but should also display the ability, in theory, to reduce the threshold of the hydrogen partial pressure below that which SRB can utilize. Methanogenesis is often believed to generate a lesser amount of cellular energy than sulfate reduction, and such a finding could remodel the microbial ecology of marine environments. Analysis of enrichments grown on methanol, trimethylamine (TMA) or betaine in the presence or absence of hydrogen indicates that methyl-reduction may occur in marine estuaries, but SRB grew more rapidly under the conditions tested.

Introduction

Using thermodynamics to predict aspects of microbial energetics and the availability of ecological niches has been a catalyst in the discovery of unsuspected types of prokaryotic physiology. Perhaps one of the most striking examples of such an application was the discovery of the anaerobic ammonium oxidizing (anammox) bacteria. Here, this model is applied to address questions arising from the catabolism of C-1 compounds in marine environments, particularly regarding the absence of the participation of sulfate reducing bacteria (SRB) in this process. Marine environments, such as marine estuaries are associated with high concentrations of sulfate compared to freshwater ecosystems. Therefore one might expect that SRB would be the dominant microorganisms involved in catabolism of C-1 compounds, given that sulfate reduction with methanol is a more energetically favorable metabolic reaction compared to methanol disproportionation by methanogenic archaea. The observation that methanogens are the microorganisms responsible for catabolism of C-1 compounds regardless of the differences in free energy of the reactions could indicate an additional component in the metabolic pathway that was previously unconsidered. Methanogenic archaea are capable of C-1 reduction by two different mechanisms. The methlyotrophic pathway uses the disproportionation of C-1 compounds as mentioned above. A second form of C-1 reduction, the methyl-reduction pathway appears to be less common among methanogenic archaea and requires molecular hydrogen as the electron donor. This pathway is known to be present in only two known isolates of methanogenic archaea, both of which were isolated from symbiotic hosts. Dr. Jaret Ledbetter proposes that SRB do not utilize C-1 compounds due to the presence of methanogenic archaea present in the marine estuary sediments that reduce C-1 compounds in the presence of hydrogen. Surprisingly the free energy calculation for the reduction of methanol with hydrogen theoretically yields more energy than sulfate reduction per mole of molecular hydrogen, and displays the ability to decrease the threshold of hydrogen partial pressure well beneath that which is required for sulfate reduction. This suggests that in the presence of certain C-1 compounds that methanogens are able to out-compete sulfate reducers for hydrogen. Sediments from Trunk River, MA. were used to inoculate media that contained one of three carbon sources (methanol, trimethylamine or betaine) which were incubated in the presence or absence of hydrogen

gas. Through direct plating and enrichment methods, this research aims to answer three main questions. Firstly, does methanogenesis of C-1 compounds in marine environments occur through the methylotrophic pathway, or the methyl-reduction pathway? Secondly, do SRB with the capability of oxidizing C-1 compounds exist in marine sediments? Finally, if reduction of C-1 compounds is occurring by the methyl-reduction pathway, can methanogens out-compete SRB for hydrogen?

Materials and Methods

Sampling

A sediment core sample was obtained from Trunk River, MA. collected at a depth of ~ 24 inches from an area that was rich in organic matter, and covered with a low level of water. Intense gas bubble formation due to external pressure exerted on the organic matter, indicated that Methanogenesis appeared to be occurring. The core sample was immediately sealed, and removed from the presence of sunlight. The sample was stored in an anaerobic glove box until ready for use. In this particular case the sample sat for three days before inoculation, which may have effected the natural population.

Media composition and incubation conditions

Dilute seawater media was used for all enrichments and agar plates. Seawater was filtered through 3 layers of Whatman filter paper, to remove large particulate matter and then through a 0.2 micron to remove the majority of the microbial biomass. *Dilute seawater media recipe:* Resazurin (0.1%) and 5 ml of trace elements were added to 750 ml filtered seawater and 300 ml of deionized water in a 3 liter round bottom flask and boiled for 10 minutes, then slightly stoppered and cooled stream of N_2/CO_2 until completely cooled, upon which the media was sealed with screw on stopper, and brought into an anaerobic chamber. Pre-weight the following chemicals and place them in the anaerobic chamber the day before. Add to cooled media in the following order: $NaHCO_3$ (4.2 g, which acts as a buffer), NH_4Cl (0.8 g), Cysteine-HCl (0.5g), KH_2PO_4 (1.0 ml, 1M anaerobic

