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

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Abstract. Controls on in situ methanotroph activity are not well understood. One potentially important parameter is copper (Cu) because it is the metal-centre of particulate methane monooxygenase (pMMO), the most active enzyme for oxidizing methane to methanol. Further, Cu-to-cell ratios influence the relative expression of pMMO versus the alternate soluble MMO (sMMO) in some species. However, most methanotroph studies only have assessed readily soluble forms of Cu (e.g. $CuCl_2$) and there is a dearth of Cu-related activity data for Cu sources more common in the environment. Here we quantified sMMO activity (as a practical indicator of Cu availability) 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 type and methanobactin (mb) might influence in situ methanotroph activity. Mb is a molecule produced by *M. trichosporium* OB3b that has a high affinity for Cu, reduces Cu toxicity, and may influence Cu availability in terrestrial systems. $CuCO_3.Cu(OH)_2$ and CuO were chosen for study based on modelling data, reflecting more and less soluble minerals, respectively, and were found to affect *M. trichosporium* OB3b activity differently. Cells grew without growth lag and with active pMMO on $CuCO_3.Cu(OH)_2$, regardless of the amount of mineral supplied (<500 µmoles Cu-total l^{-1}). The organism also grew well on CuO; however, significant sMMO activity was retained up to 50 µmoles Cu-total l^{-1}, although sMMO activity was suppressed by supplemental mb and-or direct cell-mineral contact. Mb addition 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 explains why sMMO activity has been seen in soils with high Cu and also has implications for predicting dominant MMO activity in terrestrial bioremediation applications.

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1 Introduction

Copper (Cu) is central to aerobic biological methane oxidation (Nguyen et al., 1994; Berson and Lidström, 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 (CH_4) 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) produced in some methanotroph species (Murrell et al., 2000). However, most studies on Cu and methanotrophs only have assessed readily soluble Cu sources (e.g. $CuCl_2$; Fitch et al., 1993; DiSpirito et al., 1998; Choi et al., 2006), which are rarely present in nature, and only recently has methanotroph behaviour been studied using typical environmental Cu sources, such as solid-phase minerals (Knapp et al., 2007; Kulczycki et al., 2007, 2011). Given that solid-phase conditions strongly influence metal dissolution traits for all minerals, we propose that Cu mineralogy and in situ methanotroph activity must be linked, although specific relationships between Cu-mineral dissolution and methanotroph activity are not understood.

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). Mb is a small Cu-binding molecule produced by some methanotrophs that has a very high affinity for Cu(I) (∼6–7×10^{20} M^{-1}; Ghazouani et al., 2011) and is also involved in Cu uptake, toxicity suppression and other key functions in the cells (Fitch et al., 1993; Zahn and DiSpirito, 1996; DiSpirito et al., 1998; Kim et al., 2005; Choi et al., 2006; Knapp et al., 2007; Kulczycki et al., 2007; Balasubramanian and Rosenzweig, 2008; Choi et al., 2008; Kulczycki et al., 2011). 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 methanotroph, *Methylosinus trichosporium* OB3b (Kim et al., 2004). This mb resembles a peptidic siderophore (Kim et al., 2005; Graham and Kim, 2011), has a formula of $C_{45}H_{56}N_{10}O_{16}S_5Cu^-$ 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 in the presence of Cu-Fe-oxides (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 other Cu minerals has not been assessed; especially the type of MMO activity present with differentially soluble Cu phases (Nriagu, 1979; Morton et al., 2000).

This work uses *M. trichosporium* OB3b to examine how different Cu minerals and mb might impact in situ methanotroph activity in terrestrial environments. First, Cu solubilities were calculated for different Cu minerals to predict solid-phases that are likely to be susceptible to methanotroph mediated Cu solubilisation. Second, growth patterns and sMMO activities are assessed for two Cu minerals with differing mb-Cu dissolution traits to examine how dissolution translates to observed cell activity. sMMO activity was used here as a discriminator of MMO activity because it is only expressed when inadequate Cu is available to sustain pMMO (Murrell et al., 2000). Further, sMMO activity is easy to measure (DiSpirito et al., 1998); it is often favoured over the pMMO in bioremediation because of its broad substrate range (Hanson and Hanson, 1996); and MMO activity is more closely related to actual methanotroph function than signatures of gene expression, which we have examined previously (Knapp et al., 2007). Finally, dialysis systems were used to assess whether direct cell and-or mb contact differentially enhances or restricts Cu uptake for the two minerals.

