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Interactive comment on "Kinetics of calcite precipitation by ureolytic bacteria under aerobic and anaerobic conditions" *by* Andrew C. Mitchell et al.

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We thank the reviewers for their comments on our manuscript. We were very pleased that both reviews were positive suggesting publication with revisions. We have therefore revised the manuscript according to their suggestions. The reviewer comments and responses are shown below:

Reviewer 2: The manuscript describes laboratory determinations of the urea hydrolysis (both in the presents and absence of calcite precipitation) and calcite precipitation rates for 3 microorganisms (S. pasteurii, B. sphaericus 21776, and B. sphaericus 2178) at a temperature of 30 °C. The motivation for the study was the quantification of microbial

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Discussion paper

induced calcite precipitation technology. The reported experiments were conducted under both aerobic and anaerobic conditions (screening for terminal electron acceptors). The manuscript concluded that rates (as measured by first order rate constants) for both urea hydrolysis and calcite precipitation under aerobic condition were slightly higher for B. sphaericus 21776 than for S. pasteurii.

Given the scatter among the replicate measure of rate constants (Table SI2.1 CMM+) a more defensible statement would be that there is no apparent difference in rate constants between the two microorganisms. This statement is further supported by the observation that for the calcium absent experiments the mean urea hydrolysis rate constant (Table SI2.1 CMM-) for S. pasteurii is greater than for B. sphaericus 21776; the opposite of what was observed for the calcium present experiments.

Response: We agree with the reviewer that the cell number- (i.e. OD600- and CFU/mL-) normalized rate coefficients are not significantly different. We included p-values from two sample t-test comparisons in the manuscript and modified the abstract as follows:

"All bacterial strains showed ureolytic activity inducing CaCO3 precipitation aerobically. First order rate coefficients estimated from the experiments (regardless of whether normalized to biomass concentration or not) demonstrated slightly higher rate coefficients for both ureolysis (kurea) and CaCO3 precipitation (kprecip) for B. sphaericus 21776 compared to S. pasteurii though these differences were not statistically significant. "

And also in the kinetics main section 3.1.3, page 10, line 19: 'S. pasteurii and B. sphaericus 21776 exhibited statistically insignificant differences in kurea values (t-test p-value = 0.27)'.

And page 11, line 34: 'On average, B. sphaericus 21776 had the highest kprecip (0.60 $\pm$ 0.34 h-1), although considering its high standard deviation, kprecip for S. pasteurii is not significantly different (kprecip = 0.25 $\pm$ 0.02 h-1; t-test p-value 0.21).'

Comment: The manuscript also reports (p 10; LL12-15) that calcite precipitation in-

hibits the ability of the microorganisms to hydrolyze urea (presented as the ratios of the hydrolysis rate constants constants) and proposed (based on TEM observations and solid state diffusion calculations) that precipitating calcite entombs some fraction of the microorganisms and that the entombed organisms, while still alive, do not significantly contribute to further hydrolysis. The manuscript also describes experiments assessing the potential for urea hydrolysis by S. pasteurii under anaerobic conditions. The manuscript concludes that although population growth (as measured by OD600) did not occur in the absence of oxygen, urea hydrolysis indeed occurred at rate similar to those observed under aerobic conditions. Additionally, growth resumed upon reintroduction of oxygen following anaerobic incubation indicating survival of S. pasteurii for days in the absence of oxygen.

Comment 1: The manuscript could be strengthened by additional analysis of the results. Equations 9 (and 10) represents a first order rate approximation of enzymatically catalyzed urea hydrolysis. This approach is justified because a) rates were not measured directly (rather urea [or NH4] concertation as a function of time was measured) and b) the integrated rate expression fits the observations (not surprising given the scatter in the observation both within and between experiments). However enzymatic (urease) kinetics are better describe by the Michaelis-Menten expressions that reduces to 1st order kinetics at low urea concentrations and 0th order at high urea concentrations. This results in the apparent rate varying between 1st and 0th order as a function of urea concentration potentially over the course of a single experiment. With this in mind the normalization process needs to be consider carefully especially in the comparison to results of other studies using different concentration and temperatures.

An alternative approach would be to consider a simple linear regression model of apparent kurea values in term of optical density (OD600) and temperature (Kelvin).

Using the results for S. pasteurii reported in Table 3 supplemented with the 10 and 15 °C results of Ferris et al. (2004; this is incorrectly reference in both Table 3 and the reference list in the SI as 2003) yields: log kurea = 34.3 (9.5) + 1.22 (0.47)*log OD600 –

10,070 (2,660)/TKelvin where the values in parentheses represent the standard errors for the parameters. The fit has an R value of 0.77 (n=13) with the fit parameters having p-values of 0.027 (OD600) or less. If Equation 9 in the manuscript is the proper representation of the kinetics and OD600 is the measure of cell concentration (proportional to enzyme concentration), the expect coefficient for log OD600 would be 1. The regression value of 1.22 is less than half the standard error different from 1 suggesting that the assumption of equation 9 are adequate given (or because of?) the scatter in the experimental results, although one cannot exclude the possibility that kurea may also show a dependences on urea concentration (e.g., Michaelis-Menten kinetics). In addition, the coefficient for the temperature can be used to derive an apparent activation energy thus providing a temperature depended (10 – 30 °C) expression for estimating urea hydrolysis rates.