solution). Adjust volume to 1.0L with anaerobic dH_2O. Allow media to clear before addition of Na_2S-$9H_2O$ (2 ml, 0.2M anaerobic solution). This allows the Cysteine-HCl to completely remove the remaining oxygen in the media and prevents the Na_2S-$9H_2O$ reductant from forming reactive oxygen species that can be toxic to some organisms. For plates add 7.5 grams of agar per 500ml of media (1% agar). One of three carbon sources in the form of anaerobic solutions (100X stock) was used to supplement the dilute seawater media: methanol (60mM), trimethylamine (20mM) or betaine (20mM). Media (30 mls per 160ml bottle) was dispensed into serum vials, sealed with a stopper. Gas was exchanged four times with N_2/CO_2 (80/20) to remove hydrogen and pressurized to 5 psi. All vials and bottles (agar included) were sealed with metal caps and then autoclaved with proper safety precautions (agar bottle placed in covered metal container, vials covered with another autoclave tray). A precipitate will form upon autoclaving, but will dissolve once cooled.

Enrichments and direct plating

Vials were inoculated with a ~1g of core sediment under anaerobic conditions, then gas was exchanged four times with either N_2/CO_2 (80/20) at 5 psi or H_2/CO_2 (80/20) at 7 psi. Vials were incubated at 30 °C without shaking. Because hydrogen cannot diffuse into liquid easily, this static incubation should keep the hydrogen partial pressure low within the media. Plates were inoculated with 100µl of sample from a dilution series that extended from 10^{-1} to 10^{-5} and incubated in the anaerobic incubators under sulfide gas with or without hydrogen.

Gas chromatography analysis

Methane production was measured in both primary and secondary enrichments by injecting 200µl of head space into a Shimadzu gc-14A with a flame ionization detector. A methane standard curve was calculated using known percentages of methane (0.1, 0.5, 1, 2.5. 5, 7, 10, 20). Note that a gas lock syringe was not available for methane readings, and therefore methane readings may be underestimated**.**

Sulfate assay

Sulfate was measured using the turbidimetric method. Sulfate is converted into a $BaSO_4$ suspension. The resulting turbidity is determined by measuring absorbance at 420 nm. A calibration curve with known concentrations (0.5, 1, 2.5 mM sulfate) was constructed. Due to the high concentration of sulfate in the dilute seawater media (~21 mM) 1 ml of enrichment sample was diluted in 10 ml of deionized water. Acid solution (1 ml of 6M HCL), then $BaCl_2$ (0.5g) were added to the diluted sample, then mixed vigorously to dissolve the crystals. Absorbance was taken at 420nm and sulfate concentration was determined based on the standard curve.

Results and discussion

Does methanogenesis of C-1 compounds in marine environments occur through the methylotrophic pathway, or the methyl-reduction pathway?

In the primary enrichments, methane production appears to be equal for both TMA and methanol incubated in the presence or absence of hydrogen (tables 1 and 2). However it should be noted that these measurements were made 10 days after inoculation, and differences in the rates of methane production could differ substantially between the different gas treatments. Microbial populations of primary enrichments are also very diverse, so many different types of organisms in the media both fast and slow growing could be contributing to methane production. These factors and the gram of organic matter used as inoccula play a large role in the variability associated with primary enrichments. Therefore it is probably unreasonable to determine if the methyl-reduction

pathway was responsible for catabolism of C-1 compounds in the presence of hydrogen from primary enrichments.

Regardless of this, other types of information can be gained from these values. For example very little methanogenesis occurred in those enrichments with betaine in the absence of hydrogen (table 3). Secondary enrichments of these cultures also show the same pattern (table 4), indicating that it is unlikely that betaine can be used as a substrate for methanogenesis. The methane produced in the betaine + H_2 enrichments is probably due to the reduction of CO_2 with H_2 only. Comparison between TMA and methanol enrichments (tables 1 and 2) show similar amounts of methane production, with possibly TMA being a slightly more favorable substrate for methanogenesis.