2 Materials and methods

2.1 Methanobactin production

M. trichosporium OB3b was grown in Cu-free nitrate mineral salt medium (NMS) in 5 l bioreactors (Sartorius, UK) to produce mb for subsequent 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} CH_4 (BOC, UK) to sustain ∼4 % head space CH_4 levels. Typically, cultures were grown to an optical density at 600 nm (OD_{600}) of ∼1.0 at which time 3 l of media was removed for mb harvesting. The reactor was refilled with fresh media and the 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 µm 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 and relative product purity was determined by high resolution HPLC (El Ghazouani et al., 2011).

2.2 Growth on different Cu sources

Growth of *M. trichosporium* OB3b was compared among three Cu sources; $CuCO_3.Cu(OH)_2$ (Malachite), CuO (Tenorite) and $CuCl_2$. $CuCO_3.Cu(OH)_2$ and CuO were chosen for growth studies from five possible minerals (also including Cu_2O, Cu_2S and Cu-doped SiO_2) because they are common in nature; they interact differently with mb in mineral binding experiments (Supplement; Fig. S1); and they have different aqueous Cu concentrations at equilibrium as predicted by PHREEQC geochemical modelling (see Table 1; Parkhurst and Appelo, 1999). Further, Cu_2S and Cu-doped SiO_2 were found to interfere with the sMMO activity assay (see below). $CuCl_2$ was used as the "soluble-Cu" control control for comparisons.

The growth experiments were performed using analytical grade Cu sources (Sigma-Aldrich, UK), provided at levels ranging from 0 to 500 µmol l^{-1} total-Cu in 200 ml NMS media in 1 l septum-flasks (always $n > 3$). Each flask was provided equal volumes of mid-exponential phase *M. trichosporium* OB3b with elevated sMMO activity (typically > 400 ng of napthol min^{-1} mg $cells^{-1}$) to achieve a common initial OD_{600} among treatments. sMMO activity was quantified using the standard liquid naphthalene-naphthol assay (DiSpirito et al., 1998), which was developed from the original sMMO screening assay of Brusseau et al. (1990). The flasks were then sealed, amended with reagent grade methane (4 % in the headspace) and placed on a shaker table (150 rpm) maintained at 28 °C in the dark. OD_{600} and headspace CH_4 levels were monitored over time until stationary growth was observed. No-Cu controls were maintained to confirm viability of the inoculums (data not shown).

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. CH_4 was detected by FID (flame

Table 1. Modelled dissolved Cu concentrations for aqueous solutions equilibrated with various Cu mineral phases (25 °C) calculated using the PHREEQC geochemical simulation software (Parkhurst and Appelo, 1999)[a].

	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[b]	
Formula	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}
$CuCO_3.Cu(OH)^2$	1.49×10^{-5}	2.27×10^{-12}	2.19×10^{-5}	2.40×10^{-13}	1.18×10^{-6}	7.37×10^{-16}

[a] Equilibrium equations, constants and enthalpies of reaction were derived from the llnl database (PHREEQC v2.16 for windows) and were used to determine Cu solubility when defined solutions were equilibrated with different copper mineral phrases. Equilibrium phases used were (Chalcocite, Cu_2S; Cuprite, Cu_2O; Tenorite, CuO; and Malachite, $CuCO_3.Cu(OH)^2$.

[b] Experimental conditions employed in Cu-mineral binding experiments (see Supplement).

ionization detection) and quantified on the basis of peak area calibrated using CH_4 standards (Scientific & Technical Gases Ltd, UK). The detection limit was 10 ppm CH_4 with typical relative standard deviation on the calibration curves of <1 %.

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

Preliminary experiments showed that *M. trichosporium* OB3b growth patterns differed depending upon Cu source; however, it was not clear whether cell contact and-or mb significantly 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. In these experiments, inoculate cultures were grown as in the previous experiments; however, either <1000 Da or <2000 Da pore size dialysis bags (Medicell International Ltd, UK) also were included in the culture flasks. These sieve sizes were chosen because they bracket the molecular weight of Cu-mb (i.e. ∼1215 Da), which means that they relatively exclude or allow mb to cross the membrane barrier, respectively.

