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Effects of copper mineralogy and methanobactin on cell growth and sMMO activity in *Methylosinus trichosporium* OB3b

E. Chi Fru¹, N. D. Gray¹, C. McCann¹, J. de C. Baptista¹, B. Christgen¹,
H. M. Talbot¹, A. El Ghazouani², C. Dennison², and D. W. Graham¹

¹School of Civil Engineering and Geosciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK

²Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK

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Correspondence to: D. W. Graham (d.graham@ncl.ac.uk)

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Abstract

Controls on in situ methanotroph activity are not well understood. One potentially important parameter is copper (Cu) because this metal is at the centre of particulate methane monooxygenase (pMMO), the most active enzyme involved in oxidizing methane to methanol. Furthermore, Cu-to-cell ratios influence the relative expression of pMMO versus the alternate soluble MMO (sMMO) in some species. Most methanotroph studies have only assessed readily soluble forms of Cu (e.g., CuCl_2) atypical of real methanotroph habitats and there is a dearth of activity data associated with more common environmental Cu sources. Here we quantified sMMO activity and growth kinetics in *Methylosinus trichosporium* OB3b, an organism that expresses both pMMO and sMMO, when grown on Cu-minerals with differing dissolution equilibria to assess how mineral source and methanobactin (mb) influences growth. Mb is a molecule produced by *M. trichosporium* OB3b that has a high affinity for Cu, reduces Cu toxicity, mediates Cu uptake and may be key to Cu availability in terrestrial systems. Abiotic Cu-dissolution experiments showed that Cu release is affected by mb level, although mb only enhances Cu dissolution from sparingly soluble minerals, such as CuO and to a greater extent $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$. However, the two minerals affected *M. trichosporium* OB3b growth very differently. Cells grew without growth lag and with active pMMO on $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$, regardless of the amount of mineral supplied ($< 500 \mu\text{moles Cu-total L}^{-1}$). In contrast, they also grew well with CuO ($< 50 \mu\text{moles Cu-total L}^{-1}$), but instead had active sMMO, although sMMO activity was conditionally suppressed by supplemental mb and-or direct cell-mineral contact. Mb additions significantly increased growth rates ($p < 0.05$) with both minerals. Results show mb broadly stimulates growth, but Cu mineralogy and mb dictate whether sMMO or pMMO is active in the cells. This has implications to in situ bioremediation and other studies on methanotroph function in terrestrial systems.

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1 Introduction

Copper (Cu) is central to aerobic biological methane oxidation (Nguyen et al., 1994; Berson and Lidstöm, 1996; Hanson and Hanson, 1996; Murrell et al., 2000; Knapp et al., 2007) because it is the metal-centre of particulate methane monooxygenase (pMMO) (Balasubramanian et al., 2010), the most active enzyme at converting methane (CH₄) to methanol (Hanson and Hanson, 1996). Further, Cu-to-cell mass ratio regulates the expression of pMMO versus the less efficient iron-associated soluble MMO (sMMO) in some methane-oxidizing bacteria (methanotrophs) species (Murrell et al., 2000). Therefore, relationships among Cu supply conditions, CH₄ oxidation kinetics, and methanotroph ecology must be linked, although limited data exist on activity as a function of Cu sources more common in the environment. Most studies on Cu and methanotrophs only have examined readily soluble Cu sources, which are rarely present in nature, and only recently has methanotroph function been studied with more natural Cu sources, such as solid-phase minerals (Knapp et al., 2007; Kulczycki et al., 2007, 2011).

The purpose of this study was to quantify methanotroph growth and MMO activity as a function of solid-phase Cu supply and the presence of methanobactin (mb; Kim et al., 2004); a small molecule produced by some methanotrophs that has a very high affinity for Cu (Ghazouani et al., 2011) and is involved in Cu uptake, toxicity suppression and other functions (Fitch et al., 1993; Zahn and DiSpirito, 1996; DiSpirito et al., 1998; Kim et al., 2005; Choi et al., 2006, 2008; Knapp et al., 2007; Kulczycki et al., 2007, 2011; Balasubramanian and Rosenzweig, 2008). Although other mbs have been noted (Krentz et al., 2010; El Ghazouani et al., 2011), only one complete structure has been elucidated, which is for the type II specie, *Methylosinus trichosporium* OB3b (Kim et al., 2004). This molecule resembles a peptidic siderophore (Kim et al., 2005; Graham and Kim, 2011), has a formula of C₄₅H₅₆N₁₀O₁₆S₅Cu⁻ and an exact mass of 1215.1781 Da (Behling et al., 2008). Previous work has shown that this mb mediates pMMO-related gene transcription in *M. trichosporium* OB3b grown on Cu-Fe-oxides

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(Knapp et al., 2007) and also influences growth kinetics on Cu-doped-silicates (Kulczycki et al., 2011). However, the influence of mb on methanotroph growth and activity for Cu minerals has not been assessed; especially the ability of mb to promote pMMO activity in the presence of less-soluble Cu phases (Nriagu, 1979; Morton et al., 2000), which are common across terrestrial ecosystems.

