Integrating genome-scale metabolic models into the prediction of microbial kinetics in natural environments

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Abstract

We propose a new method to predict microbial metabolic rates in natural environments using genome-scale metabolic models. This method is a hybrid of existing approaches, i.e., rate laws and flux balance analysis (FBA). It accounts for the availabilities of chemical energy and growth nutrients in the environment, and applies FBA independently to the respiration and biosynthesis pathways of genome-scale metabolic models. We illustrate the new method by modeling the metabolism of a representative methanogen – *Methanosarcina barkeri* – in laboratory reactors and in pristine and biostimulated aquifers. The laboratory application demonstrates that the hybrid method predicts the rates of individual biochemical reactions within overall cell metabolism and tracks, explicitly, cellular fluxes of carbon and energy. The aquifer applications reveal that the growth of methanogens in natural systems can be limited by multiple factors, including energy sources and growth nutrients, and that the limitations are subject to Liebig's Law of the Minimum. These results highlight the improvements of the new method in biogeochemical reaction modeling, including its applicability to diverse environments, from eutrophic to oligotrophic.

Keywords: genome-scale metabolic model, biogeochemical reaction modeling, microbial kinetics, flux balance analysis, nutrient limitation, methanogenesis

1. Introduction

Biogeochemical reaction modeling simulates, numerically, concurrent geochemical reactions and microbial metabolisms in natural environments (Bethke, 2008). This method combines geochemical and microbial reaction models. Geochemical models focus on chemical speciation, redox reactions, mineral precipitation and dissolution, and other abiotic reactions. Microbial models describe microbial reactions and the development of microbial populations. By coupling microbial and geochemical models, biogeochemical reaction modeling offers a quantitative assessment of microbial processes in natural environments, and has become a routine tool for both theoretical and practical applications, such as element cycling and the contamination and remediation of groundwater (Druhan et al., 2012; Jin and Roden, 2011; Johannesson and Neumann, 2012).

Classical approaches simulate microbial metabolisms using black-box models (Jin et al., 2013). These models bypass biochemical pathways and metabolic regulation, and compute rates of respiration and growth directly from environmental concentrations of energy sources and growth nutrients using rate laws (Monod, 1949; Simkins and Alexander, 1984). For example, they calculate respiration rates r'_R (mol·g^{-1}·s^{-1}) using the thermodynamically-consistent Monod equation (Jin and Bethke, 2002; 2003),

$$r'_R = k \cdot \frac{m_D}{m_D + K_D} \cdot \frac{m_A}{m_A + K_A} \cdot \left[1 - \exp\left(-\frac{\Delta G_A - \Delta G_C}{\chi RT} \right) \right], \quad (1)$$

where k is the rate constant (respiration rate per unit biomass, mol·g^{-1}·s^{-1}), m_D and m_A are the molal concentrations of electron donors and acceptors, respectively, K_D and K_A are the molal half-saturation constants, ΔG_A is the energy available from redox reactions – the negative of the Gibbs free energy change (J·mol^{-1}), ΔG_C is the energy conserved by respiration (J·mol^{-1}), χ is

the average stoichiometric number, R is the gas constant (8.3145 J·mol^{-1}·K^{-1}), and T is the temperature in Kelvin. They calculate biosynthesis rate r'_X – biomass production rate per unit biomass (s^{-1}) – from respiration rate,

$$r'_X = Y_{X/i} \cdot \nu_{i,R} \cdot r'_R \ , \tag{2}$$

and calculate microbial growth rate (g·kg^{-1}·s^{-1}),

$$\frac{d[X]}{dt} = \left(r'_X - r'_M\right) \cdot [X] \ , \tag{3}$$

as the difference between the biosynthesis rate r'_X and the maintenance/death rate r'_M (s^{-1}) (Jin and Roden, 2011; Jin et al., 2013). Here [X] is the biomass concentration in dry weight per unit water mass (g·kg^{-1}), $Y_{X/i}$ is the biomass yield per chemical compound i (g·mol^{-1}), and $\nu_{i,R}$ is the stoichiometric coefficient of compound i in respiration reaction equation. These equations are simple in implementation and fast in computation, but their application to natural environments has not always been successful. For instance, their predictions can deviate from field observations by orders of magnitude (Brown et al., 2000; Chapelle and Lovley, 1990; Murphy and Schramke, 1998). Previous studies have questioned the applicability of the rate equations, and ascribed the discrepancies to the differences in growth conditions and microbial metabolisms between laboratory reactors and natural environments (Jannasch, 1967; Jin and Bethke, 2005; Jin et al., 2013).

Recently, genome-scale metabolic models have emerged as a solution to address the challenges of the standard modeling approach (Mahadevan et al., 2011). These models use a series of stoichiometric equations to describe biochemical reactions of metabolic pathways, from nutrient uptake, to respiration, and to biosynthesis (Bordbar et al., 2014; Reed and Palsson, 2003). They make possible the integration of metabolic pathways into the prediction of microbial

kinetics.