Three types of dialysis experiments were performed. In all cases, CuO or $CuCO_3.Cu(OH)_2$ was provided at 50 µmoles total-Cu l^{-1} inside the dialysis bag and the culture placed outside of the bag within the flasks. 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, OD_{600}, and CH_4 levels over time (∼60 h) in OB3b cultures that initially had sMMO activity. The cells were inoculated outside of <1000 Da dialysis bags, whereas minerals were placed inside or outside the bags. The second experiment was the same as the first except the inoculate culture had no detectable sMMO activity (< 10 ng of napthol min^{-1} mg cells^{-1}), implicitly meaning it had pMMO activity (Murrell et al., 2000). The goal here was to assess whether direct cell-mineral contact affected growth and sMMO activity without supplementary mb.

The third experiment used the same 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 experiments (∼24 h) because the goal was to assess the role of additional mb on sMMO activity, and it was shortened to reduce the impact of new mb production on cell activity during the experiment. Supplemental mb was provided at a ∼1:1 mb:total Cu molar ratio to half of the flasks and no additional mb provided to the others, and sMMO activity, CH_4 levels, and OD_{600} were monitored over time.

In this latter experiment, soluble Cu versus cellular Cu was 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 was assumed to be 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. "Cellular Cu" was defined as the surfaced-associated Cu plus the Cu present in the final dried pellet.

Cu from each fraction was analysed using a Varian Vista MPX Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES) (Agilent Technologies, UK) and levels were normalised to the ambient cell dry mass at the time of harvesting. Calibration standards (0.0, 3.1, 6.3, 9.4 and 15.7 µmol l^{-1} Cu) were checked against a river water reference material containing 2.4 µmol l^{-1} Cu (LGC, UK). The detection limit for dissolved Cu was 0.16 µmol l^{-1} Cu with a typical relative standard deviation for the reference material of <10 %.

3 Results and discussion

3.1 Affect of different Cu sources on growth patterns

CuO and $CuCO_3.Cu(OH)_2$ were chosen for growth and activity studies based on Table 1 data and previous results (Knapp et al., 2007; Kulczycki et al., 2007; Kulczycki et al., 2011). *M. trichosporium* OB3b cultures with active sMMO were transferred to replicate vials containing CuO, $CuCO_3.Cu(OH)_2$ and $CuCl_2$ containing up to 500 µmol total-Cu l^{-1} and growth was monitored over time. Growth rate was very similar for CuO and $CuCO_3.Cu(OH)_2$ and no lag phase was observed (Fig. 1a and b). However, only 2.5 µmol total-Cu l^{-1}as $CuCl_2$ caused a significant growth lag in the culture (Fig. 1c), which has been seen previously in *M. trichosporium* OB3b with active sMMO and soluble Cu (Kim et al., 2005). Clearly, the two solid-phase Cu sources restrict Cu release into solution allowing unaffected cell growth at very high Cu levels with no sign of toxicity.

To determine how sMMO activity was influenced by each Cu addition, single sMMO activity assays were performed on each culture during late-exponential phase. sMMO activity was not detectable in any cultures supplemented with $CuCO_3.Cu(OH)_2$ or $CuCl_2$, but sMMO activity was prevalent with CuO up to 50 µmol total-Cu l^{-1}. Cu dissolution from CuO was, therefore, not sufficient to repress sMMO activity (i.e. to provide Cu for switchover to pMMO activity), whereas Cu release from $CuCO_3.Cu(OH)_2$ and $CuCl_2$ was sufficient to repress sMMO activity, which is consistent with Cu dissolution trends predicted for the two mineral phases (Table 1).

3.2 Effect of mineral-cell contact on sMMO activity

Further growth and activity experiments were performed to verify the previously observed influence of the two Cu minerals on sMMO activity (Sect. 3.1). Specifically, activity was assessed under different culture conditions, including systems that restrict direct cell-mineral contact and also with supplemental mb. Previous results showed that mb interactions with Cu silicates quenched the surface of the mineral, suppressing Cu release and allowing sMMO-related gene expression, even when Cu levels were high (Knapp et al., 2007; Kulczycki et al. 2007; Kulczycki et al., 2011). Therefore, we speculated that mb mediation and cell surface interactions might also explain differences between $CuCO_3.Cu(OH)_2$ and CuO relative to sMMO activity. As such, *M. trichosporium* OB3b was grown in flasks containing selectively permeable barriers (i.e. dialysis bags) that allowed the cells, mb and the minerals to be separated in combinations to delineate how mb, mineral, and surface contact affect activity.