This work intersects three basic issues and uses *M. trichosporium* OB3b to examine how different Cu minerals and mb might impact in situ methanotroph activity in terrestrial settings. First, abiotic Cu dissolution rates are compared as a function of mb supply level for five different Cu minerals to determine solid-phases that are susceptible to mb-mediated Cu solubilisation. Second, growth patterns and rates, and sMMO activities are assessed with a sub-set of Cu minerals with differing mb-Cu dissolution characteristics to examine how dissolution traits relate to observed MMO activity. sMMO activity was used as a discriminator of MMO activity because it is only expressed when there is inadequate available Cu to sustain pMMO (Murrell et al., 2000); sMMO activity is easy to measure (DiSpirito et al., 1998); and MMO activity is more closely related to actual microbial function than signatures of gene expression, which was examined previously (Knapp et al., 2007). Finally, dialysis systems were used to assess whether direct cell and-or mb contact facilitate Cu uptake from different solid-phase minerals.

2 Materials and methods

2.1 Methanobactin production

M. trichosporium OB3b cultures were grown in Cu-free nitrate mineral salt medium (NMS; 11) in 5 L bioreactors (Sartorius, UK) to produce adequate mb for Cu dissolution and microbial growth experiments (Fox et al., 1990; Tellez et al., 1998). The organisms were grown as batch cultures at 28°C, mixed at 200 rpm, and supplied with ~ 60 mL min⁻¹ CH₄ (BOC, UK) to sustain ~ 4% head space CH₄ levels. Typically, cultures were grown to an optical density at 600 nm (OD₆₀₀) of ~ 1.0 at which time 3 L of media was removed for mb harvesting, the reactor refilled with fresh media, and the

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culture re-grown for harvesting again. Spent media were centrifuged (Cryofuge 5500i, DJB Labcare Ltd, UK) at $5600 \times g$ for 60 min and the supernatant was vacuum filtered through a $0.45 \mu\text{m}$ membrane filter (Gelman, USA) to remove residual solids. The mb fraction was separated using reversed-phase C_{18} solid-phase extraction (SPE) cartridges (SEP-Pak plus 55–105 μm , Waters, UK), eluted with 60% acetonitrile (99.9%, Fisher Scientific, UK), and lyophilised for storage and use. Mb quality was verified during processing using UV-Vis spectrophotometry (Graham and Kim, 2011) and relative product purity was determined by high resolution HPLC (El Ghazouani et al., 2011).

2.2 Abiotic Mb-Cu-mineral dissolution experiments

The minerals assessed included analytical-grade $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ (Malachite), CuO (Tenorite), Cu_2O (Cuprite), and Cu_2S (Chalcocite) (all from Sigma-Aldrich, UK) as well as Cu-doped SiO_2 synthesized to 1000 ppm Cu (Knapp et al., 2007; Kulczycki et al., 2007). These minerals were chosen to span different aqueous phase Cu equilibrium characteristics. Table 1 summarises soluble Cu estimates for four of the five minerals tested (under different assumed aqueous conditions) using the geochemical model PHREEQC (Parkhurst and Appelo, 1999). The solubility of the synthesised Cu-doped silicate was not modelled because appropriate equilibrium constants and enthalpy of reactions were not available.

The abiotic Cu dissolution experiments used acid-washed 50 mL glass vials (5% HNO_3) that contained one mineral, mb (as needed), and 20 mL of 5 mM carbonate-buffered deionised water of pH 8.0. Experiments were performed in duplicate and mineral supplies were always $16.55 \mu\text{moles-total Cu}$ per vial. Minerals were pre-crushed and sieved to $< 90 \mu\text{m}$ grain size to ensure consistent surface-to-volume ratios among minerals. Cu-free crude mb ($\sim 20\%$ purity) was added at either 0.33 or 1.65 mg per vial, which were designated as the $1\times$ and $5\times$ treatments, respectively. Mb and Cu mineral supply levels were chosen such that Cu was always in excess to compare the impact of different mb levels on Cu release rates. The Cu(I) mineral treatments were prepared using solutions bubbled with oxygen-free N_2 for one hour.

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Assays were performed by placing vials on a shaker table (30 rpm) at room temperature and collecting 2-mL samples for soluble Cu analysis after 0, 3 and 6 h of contact. Mineral only and mb-only (at 5×) controls were maintained to compare Cu dissolution rates with and without mb added. Samples were filtered immediately through 0.45 µm membrane filters, acidified to < pH 2.0 with HCl (37.5% w/w) and soluble Cu was analysed using a Varian Vista MPX Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES) (Agilent Technologies, UK), using 0, 3.1, 6.3, 9.4 and 15.7 µmol L⁻¹ Cu calibration standards checked against river water reference material containing 2.4 µmol L⁻¹ Cu (LGC, UK).