Secondary enrichments, while still not as favorable as a pure cultures may provide more information with regards to the methyl-reduction pathway. The values for methane production obtained for TMA and betaine secondary enrichments were taken 3 days after inoculation (table 4). Here we can see that growth on TMA in the absence of H_2 produces less methane than in the presence of H_2, although this does not clearly indicate that TMA is being utilized by the methyl-reduction pathway. The possible presence of methanogens reducing H_2/CO_2 may be contributing to the larger amounts of methane. Remember that it appears unlikely that betaine can be used as a substrate for methanogenesis. The methane production for the betaine + H_2 enrichments can be used as a control for this alternative pathway. If the methane produced from TMA alone is combined with that of H_2/CO_2 , there is excess methane in some of the TMA + H_2 enrichments that may be attributed to the methyl-reduction pathway. This information taken along with the ability of the methyl-reduction pathway to reduce the hydrogen partial pressure below that which ca be used by CO_2 reducing methanogens, provide evidence for the presence of the methyl-reduction pathway.

Do SRB with the capability of oxidizing C-1 compounds exist in marine sediments?

In the primary enrichments incubated in the presence of H_2 the sulfate was almost completely consumed, however in the absence of H_2 this was not true, indicating that sulfate reduction was most likely the result of H_2 consumption (tables 6,7,8). The levels

of sulfate reduction present in those enrichments without H_2 can be contributed to the presence of organic mater in the primary enrichments and also the production of H_2 that occurs from the methylotrophic pathway. Interestingly, nearly all the sulfate was consumed in the betaine enrichments, indicating that this carbon source may be being used for sulfate reduction (table 9). Given that it is unlikely that betaine can be used as a substrate for methanogenesis, there is no source of hydrogen for sulfate consumption in the enrichment, providing more evidence for sulfate reduction with betaine. Perhaps Betaine secondary enrichments do not show above average levels of sulfate reduction, however these cultures did show growth, and if given enough time, may have consumed more sulfate.

If reduction of C-1 compounds is occurring by the methyl-reduction pathway, can methanogens out-compete SRB for hydrogen?

If methanogens were out-competing SRB for hydrogen it would be expected that methanogens would dominate the secondary enrichments incubated in the presence of hydrogen. While it was true that under fluorescence microscopy for F_{420} many methanogens were present in high numbers, other non-methanogen type cells were frequently present in secondary enrichments. Methanol secondary enrichments were incubated for a week longer than the TMA and betaine enrichments, and sulfate was almost completely consumed after 10 days of incubation, indicating that these organisms were thriving under the incubation conditions (table 10). Using values for methane production, calculation of the moles of methanol converted into methane show that only roughly 1/4 of the methanol added to the enrichments was consumed for methane production. From this one of two conclusions can be made. The first is that methyl-reduction pathway is not occurring in marine environments, and therefore SRB are out-competing the methanogens for hydrogen. The ability of methanogens using the methyl-reduction pathway to out-compete SRB for hydrogen hinges on the theory that they are able to pull the hydrogen partial pressure lower than that which the SRB can utilize. The hydrogen partial pressure in the environment is $\sim 10^{-5}$ atm, one which is difficult to simulate in culture. We chose a static incubation for hydrogen, in which the vials were

pressurized, and not shaken. Given that hydrogen cannot diffuse readily in liquid, it was expected that the partial pressure in the media would remain low. However it is possible that this was not the case, and that there was an excess of hydrogen in the enrichments. In this case we would expect both organisms to thrive.