The two initial experiments were performed using dialysis flask systems without amended mb. The first experiment inoculated *M. trichosporium* OB3b cultures with strong sMMO activity (~600 ng of napthol min^{-1} mg $cells^{-1}$) into

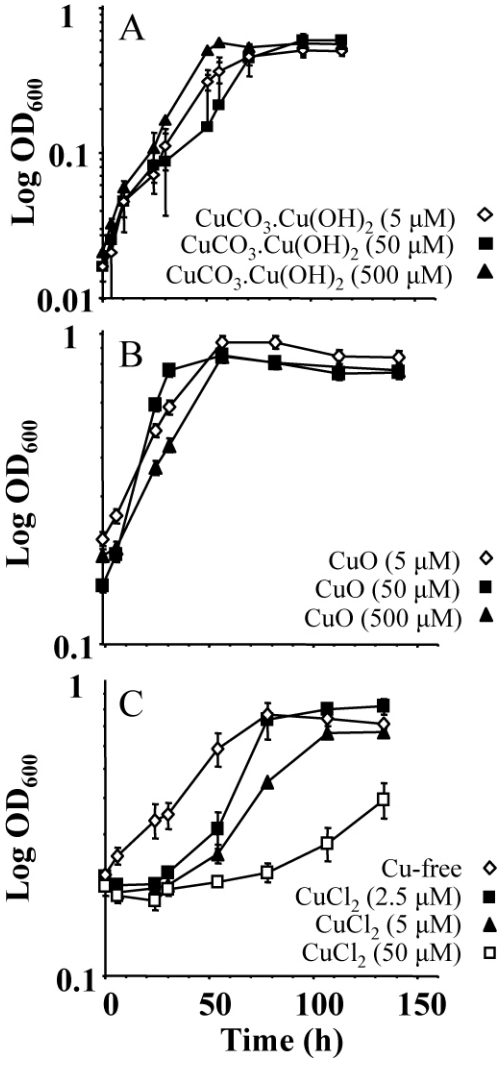

Fig. 1. Effect of Cu source and concentration on the growth of *Methylosinus trichosporium* OB3b. Error bars represent standard deviations ($n = 3$).

duplicate flasks containing each mineral at 50 µmol total-Cu l^{-1}, where the mineral was located inside and outside of dialysis bags (pore size <1000 Da) . In theory, if direct contact between the cells and the mineral was needed for Cu uptake, sMMO activity would be retained when the minerals and cells were separated because mineral-Cu would not readily cross a <1000 Da barrier. The <1000 Da dialysis bags were explicitly used to block new mb produced by the cells during the experiment that might confound any cell-mineral surface dependent interactions. Preliminary tests were performed on the dialysis bags to verify that the bags themselves did not affect sMMO activity and no significant difference in sMMO activity was measured with just bags present (Supplement, Fig. S2).

When the cells and CuO were separated by the dialysis bags, significant sMMO activity was perpetuated over time