2.3 Growth on different Cu sources

Growth behaviour of *M. trichosporium* OB3b was compared on three Cu sources; CuO, CuCO₃·Cu(OH)₂ and CuCl₂. These sources were chosen because abiotic data indicated CuO and CuCO₃·Cu(OH)₂ responded differently to mb exposure and CuCl₂ was retained as a growth control. Each Cu source was added at levels ranging from 0 to 500 µmol L⁻¹ total-Cu to 1-L replicate flasks (*n* > 3) containing 200 mL NMS growth media. Equal volumes of mid-exponential phase *M. trichosporium* OB3b with elevated sMMO activity were inoculated into each flask to achieve common OD₆₀₀ among treatments. The flasks were then sealed with septum-lids, amended with reagent grade methane (4% in the headspace) and placed on a shaker table (150 rpm) maintained at 28 °C in the dark. OD₆₀₀ and headspace CH₄ levels were monitored over time until stationary growth was observed. No-Cu controls were maintained to confirm viability of the inoculums.

Methane was measured by gas chromatography using a Carlo ERBA HRGC 5160 (CE instruments, UK) fitted with a Chrompak (Kinesis Ltd, UK) pot-fused silica capillary column (30 m × 0.32 mm). The carrier gas was helium, and the injector (250 °C) and oven (35 °C) were maintained at constant temperature. CH₄ was detected by FID (flame ionization detection) and quantified on the basis of peak area calibrated using

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CH₄ standards (Scientific & Technical Gases Ltd, UK). sMMO activity was assessed using the liquid naphthalene-naphthol assay (DiSpirito et al., 1998).

2.4 Assessing the influence of cell-mineral contact and mb on cell growth and sMMO activity

Preliminary experiments showed that *M. trichosporium* OB3b MMO activity and growth differed depending upon Cu source; however, it was not clear how cell contact or mb influenced observed behaviour. Therefore, further experiments were performed using molecular sieve dialysis bags to assess the need for contact among mb, the cells and the minerals to sustain growth and activity. Inoculate cultures were grown the same as in previous experiments. However, either 1000 Da or 2000 Da pore size dialysis bags (Medicell International Ltd, UK) also were included in the cultures flasks. These sieve sizes were chosen because they bracket the molecular weight of mb (i.e., ~ 1215 Da), which means that they either exclude or allow mb to cross the membrane barrier, respectively.

Three types of dialysis experiments were performed. In all cases, CuO or CuCO₃·Cu(OH)₂ was provided at 50 μmoles total-Cu L⁻¹ inside the dialysis bag and the culture placed outside of the bag within each flask. Depending upon the experiment, supplemental mb also was provided to the culture and appropriate controls were maintained for comparison (e.g., no mineral, no mb). The first experiment assessed sMMO activity, OD₆₀₀ and CH₄ levels over time (~ 60 h) in OB3b cultures that initially had sMMO activity with the cells outside and the minerals inside 1000 Da dialysis bags (in duplicate). The second experiment was the same as the first except the inoculate culture had no sMMO activity, implicitly meaning it had pMMO activity. The purpose of these experiments was to assess whether direct cell-mineral contact affected growth and sMMO activity for minerals with different Cu dissolution characteristics.

The third experiment used the same basic design as the first (i.e., cells with initial sMMO activity) except 2000 Da dialysis bags were used and supplemental mb was provided outside the bags in some flasks. This experiment was shorter than the earlier

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experiments (~24 h) because the goal was to assess the role of mb addition on sMMO activity, and it was desired to minimize the impact of new mb production on cell activity during growth. Extra Mb was provided at a ~1 : 1 mb : total Cu molar ratio to half of the flasks (in duplicate) and no additional mb was provided to the others, and sMMO activity, CH₄ levels, and OD₆₀₀ were monitored over time.

In this latter experiment, soluble Cu, cell surface-associated Cu, and intracellular Cu also were quantified at the end of the experiment according to previous methods (Fitch et al., 1993; Yu et al., 2009). Briefly, 10 mL of bulk solution was centrifuged at 12 000 × *g* for 10 min and the supernatant was retained as “soluble Cu”. The pellet was resuspended in 10 mL of Cu-free, 10 mM EDTA solution (pH 7.0) and agitated at 200 rpm for 1 h at 28°C. This solution was centrifuged again and the supernatant identified as “surface-associated Cu”. The resulting pellet was washed and centrifuged three times in deionised water and then suspended in 3-mL deionised water and freeze-dried. The dried cells were dissolved in HCl (37.5% w/w; 1 mL) and diluted to 10 mL again in deionised water. Cu associated with the three dissolved fractions were analysed by ICP-OES and normalised to the ambient cell dry mass at the time of harvesting.