Acknowledgements

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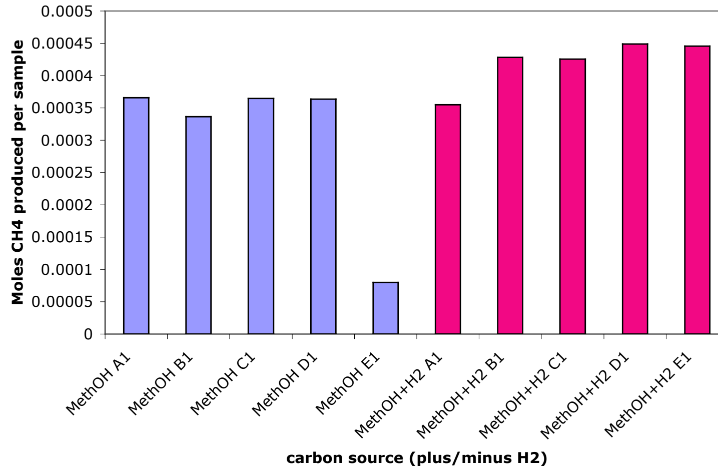
Table 1: Methane production in methanol primary enrichments
Moles CH4 produced per sample
0.0005
0.00045
0.0004
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
MethOH A1
MethOH B1
MethOH C1
MethOH D1
MethOH E1
MethOH+H2 A1
MethOH+H2 B1
MethOH+H2 C1
MethOH+H2 D1
MethOH+H2 E1
carbon source (plus/minus H2)

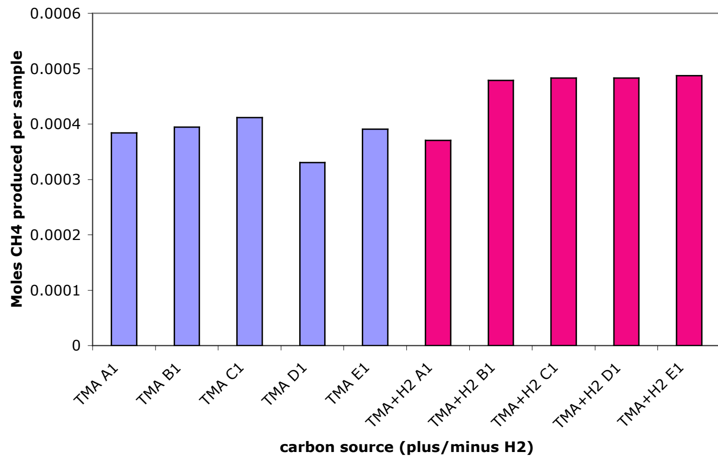
Table 2: Methane production in TMA primary enrichments
Moles CH4 produced per sample
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
TMA A1
TMA B1
TMA C1
TMA D1
TMA E1
TMA+H2 A1
TMA+H2 B1
TMA+H2 C1
TMA+H2 D1
TMA+H2 E1
carbon source (plus/minus H2)

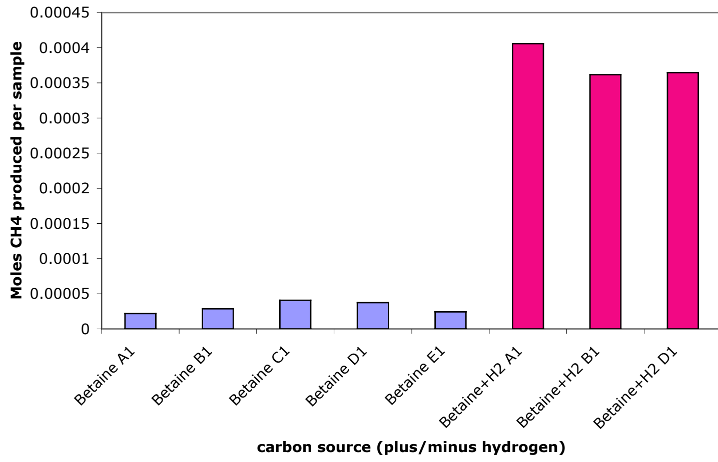
Table 3: Methane production in betaine primary enrichments
Moles CH4 produced per sample
0.00045
0.0004
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
Betaine A1
Betaine B1
Betaine C1
Betaine D1
Betaine E1
Betaine+H2 A1
Betaine+H2 B1
Betaine+H2 D1
carbon source (plus/minus hydrogen)