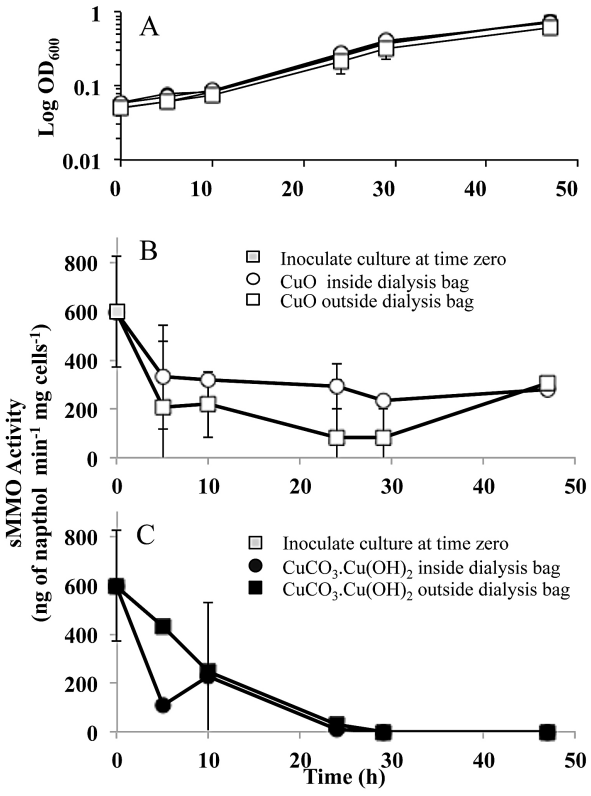

Fig. 2. Affect of direct mineral contact on the maintenance of sMMO activity (ng of napthol min^{-1} mg cell dry weight^{-1}) when the initial culture has sMMO activity. **(A)** Growth curves for CuO and $CuCO_3.Cu(OH)_2$ at 50 µmol total-Cu l^{-1} where the mineral was inside and outside of $<$ 1000 Da sieve size dialysis bags in the culture flask ($n = 2$ per treatment). **(B)** sMMO activity over time as function of location of CuO; i.e. inside and outside of the dialysis bags. **(C)** sMMO activity over time as function of location of $CuCO_3.Cu(OH)_2$; i.e. inside and outside of the dialysis bags. Error bars represent standard deviations ($n = 3$).

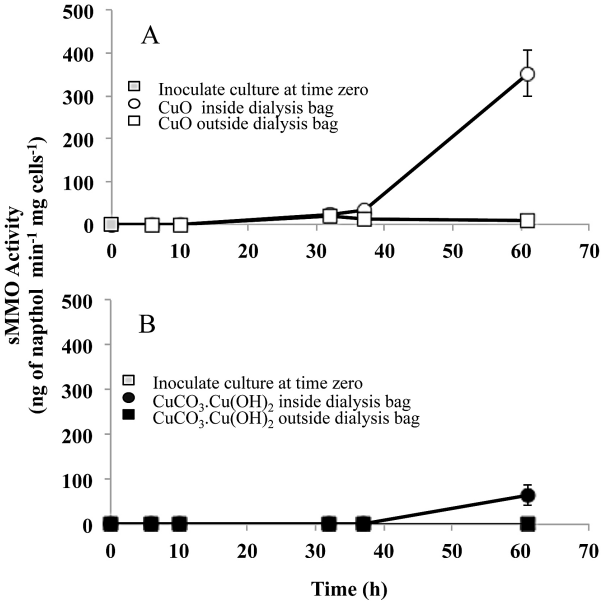

Fig. 3. Affect of direct mineral contact on the formation of sMMO activity (ng of napthol min^{-1} mg cell dry weight^{-1}) when the initial culture does not have sMMO activity. sMMO activity presented over time as function of location of the two minerals, **(A)** CuO and **(B)** $CuCO_3.Cu(OH)_2$; i.e. inside and outside of $<$1000 Da sieve size dialysis bags. sMMO activity is initially not present, but sMMO activity appears as cell density increases, especially for CuO without direct cell-mineral contact. Mean specific growth rates for the cultures range from 0.45 to 0.50 h^{-1}. Error bars represent standard deviations ($n = 3$).

in the cells, implying Cu is not readily available from this mineral without intimate contact (Fig. 2b). However, when direct contact was permitted, sMMO activity initially declined, but after 47 h, activity recovered to the same level as when contact was not allowed. This implies that Cu is more available from CuO with direct cell-mineral contact, but as cell densities increase (Fig. 2a), Cu dissolution is likely too slow to supply sufficient Cu for the growing culture. In contrast, sMMO activity was completely suppressed by $CuCO_3.Cu(OH)_2$ after 30 h, regardless of cell-mineral contact (Fig. 2c). Apparently, Cu dissolution from this mineral is sufficiently rapid to supply available Cu across the dialysis barrier, suggesting direct contact is not as critical for cells to obtain Cu from $CuCO_3.Cu(OH)_2$.