3 Results and discussion

3.1 Abiotic Cu mineral-Mb dissolution experiments

Figure 1 presents the relative levels of Cu released from the five minerals with and without mb added (at two different mb levels) after three and six hours exposure. Reported values are the differences in soluble Cu with and without mb present; positive values indicate mb enhanced Cu release, whereas negative values indicate mb quenched Cu release. Background soluble Cu levels for minerals without mb added were always < 0.2 mg-Cu L⁻¹, whereas mb-only controls always had < 0.08 mg-Cu L⁻¹.

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Both Cu-doped SiO₂ and Cu₂S released less Cu with mb present than when it was absent, which is consistent with Kulczycki et al. (2007), who showed mb tends to coat such surfaces and quench Cu release, similar to other complexing agents with hydrated Cu silicate minerals (Fuerstenau et al., 2000). Whereas, CuO and Cu₂O had lower Cu release levels with mb present after three hours, but higher levels of Cu release (compared with no mb controls) after six hours with 5× mb. In contrast, CuCO₃·Cu(OH)₂ always released higher levels of Cu when mb was provided at 5× and was the only mineral with elevated Cu levels at 1× mb. These Cu release patterns are consistent with MMO-related gene expression patterns observed in *M. trichosporium* OB3b with Cu-Fe oxides and Cu-doped SiO₂ (Knapp et al., 2007), which indicated mb only increases Cu uptake from some minerals. Further, Cu release patterns in Fig. 1 broadly parallel predicted aqueous phase Cu equilibrium patterns shown in Table 1.

3.2 Affect of different Cu sources on growth patterns

Based on Fig. 1 data and previous results (Knapp et al., 2007; Kulczycki et al., 2007, 2011), CuO and CuCO₃·Cu(OH)₂ were selected for growth and activity studies because they displayed distinctly different patterns of Cu release in the presence of mb. Figure 2 shows that *M. trichosporium* OB3b cultures, with initially active sMMO, grew without a lag phase with CuO or CuCO₃·Cu(OH)₂ as the Cu source up to 500 μmol total-Cu L⁻¹. In contrast, only 2.5 μmol total-Cu L⁻¹ as CuCl₂ resulted in a significant growth lag, which has been observed previously in *M. trichosporium* OB3b (with active sMMO) when provided readily soluble Cu levels (Kim et al., 2005).

Although only a fraction of Cu from the solid-phase minerals is accessible to the aqueous phase, these data show that some solid Cu sources are superior to soluble Cu sources, being capable of sustaining growth at very high total Cu levels. However, Table 1 data predicts that equilibrium aqueous phase Cu levels should be > 10 times greater for CuCO₃·Cu(OH)₂ than CuO in this growth media, therefore ad hoc sMMO activity assays were performed on selected cultures to assess Cu availability (data not shown). Assays showed that sMMO activity disappeared when the cultures were

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provided $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ or CuCl_2 , but sMMO activity was sustained with CuO , even at levels up to $50 \mu\text{mol total-Cu L}^{-1}$. This implies Cu dissolution rates from CuO are too slow to provide adequate Cu to suppress sMMO activity, although how this relates to mb supply and physical factors was not initially clear.

3.3 Effect of mineral-cell contact on sMMO activity

Preliminary assays showed that sMMO activity was retained in *M. trichosporium* OB3b in the presence of similar and elevated levels of CuO , but not $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$. This was somewhat surprising because abiotic experiments showed that mb could sequester Cu from both minerals, although $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ was clearly more susceptible to mb attack. Therefore, to explain the differences in sMMO activity patterns between CuO and $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ as Cu sources, further growth experiments were performed to delineate the roles of mb and cell-mineral contact on Cu availability with solid minerals. It was hypothesized that for a less soluble mineral like CuO , increased mb exposure and direct physical contact might enhance Cu uptake, which was tested using selectively permeable barriers (i.e., dialysis bags) that separated the cells, mb and the minerals in different ways during growth.

Two initial experiments were performed with the dialysis systems. The first experiment inoculated *M. trichosporium* OB3b with strong initial sMMO activity into flasks containing the two minerals at $50 \mu\text{mol total-Cu L}^{-1}$ located inside and outside $< 1000 \text{ Da}$ dialysis bags (in duplicate). In theory, if direct contact between the cells and the mineral were essential to Cu uptake, sMMO activity should be retained when the cells and mineral are separated, but lost when contact is allowed. Further, in this experimental design, mb produced by the cells should not play a direct role in Cu acquisition from the separated minerals because mb does not readily cross the dialysis barrier. As background, preliminary tests were performed on the dialysis bags to verify the bags themselves did not affect sMMO activity and no significant difference in sMMO activity was seen with just bags present (see supplementary Fig. S1).

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Figure 3 shows that when the cells and CuO (or mb) are not allowed direct contact, sMMO activity is retained in the cells, implying Cu is functionally unavailable. However, direct cell-CuO contact initially suppressed sMMO activity when contact was allowed, although as cell densities increased, sMMO activity reappeared. In contrast, sMMO activity was completely suppressed by $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ regardless of physical contact. Apparently, Cu dissolution rates from this mineral are sufficiently rapid to supply Cu across the barrier to the cells, implying direct contact is not critical to obtain Cu for growth.