For example, dynamic flux balance analysis predicts microbial rates by combining flux balance analysis (FBA) with the Michaelis-Menten equation (fig 1A) (Mahadevan et al., 2002; Vargas et al., 2011). The Michaelis-Menten equation calculates cellular fluxes of nutrient uptake from the environment. FBA is a standard method for analyzing genome-scale metabolic models (Feist and Palsson, 2008; Orth et al., 2010). It takes one or more uptake fluxes as the input, and maximizes growth rates by optimizing the rates of biochemical reactions in genome-scale metabolic models. This method only requires the kinetic parameters for nutrient uptake, and is especially attractive where critical information, such as enzyme kinetic parameters, is not available.

Previous studies applied dynamic FBA to both bioreactors and natural environments (Henson and Hanly, 2014; Meadows et al., 2010; Tartakovsky et al., 2013; Zhao et al., 2011; Zhuang et al., 2010). However, the method may not be suitable for many environmental applications. For instance, in order to apply the genome-scale metabolic model of a ferric-iron respirer – *Geobacter sulfurreducens* – to an aquifer, the rate predictions had to be scaled down by an *ad hoc* factor of 10 (Fang et al., 2011; Scheibe et al., 2009).

Several factors have been proposed to account for the rate overestimation by dynamic FBA. Specifically, genome-scale metabolic models are under-determined – they have more metabolite fluxes than biochemical reactions (Herrgård et al., 2006). As a result, their solutions might be mathematically correct, but physiologically infeasible. In addition, FBA maximizes microbial growth rates at given nutrient fluxes, which may not work for microbes whose metabolic performance is suboptimal (Feist and Palsson, 2010; Wintermute et al., 2013). Also important is the utilization of the standard Michaelis-Menten equation. This equation does not

consider thermodynamic controls on microbial metabolism, and may not capture the full complexity of nutrient transport from the environment to the cytoplasm (Button, 1985; Jin and Bethke, 2007). Furthermore, growth rates and other model predictions are highly sensitive to the enzyme parameters in the Michaelis-Menten equation, which makes dynamic FBA prone to error (Klier, 2012).

Here we propose a new method for applying genome-scale metabolic models to microbial kinetics in natural environments (Jin et al., 2013). This method is a hybrid of FBA and the rate laws for microbial respiration and nutrient uptake (Jin and Bethke, 2003). It accounts for microbial energy conservation and the uptake of growth nutrient, and applies FBA independently to the respiration and biosynthesis pathways of genome-scale metabolic models.

We illustrate the hybrid method using a representative methanogen –*Methanosarcina barkeri*. *M. barkeri* and its relatives live in diverse environments, from surface sediments to aquifers (Hedderich and Whitman, 2006; Smith et al., 2015). They can make methane from acetate – a major contributor to global methane production and a key process of biogeochemical carbon cycling (Ferry, 2010). We apply the hybrid method to simulate the metabolism of *M. barkeri* in laboratory reactors, as well as to analyze microbial growth under energy- vs. nutrient-limiting conditions in pristine and biostimulated aquifers.

2. Methods

2.1. Flux balance analysis

Genome-scale metabolic models represent the biochemical reactions of an entire metabolism using a stoichiometric matrix, **S**, of size $m \times n$. Here, n is the number of biochemical reactions and m is the number of metabolites – chemical compounds consumed and produced by biochemical reactions. Element S_{ij} is the stoichiometric coefficient of metabolite i in reaction j.

These biochemical reactions are derived from annotated genomes, and drive respiration, biosynthesis, detoxification, and other key metabolic functions (Thiele and Palsson, 2010). In addition, genome-scale metabolic models also contain two hypothetical reactions. One is the biomass production reaction that produces new cells from amino acids, nucleic acids and other biomass precursors, and the other is an ATP hydrolysis reaction that accounts for the energy consumption by cellular maintenance – metabolic processes that maintain the integrity and function of cell components and structures, but do not contribute to cell reproduction (Hoehler and Jørgensen, 2013).

FBA assumes that metabolism is at steady state, and applies the principle of mass balance to metabolite fluxes. The result is a linear algebra equation,

$$\mathrm{J} = \mathbf{S}\cdot\mathrm{R} \tag{4}$$

where J and R are column vectors; element J_i is the net flux or production rate of metabolite i per unit dry weight of biomass ($mol\cdot g^{-1}\cdot s^{-1}$), and element R_j is the rate of biochemical reaction j per unit biomass ($mol\cdot g^{-1}\cdot s^{-1}$).

Different modeling disciplines treat under-determined systems differently. For instance, geochemical reaction modeling brings under-determined systems to balance using the principle of mass action (Bethke, 2008). But this approach is not feasible for genome-scale metabolic models because kinetic parameters are not available for most biochemical reactions. Instead, FBA takes one or more uptake fluxes of energy sources and growth nutrients as the input, and optimizes the rates of individual biochemical reactions in order to maximize the rates of growth (Feist and Palsson, 2010).