To corroborate Fig. 2 results, a second experiment was performed with $<$1000 Da dialysis bags, but in this case, the inoculate OB3b culture had no detectable sMMO activity. Here we monitored the development of sMMO activity over time as the culture densities increased as an indicator of Cu non-availability. Initially, no sMMO activity was apparent in any culture (with or without direct cell-mineral contact; Fig. 3). However, Fig. 3a shows that strong sMMO activity ultimately developed as the culture density increased with CuO and no direct cell contact (curves not shown), which is consistent with Fig. 2b. However, when $CuCO_3.Cu(OH)_2$ was the Cu source, no sMMO activity was detectable until the late growth phase of the experiment (Fig. 3b). Interestingly, a trace level of sMMO activity became detectable when $CuCO_3.Cu(OH)_2$ was inside the dialysis bag, suggesting cell mineral contact may slightly enhance Cu availability, although this influence is less pronounced than for CuO. Typical cell growth curves for all dialysis experiments are provided in Fig. 2a, showing that growth lags were not observed for either mineral at 50 µmol total-Cu l^{-1}. In addition, growth rates were similar among treatments.

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

The previous experiments showed that direct cell-mineral contact increased Cu availability, especially with CuO. However, the use of <1000 Da dialysis bags did not allow mb to directly mediate Cu acquisition. To assess the possible role of extracellular mb in Cu supply, a third experiment was performed in which <2000 Da dialysis bags were substituted for the <1000 Da bags, and supplemental mb was provided to some flasks. sMMO activity declined after 6 h with both minerals with and without supplemental mb (Fig. 4). However, cells exposed to CuO without supplemental mb retained three times greater sMMO activity after 24 h relative to the other treatments. Further, when no excess mb was provided to CuO, cellular Cu was not detected (<0.1 µg-Cu/mg-cell dry weight), implying that little Cu was being internalised by the cells. In contrast, when $CuCO_3.Cu(OH)_2$ was the Cu source, sMMO activity decreased independent of mb supply, and cellular Cu levels increased substantially (Fig. 4). $CuCO_3.Cu(OH)_2$ readily provides Cu to *M. trichosporium* OB3b, both suppressing sMMO activity and facilitating Cu uptake into the cell, whereas mb mediation and direct contact are important for Cu uptake from CuO.

3.4 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 in the presence of CuO or $CuCO_3.Cu(OH)_2$ (50 µmol total-Cu l^{-1}). Overall, excess mb did not alter cell yields for the minerals (0.011 ± 0.005 and 0.010 ± 0.003 mg cell dry weight/mg CH_4 with and without mb, respectively; mean ± 95 % confidence interval). However, mb significantly increased growth rates ($p < 0.05$, Wilcoxon signed-rank test). The mean specific growth rate coefficient with mb was $0.055 \pm 0.007\ h^{-1}$, whereas the coefficient was $0.044 \pm 0.006\ h^{-1}$ without mb, which implies that mb enhances cell growth on these solid-phase Cu-minerals, regardless of whether sMMO or pMMO activity prevails in the cells.

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

Mb is known to play multiple roles relative to Cu supply in *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; Balasubramanian and Rosenzweig, 2008; Choi et al., 2008; Kulczycki et al., 2011). However, results here show that the influence of mb on cell growth in the natural environment depends on solid-phase Cu conditions. For example, for relatively insoluble Cu sources, such as some silicates, mb does not enhance Cu acquisition and may actually quench the release of Cu from such surfaces (Kulczycki et al., 2007, Kulczycki et al., 2011). Whereas, *M. trichosporium* OB3b readily acquires Cu from more soluble minerals, such as $CuCO_3.Cu(OH)_2$. For such minerals it is not yet clear whether mb is actually needed for Cu uptake; however, we suspect mb must play some role, possibly through toxicity suppression by binding to free Cu(I) (Kim et al., 2005; El Ghazouani et al., 2011). In fact, *M. trichosporium* OB3b grows well on $CuCO_3.Cu(OH)_2$, particularly at very high Cu levels; the mineral appears to act as a "controlled release" Cu source, allowing the cell to obtain enough Cu for growth, but at a supply rate that does not cause toxicity.