To corroborate Fig. 3 results, a second experiment was performed with < 1000 Da dialysis bags. However, in this case, the initial culture did not have sMMO activity and we monitored the formation of sMMO activity as the culture grew as an indicator of Cu availability. Figure 4 shows that even with a culture without initial sMMO activity, strong sMMO activity develops over time with CuO as the Cu source and direct contact between the cells and CuO are not permitted. As before, when $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ was the Cu source, no sMMO activity was detectable with this mineral until high cell densities were achieved. Trace sMMO activity was detected when $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ was inside the dialysis bag at the highest cell densities, suggesting that cell contact might slightly enhance Cu availability from $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$. As an aside, Fig. 3a describes typical cell growth curves for all dialysis experiments (other data not shown); i.e., no grow lags were observed and differences in growth rates were not significant among treatments.

3.4 Effect of mb on sMMO activity and Cu uptake from different minerals

The previous experiments showed that direct contact between the cells and CuO (and to a much lesser degree, $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$) was important to increased Cu availability from this mineral. However, the experimental design did not physically allow mb to act remotely and potentially mediate Cu acquisition. To assess the extracellular role of mb in Cu supply, < 2000 Da dialysis bags were substituted for the < 1000 Da bags in the experiment, and supplemental mb was provided to some flasks.

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Figure 5 shows that sMMO activity initially declined when the cells were exposed to CuO, but the cells sustained higher sMMO activity after 24 h when no excess mb was provided. Furthermore, when excess mb was not provided, no intracellular Cu was detectable (i.e., $< 0.1 \mu\text{g-Cu/mg-cell dry weight}$), implying Cu was not being readily acquired by the cells. In contrast, when $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$ was the Cu source, Fig. 5 shows sMMO activity was suppressed rapidly and intracellular Cu levels increased substantially, which did not differ as a function of mb supplementation.

3.5 Growth kinetics of *M. trichosporium* OB3b on mineral Cu sources with and without mb

Specific growth rates and cell yields were calculated for all experiments with and without excess mb for *M. trichosporium* OB3b grown on CuO or $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$ ($50 \mu\text{mol total-Cu L}^{-1}$). Overall, excess mb did not alter cell yields for the minerals (data not shown); however, mb significantly ($p < 0.05$, Wilcoxon signed-rank test) increased growth rates. The mean specific growth rate with mb was $0.055 \pm 0.007 \text{ h}^{-1}$ (mean $\pm 95\%$ confidence interval), whereas without mb was $0.044 \pm 0.006 \text{ h}^{-1}$, which implies mb broadly enhances cell growth on solid-phase Cu-minerals, regardless of whether sMMO or pMMO activity prevails in the cells.

3.6 Role of mb in cell growth on solid-phase mineral Cu sources

Mb appears to play multiple roles relative to Cu in the growth of *M. trichosporium* OB3b (Fitch et al., 1993; Zahn and DiSpirito, 1996; DiSpirito et al., 1998; Kim et al., 2005; Knapp et al., 2007; Kulczycki et al., 2007, 2011; Balasubramanian and Rosenzweig, 2008; Choi et al., 2008). Although other mbs have been noted (Krentz et al. 2010; El Ghazouani et al., 2011), results here suggest their prominent role(s) depend on the Cu source. For example, for highly insoluble Cu sources, such as silicates and sulphides, mb does not appear to enhance Cu acquisition and may actually quench the release of Cu from such surfaces (Kulczycki et al., 2007, 2011). Alternately,

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M. trichosporium OB3b readily acquires Cu from more soluble mineral sources, such as $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$, and it is not clear that mb is actually needed for Cu uptake. However, we strongly suspect mb plays a mediating role in Cu uptake (even with such Cu sources) and also toxicity suppression due to mb binding to Cu(I) (Kim et al., 2005; El Ghazouani et al., 2011). In fact, *M. trichosporium* OB3b grows very well on $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$, particularly at very high Cu levels; i.e., the mineral appears to act as a “controlled release” Cu source, allowing the cell to obtain enough Cu for growth, but at a Cu supply rate that does not cause toxicity.

In contrast, mb appears to play a more central role in Cu supply for growth with CuO, which is intermediate in Cu solubility (Table 1). Specifically, Fig. 5 shows that mb can remotely obtain Cu from this mineral and returns Cu to the cell, suppressing sMMO activity and increasing intracellular Cu levels. Further, Figs. 3 and 4 show that direct cell contact with this mineral also enhances Cu availability. Although mechanistic details for each mineral are still needed, these observations generally show that the nature of each mineral Cu influences how the cells obtain Cu and that the relative role of mb in that process varies from mineral to mineral. As such, Cu mineralogy must be very important to in situ MMO activity (i.e., sMMO vs. pMMO) observed in terrestrial settings.