Response: We agree with the reviewer that Michaelis Menten-type of kinetics are most appropriate for describing enzyme kinetics over a wide range of concentrations and that the observed catalysis rate (v) indeed would be concentration-dependent. However, it has been demonstrated in the literature repeatedly that a first order first-order ureolysis rate model is appropriate in this study, rather than a the Michaelis-Menten model, specifically because urea concentrations are at concentration of 330mM or below. We have therefore included a paragraph justifying the use of the first-order ureolysis rate model over the Michaelis-Menten model in our study (Page 7, lines 2-5):

"Indeed, while the Michaelis-Menten model has been used when evaluating ureolysis, studies of ureolysis-induced calcium carbonate precipitation have demonstrated that the first-order ureolysis rate model fits well for urea concentrations of approximately 330 mM or below (Lauchnor et al., 2015; Connolly et al., 2015), which is the concentration range used in this study."

A first order approach indeed facilitates communication of the observed results since only one parameter (the first order kinetic rate coefficient) has to be compared and the overall goal of this manuscript was to compare the achievable urea hydrolysis and cal-

cite precipitation rates for the three different strains (S. pasteurii, B. sphaericus 21776, and B. sphaericus 21787) and for S. pasteurii under aerobic and anaerobic conditions.

As reviewer 2 points out here, Michaelis Menten-type kinetics can be approximated with first or zero kinetics for concentration ranges well below or above the half saturation constant (kM), respectively. As stated above, there is ample evidence in the literature (e.g. Lauchnor et al., 2015; Connolly et al., 2015) that first order kinetic approaches describe the kinetic behavior of urea hydrolysis for urea concentrations at 330mM or below quite well (see also Mitchell and Ferris, 2005; 2006a, b; Ferris et al 2004; Tobler et al., 2011; Cuthbert et al 2011) and the overall goal of this manuscript was to compare the achievable urea hydrolysis and calcite precipitation rates for the three different strains (S. pasteurii, B. sphaericus 21776, and B. sphaericus 21787) and for S. pasteurii under aerobic and anaerobic conditions. We indeed agree that additional analyses (temperature dependencies, OD dependencies, etc.) could be wrapped into a more widely valid kinetic equation but we are uncomfortable doing so at the current time since there are too many uncertainties regarding the exact manner in which data by other groups (Tobler et al 2011; Ferris et al., 2004) were acquired.

We also in response to reviewer 1's comment 1 addressed the normalization to biomass concentration and outlined the changes made in this section.

Ferris et al. (2004) reference has been corrected in both, Table 3 and the reference list in the supplementary information.

Comment 2: Page 7 line 5 – assuming the reaction is zero order with respect to biomass is not the same as stating that X=0 (if X=0 then d[Urea]/dt in equation 9 is also 0). Perhaps this section could be revised to consider this a pseudo-first order reaction where the apparent rate constant k*urea = kurea [X] and d[X]/dt = 0.

Response: As stated in the response to comment 1 of reviewer 1: We apologize for the oversight and thank both reviewers for pointing out this issue. This should have stated X = X0 and not X = 0. As outlined in our response to reviewer 1, we have now modified

the text as follows (Page 7, line 11) :

“Biomass-normalized ureolysis rates were calculated. Firstly, it was assumed that biomass (X) is constant in Eqs. 10 and 11, as performed in other studies of ureolysis kinetics (Cuthbert et al., 2012;Ferris et al., 2004;Mitchell and Ferris, 2005, 2006;Schultz et al., 2011;Tobler et al., 2011). Secondly, the obtained first order rate coefficients with respect to urea concentration (kurea) were normalized to the biomass concentration by dividing the ureolysis rate coefficient by the biomass at the onset of precipitation (i.e. urea hydrolysis rates were normalized to the absorbance reading of initial biomass, OD600, and CFU mL-1; SI section 2.2), which was equivalent to the initial biomass in each system (X = X0). This is an appropriate choice of model, since the biomass analysis indicated that the cell density was constant for the duration of CaCO3 precipitation and was equivalent to the initial biomass in the systems, as presented in the results section. The biomass-normalized ureolysis rates were compared to other parameters previously published (Ferris et al., 2004;Fujita et al., 2000;Stocks-Fischer et al., 1999;Tobler et al., 2011).”

Comment 3: Page 8 line 16 (and SI page 4 line 6) – the statement that approximately 25.5 mM of urea need to hydrolyze in order to achieve super-saturation is incorrect. Figure SI1.1 shows that a DIC concertation of 25.45 mM is required to reach saturation with respect to calcite. Given that the medium starts with DIC concentration of 25 mM (as NaHCO3; Table SI1.1) only 0.45 (0.5) mM of urea hydrolysis is required to achieve calcite saturation.

Response: We agree with the reviewer and truly appreciate the thorough review. Based on the modeling It indeed only takes an additional 0.45 mM of urea hydrolyzed in order to reach a SI = 0 for calcite since the medium already contains 25 mM bicarbonate. – the overall bicarbonate concentration is predicted to be 25.5 mM at that point. – We corrected the statement in the manuscript and in the supplementary information: “Geochemical modelling suggested that no CaCO3 precipitation should occur in the absence of ureolysis and that approximately 0.45 mM of urea would have had to be

hydrolyzed to achieve supersaturation and for CaCO3 precipitation to commence (see SI1.4).”

We thank the reviewers again for their valuable comments and look forward to hearing back.

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