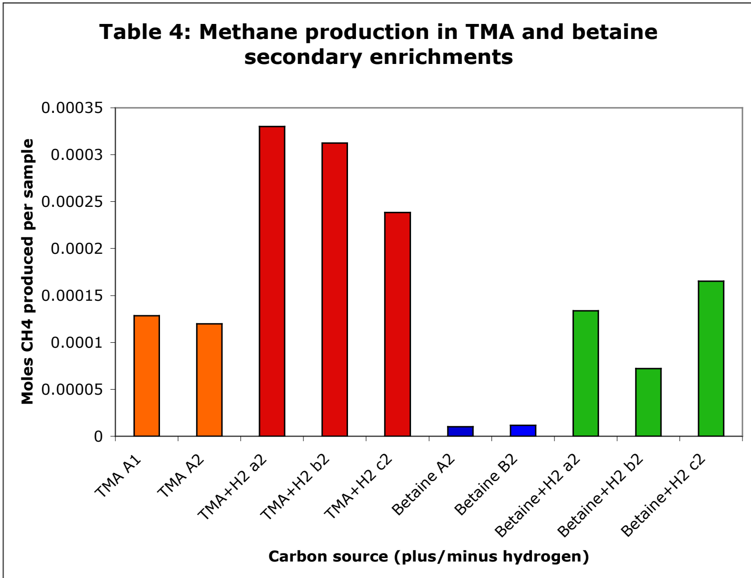
Table 4: Methane production in TMA and betaine secondary enrichments
Moles CH4 produced per sample
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
TMA A1
TMA A2
TMA+H2 a2
TMA+H2 b2
TMA+H2 c2
Betaine A2
Betaine B2
Betaine+H2 a2
Betaine+H2 b2
Betaine+H2 c2
Carbon source (plus/minus hydrogen)

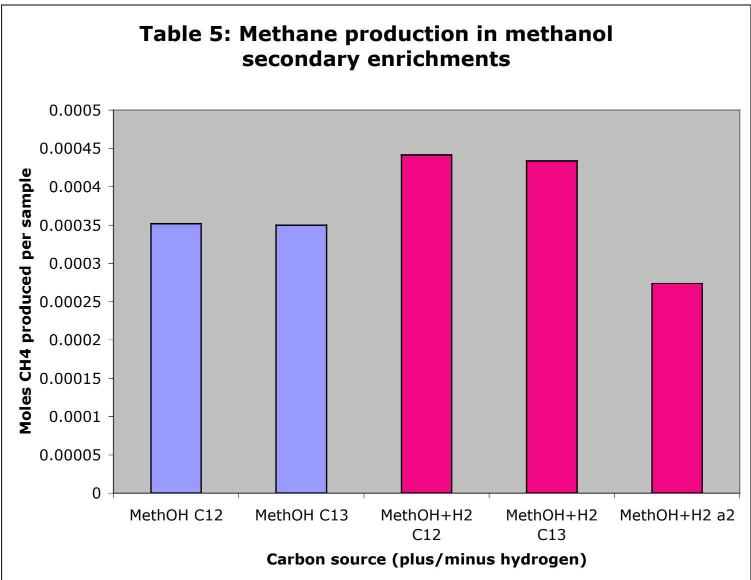
Table 5: Methane production in methanol secondary enrichments
Moles CH4 produced per sample
0.0005
0.00045
0.0004
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
MethOH C12
MethOH C13
MethOH+H2 C12
MethOH+H2 C13
MethOH+H2 a2
Carbon source (plus/minus hydrogen)

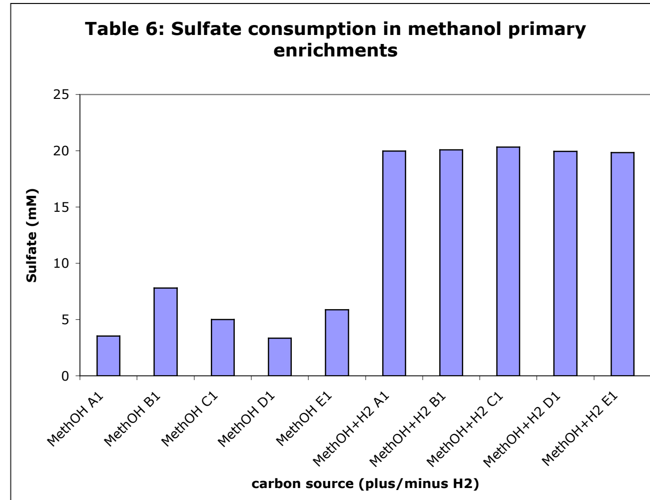
Table 6: Sulfate consumption in methanol primary enrichments
Sulfate (mM)
25
20
15
10
5
0
MethOH A1
MethOH B1
MethOH C1
MethOH D1
MethOH E1
MethOH+H2 A1
MethOH+H2 B1
MethOH+H2 C1
MethOH+H2 D1
MethOH+H2 E1
carbon source (plus/minus H2)