Although Figs. 3–5 show mb affects actual Cu uptake mechanisms differently among minerals, mb itself seems to act as general stimulant when *M. trichosporium* OB3b is grown on solid-phase CuO and $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$. Interestingly, this stimulatory effect does not appear to depend on whether pMMO or sMMO is the active MMO. Whether this effect results from subtle Cu toxicity suppression, enhanced Cu bioavailability after release, or something more speculative is not yet known (e.g., quorum signalling seen with similar molecules; Miller and Bassler, 2001; D’Onofrio et al., 2010).

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4 Conclusions

In summary, this work shows that some (probably many) solid-phase Cu minerals readily support growth in *M. trichosporium* OB3b and that mb conditionally plays an important role in that activity. In fact, data suggest some solid Cu sources are superior to soluble sources (CuCl₂) for growth, which is actually not surprising because methanotrophs naturally reside in settings where Cu tends to be less available, such as soils. However, the specific active MMO in the cells differs depending upon Cu source; for CuCO₃·Cu(OH)₂, pMMO was implicitly sustained, whereas for CuO, sMMO was sustained unless Cu levels were high or the mineral was proximally close to the cells.

Although not central to this work, this observation has significant practical implications because it might be possible to promote sMMO activity in biotechnical applications, such as contaminant degradation (Oldenhuis et al., 1989; Kim and Graham, 2003; Lee et al., 2006), by employing Cu sources like CuO in the system. Regardless, here we show that in addition to CH₄, O₂ and other known regulators of aerobic CH₄ metabolism (Graham et al., 1993; Hanson and Hanson, 1996), mb and Cu mineralogy also are key drivers of methanotroph growth and presumably ecology, which must be considered in future studies, especially in terrestrial settings. Specifically, mb broadly stimulates growth in solid-phase Cu systems, whereas Cu mineralogy dictates whether sMMO or pMMO is the active form in the cells.

Supplementary material related to this article is available online at:

<http://www.biogeosciences-discuss.net/8/2851/2011/bgd-8-2851-2011-supplement.zip>.

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Table 1. Modelled dissolved copper concentrations for aqueous solutions equilibrated with various Cu mineral phases (25 °C) calculated using the PHREEQC geochemical simulation software (Parkhurst and Appelo, 1999). Equilibrium equations, constants and enthalpies of reaction were derived from the Iln database (PHREEQC v2.16 for windows) and were used to determine Cu solubility when defined solutions were equilibrated with different copper mineral phases. Equilibrium phases used were (Chalcocite, Cu_2S ; Cuprite, Cu_2O ; Tenorite, CuO ; and Malachite, $\text{Cu}(\text{OH})_2 \cdot \text{CuCO}_3$).

Formula	Cu solubility (mol dm^{-3}) in pure water pH 7		Cu solubility in growth medium pH 7		Cu solubility in carbonate buffered solution pH 8	
	Cu^{2+}	Cu^{1+}	Cu^{2+}	Cu^{1+}	Cu^{2+}	Cu^{1+}
Cu_2S	1.65×10^{-17}	2.01×10^{-12}	3.38×10^{-10}	1.50×10^{-7}	2.16×10^{-17}	1.65×10^{-13}
Cu_2O	5.13×10^{-13}	1.55×10^{-8}	5.74×10^{-7}	2.14×10^{-8}	4.04×10^{-13}	1.82×10^{-9}
CuO	9.71×10^{-7}	5.52×10^{-16}	2.07×10^{-6}	2.25×10^{-14}	8.80×10^{-7}	1.23×10^{-16}
$\text{Cu}_2\text{CO}_3(\text{OH})_2$	1.49×10^{-5}	2.27×10^{-12}	2.19×10^{-5}	2.40×10^{-13}	1.18×10^{-6}	7.37×10^{-16}

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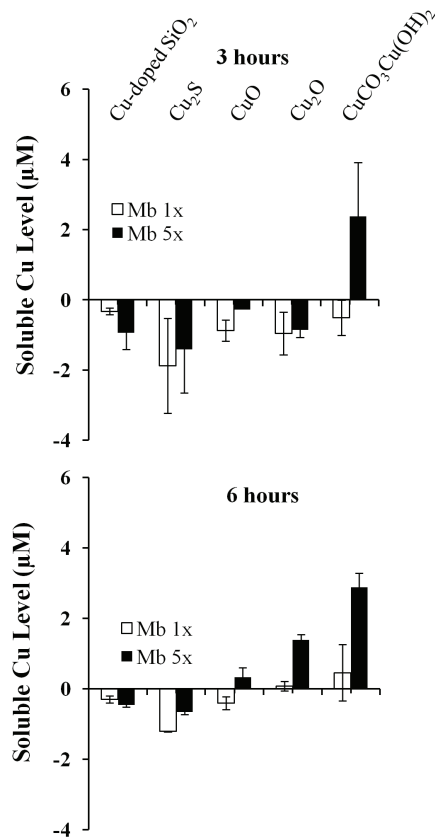


Fig. 1. Effect of mb level on Cu release from different Cu solid-phase mineral. Black bars denote experiments with 0.33 mg mb/20 mL (1×) and white bars denote 1.65 mg mb/20 mL (5×). Values are the “relative” dissolved Cu levels obtained by subtracting Cu released from each mineral without mb from the Cu released when mb is provided. Error bars present the range of values from two independent experiments.