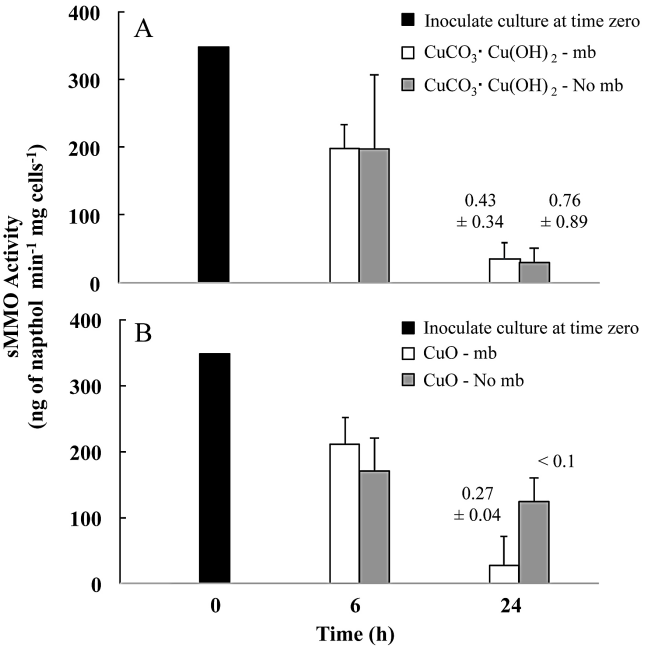

Fig. 4. sMMO activity (ng of napthol formed min^{-1} mg cell dry $weight^{-1}$) for *M. trichosporium* OB3b with and without supplemental methanobactin in the presence of **(A)** $CuCO_3.Cu(OH)_2$ and **(B)** CuO confined in <2000 Da sieve size dialysis bags. Concentration of cellular Cu after 24 h is annotated above columns (as µg-Cu/mg cell dry weight ± 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}.

In contrast, mb appears to play a more central role in Cu supply for growth with CuO, a more sparingly soluble mineral (Table 1). Mb remotely obtains Cu from this mineral, returning Cu to the cell, suppressing sMMO activity, and increasing cellular Cu levels (Fig. 4b). Further, direct cell contact with CuO enhances Cu availability (Figs. 2a and 3a). Clearly, the two minerals investigated differ in their capacity (and maybe mechanism) for Cu supply to *M. trichosporium* OB3b, which indicates differences in Cu mineralogy influence in situ MMO activity (i.e. sMMO vs. pMMO).

Although mb affects Cu-related responses differently among minerals (Figs. 2–4), mb also appears to act as a mild growth stimulant in the presence of solid-phase CuO and $CuCO_3.Cu(OH)_2$. Interestingly, this stimulatory effect does not appear to depend on whether pMMO or sMMO is the active MMO in the cell. Whether this effect results from Cu toxicity suppression, enhanced Cu bioavailability after release, or something more speculative is not known (e.g. quorum signalling seen with similar molecules; Miller and Bassler, 2001; D'Onofrio et al., 2010).

4 Conclusions

This work shows that solid-phase Cu minerals readily support growth in *M. trichosporium* OB3b and that mb conditionally plays an important role in that activity. In fact, new data here suggest solid Cu sources maybe be superior to more soluble sources, which is actually not surprising because methanotrophs usually reside in environments where Cu is less available, such as soils. However, the active MMO in the cells differs depending upon the Cu source; pMMO activity is sustained with $CuCO_3.Cu(OH)_2$, whereas sMMO activity prevails with CuO unless available Cu:cell ratios are high or the mineral is proximally close to the cells.

Observations here have significant practical implications. First, results suggest a strategy for promoting (or explaining) sMMO activity in biotechnical applications, such as contaminant degradation (Oldenhuis et al., 1989; Kim and Graham, 2003; Lee et al., 2006). If Cu is provided or is presented in a sparingly soluble form, favoured sMMO activity will prevail. Furthermore, previous field work has not found correlations between in situ Cu level and methanotroph activity, despite obvious links between Cu and methanotroph function. However, our data show that differences in Cu mineralogy potentially explain why soil-Cu and methanotroph activity have not been correlated in past studies. Therefore, our results suggest mineralogical assessment is essential for explaining MMO activity in natural systems. Regardless, mb and Cu mineralogy now should be added to CH_4, O_2 and nitrogen as key regulators of in situ aerobic CH_4 metabolism (Graham et al., 1993; Hanson and Hanson, 1996), and should be considered in future field investigations, especially in terrestrial environments. Mb broadly stimulates growth in solid-phase Cu systems, whereas Cu mineralogy influences whether sMMO or pMMO is the active form in resident cells.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/8/2887/2011/bg-8-2887-2011-supplement.pdf.

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