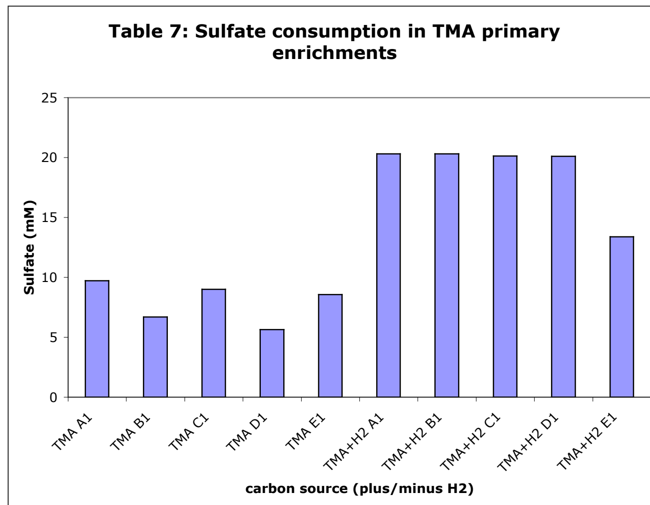
Table 7: Sulfate consumption in TMA primary enrichments
Sulfate (mM)
25
20
15
10
5
0
TMA A1
TMA B1
TMA C1
TMA D1
TMA E1
TMA+H2 A1
TMA+H2 B1
TMA+H2 C1
TMA+H2 D1
TMA+H2 E1
carbon source (plus/minus H2)

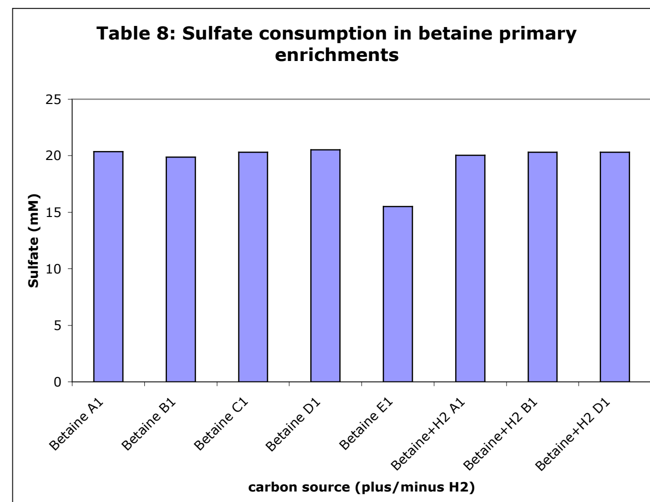
Table 8: Sulfate consumption in betaine primary enrichments
Sulfate (mM)
25
20
15
10
5
0
Betaine A1
Betaine B1
Betaine C1
Betaine D1
Betaine E1
Betaine+H2 A1
Betaine+H2 B1
Betaine+H2 D1
carbon source (plus/minus H2)