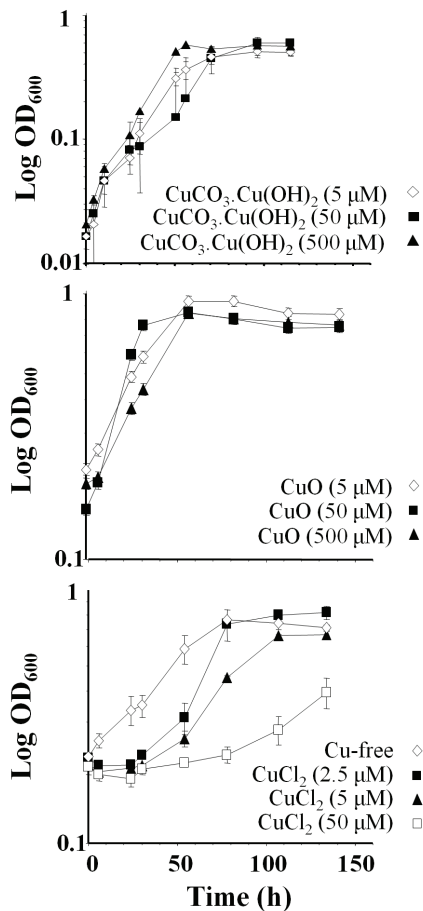


Fig. 2. Effect of Cu source and concentration on the growth pattern of *Methylosinus trichosporium* OB3b. Error bars represent standard deviations ($n = 3$).

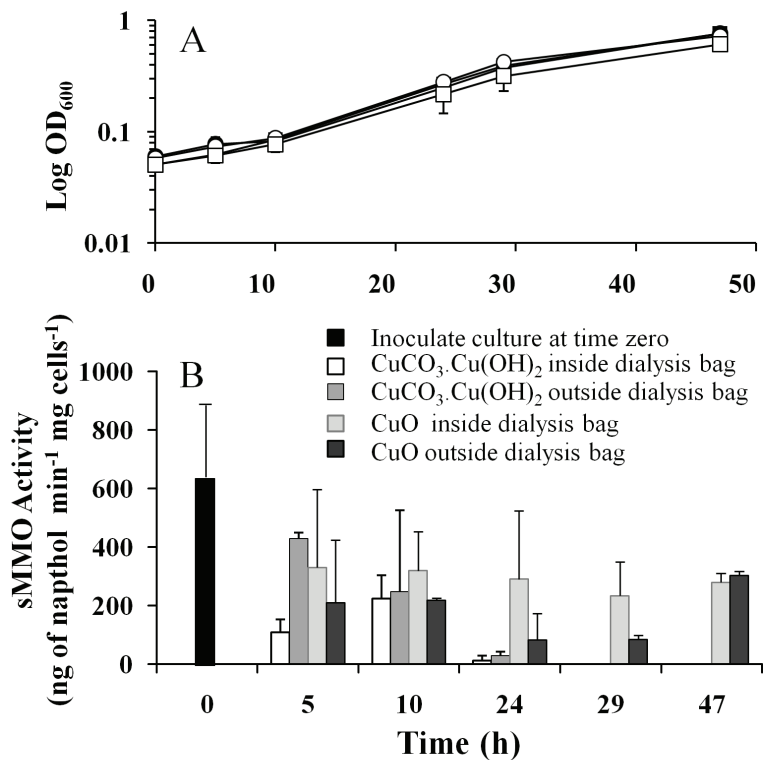


Fig. 3. Affect of direct mineral contact on the maintenance of sMMO activity ($\text{ng of naphthol min}^{-1} \text{ mg cell dry weight}^{-1}$) when the initial culture has sMMO activity. **(A)** Growth curves for CuO and $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$ at $50 \mu\text{mol total-Cu L}^{-1}$ where the mineral was inside and outside of $< 1000 \text{ Da}$ sieve dialysis bags in the culture flask ($n = 2$ per treatment). **(B)** sMMO activity over time as function of location of the two minerals, CuO and $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$; i.e., inside and outside of the dialysis bags. sMMO activity drops initially in all treatments, but increases as cell density increases, especially with CuO as the Cu source and direct cell-mineral is not permitted. Error bars represent standard deviations.

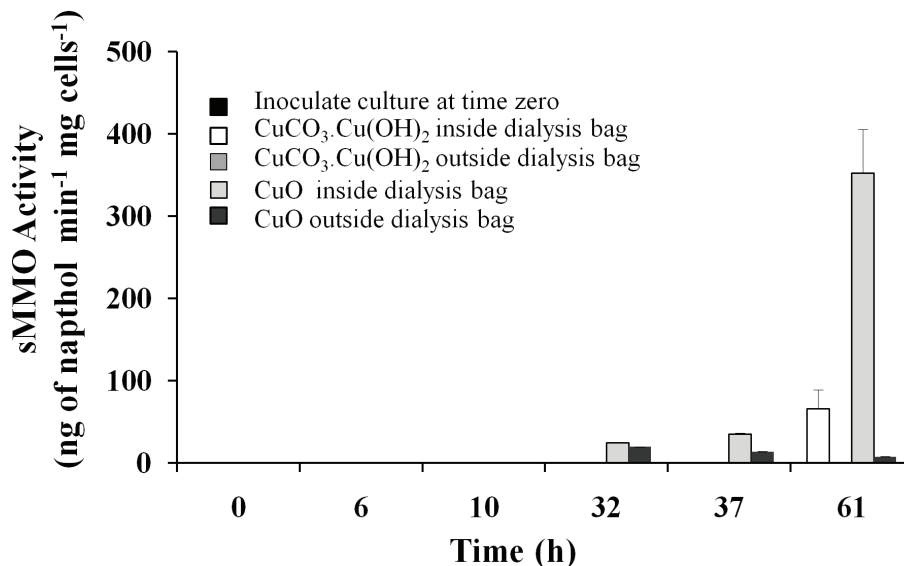


Fig. 4. Affect of direct mineral contact on the formation of sMMO activity (ng of naphthol min^{-1} mg cell dry weight $^{-1}$) when the initial culture does not have sMMO activity. sMMO activity presented over time as function of location of the two minerals, CuO and $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$; i.e., inside and outside of < 1000 Da sieve size dialysis bags. sMMO activity is initially not present, but increases as cell density increases sMMO activity appears, especially with CuO as the Cu source and direct cell-mineral is not permitted. Mean specific growth rates for the cultures range from 0.45 to 0.50 h^{-1} . Error bars represent standard deviations.

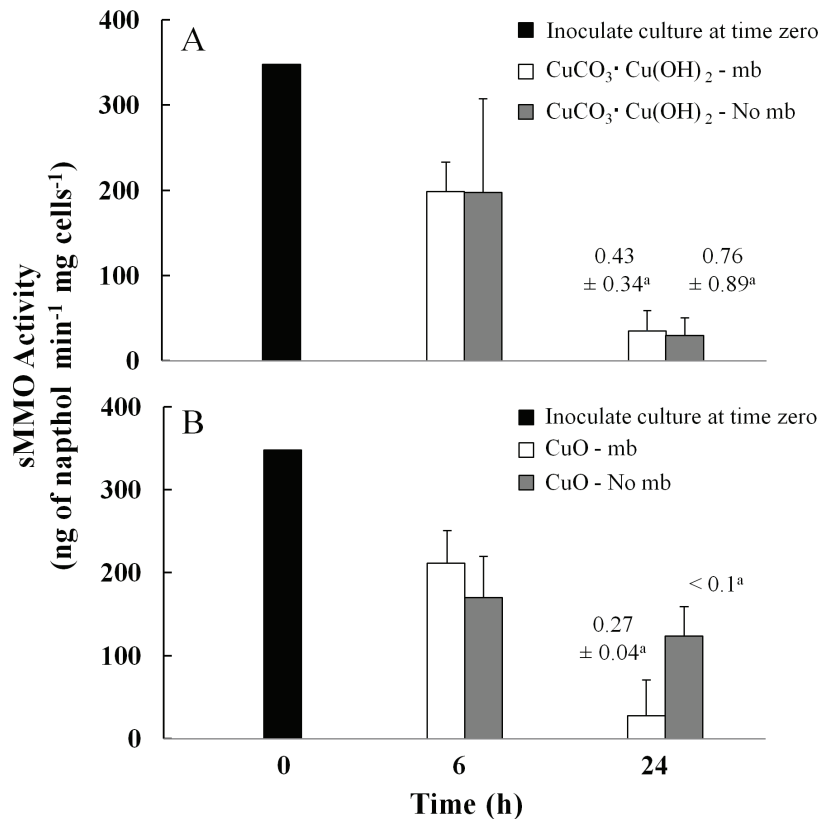


Fig. 5. sMMO activity (ng of naphthol formed min^{-1} mg cell dry weight $^{-1}$) for *M. trichosporium* OB3b with and without supplemental methanobactin in the presence of **(A)** $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$ and **(B)** CuO confined in 2000 Da sieve size dialysis bags. Concentration of intracellular Cu in the cells after 24 h of growth is inset ($\mu\text{g-Cu/mg-cell mass} \pm$ standard deviation). Error bars represent standard deviation ($n = 3$). Mean specific growth rates for the cultures range from 0.36 to 0.50 h^{-1} .