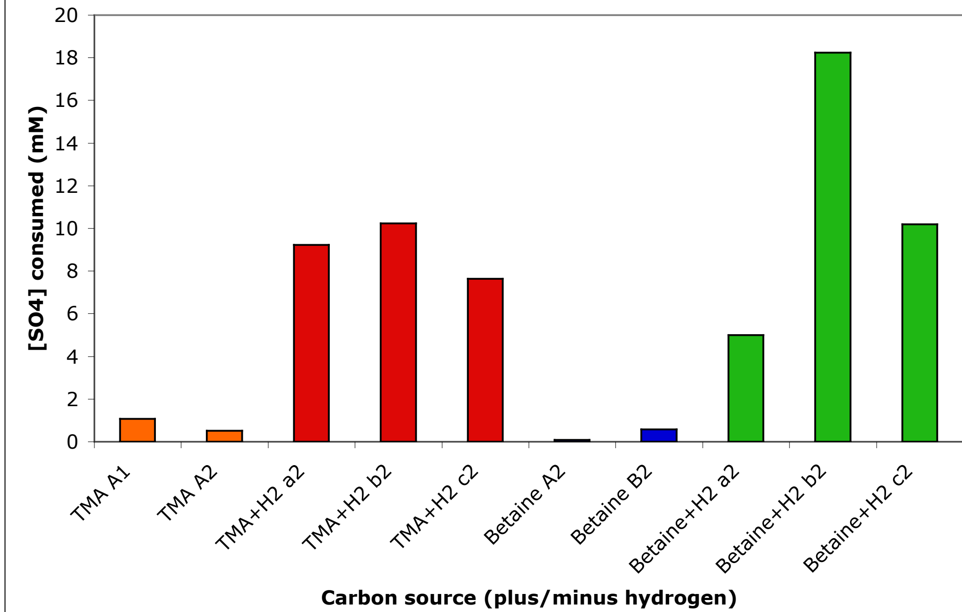
Table 9: Sulfate consumption for TMA and betaine primary transfers
[SO4] consumed (mM)
20
18
16
14
12
10
8
6
4
2
0
TMA A1
TMA A2
TMA+H2 a2
TMA+H2 b2
TMA+H2 c2
Betaine A2
Betaine B2
Betaine+H2 a2
Betaine+H2 b2
Betaine+H2 c2
Carbon source (plus/minus hydrogen)

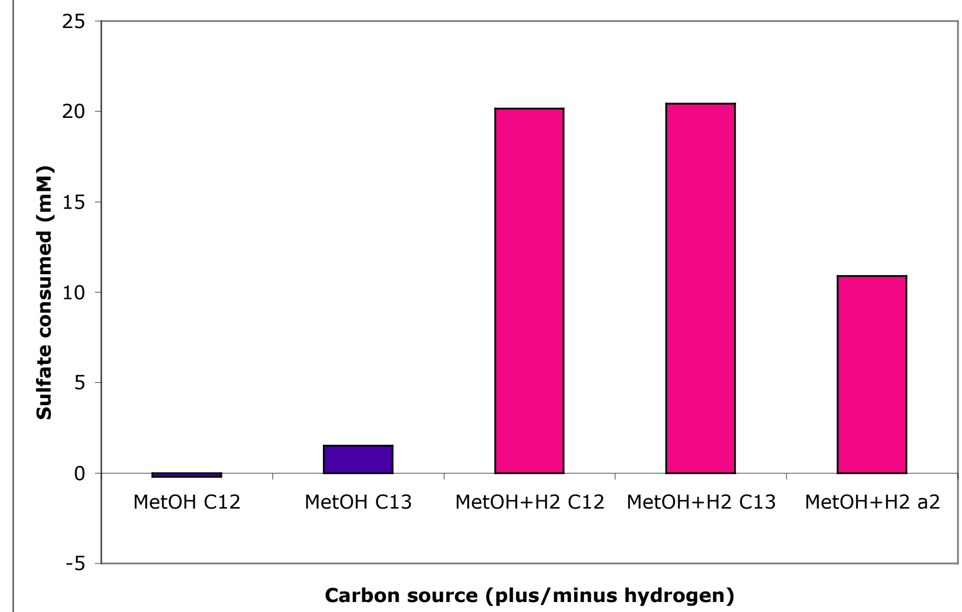
Table 10: Sulfate consumption for Methanol primary transfers
Sulfate consumed (mM)
25
20
15
10
5
0
-5
MetOH C12
MetOH C13
MetOH+H2 C12
MetOH+H2 C13
MetOH+H2 a2
Carbon source (plus/minus hydrogen)