2.2. Dynamic FBA

Dynamic FBA applies FBA to simulate microbial metabolisms, assuming that the

metabolisms are at quasi steady-state (Becker et al., 2007). At each time step, this method assumes that cell metabolisms are at steady state, and predicts microbial rates and chemical fluxes in two steps (fig 1A) (Mahadevan et al., 2002). It first applies the classical Michaelis-Menten equation to compute the uptake fluxes of energy sources and growth nutrients. For example, the uptake flux F_{N} of a nutrient is calculated according to,

$$F_{\mathrm{N}} = V_{\max} \cdot \frac{m_{\mathrm{N,env}}}{m_{\mathrm{N,env}} + K_{\mathrm{N,env}}}, \tag{5}$$

where $V_{\max}$ is the maximum flux ($\mathrm{mol \cdot g^{-1} \cdot s^{-1}}$), $m_{\mathrm{N,env}}$ is the molal concentration of nutrient N in the environment, and $K_{\mathrm{N,env}}$ is the molal Michaelis constant (M). It then takes one or more uptake fluxes as the input, and applies FBA to genome-scale metabolic models to predict microbial growth rates and the fluxes of nutrient consumption and waste production by cell metabolisms. At the end of the time step, the growth rates and chemical fluxes are applied to update the concentrations of biomass and chemical compounds, respectively.

2.3. Hybrid method

We propose a hybrid method that combines FBA with microbial rate laws. This method builds on current frameworks of biogeochemical reaction modeling, and accounts for the availabilities of chemical energy and growth nutrients in the environment (fig 1B).

2.3.1. Nutrient uptake

A unique feature of the hybrid method is the consideration of cytoplasmic nutrient accumulation. Microbes can accumulate nutrient in the cytoplasm by releasing proton motive force or by hydrolyzing ATP – a process called active transport (Tanford, 1983). By coupling to the release of proton motive force, nutrient transport can be described as

$$\mathrm{N_{env}} + \nu_{\mathrm{H}} \mathrm{H^{+}_{env}} \rightleftarrows \mathrm{N_{cyt}} + \nu_{\mathrm{H}} \mathrm{H^{+}_{cyt}}, \tag{6}$$

where N_{env} and N_{cyt} are the nutrient in the environment and the cytoplasm, respectively, H^+_{env} and H^+_{cyt} are the protons in the environment and the cytoplasm, respectively, and ν_H is the number of protons transported together with the nutrient. We calculate the uptake flux F_N using the thermodynamically-consistent Michaelis-Menten equation,

$$F_N = V_{max} \cdot \frac{m_{N,env}}{m_{N,env} + K_{N,env}} \cdot F_T , \quad (7)$$

in order to account for the feedback inhibition of nutrient accumulation in the cytoplasm (Jin and Bethke, 2007). Here F_T is the thermodynamic factor,

$$F_T = 1 - \frac{a_{N,env}}{a_{N,cyt}} \exp\left[\frac{\left(z_N + \nu_H\right) \cdot F \Delta p}{RT} + z_N \cdot \ln(10) \cdot \Delta pH \right], \quad (8)$$

where a represents chemical activity – the product of molal concentration and activity coefficient, z_N is the electrical charge of the nutrient, Δp is the proton motive force (V), ΔpH is the pH difference between the environment and the cytoplasm, and F is the Faraday constant (Jin, 2012).

Evaluating the thermodynamic factor (eq 8) requires knowledge of the nutrient concentration in the cytoplasm – a parameter not available for most microbes. We note that cytoplasmic nutrient concentration influences not only nutrient transport (eq 8), but also the activities of cytoplasmic enzymes that consume nutrients. The impact on nutrient consumption can be quantified using a kinetic factor F_K,

$$F_K = \frac{m_{N,cyt}}{m_{N,cyt} + K_{N,cyt}} , \quad (9)$$

where m and K are the molal concentration and Michaelis constant of nutrients, respectively (Jin and Bethke, 2007).

From equation 8 and 9, we see that high cytoplasmic concentrations inhibit nutrient assimilation by lowering the F_T value and hence the uptake flux F_N. On the other hand, high concentrations raise the kinetic factor F_K and the activities of nutrient-consuming enzymes, thereby promoting nutrient assimilation. We thus assume that to effectively assimilate nutrients, microbes need to maximize the product of the thermodynamic factor F_T and the kinetic factor F_K,

$$\max\left(F_T \cdot F_K^n\right). \tag{10}$$

Here, the exponent n accounts for the likelihood that the thermodynamic and the kinetic factors may influence nutrient consumption and assimilation to different extents. Solving equation 10 gives the optimum cytoplasmic nutrient concentration, which is then applied to compute the nutrient uptake flux (eq 7).

2.3.2. Respiration

The hybrid method calculates respiration rate r'_R according to the thermodynamically-consistent Monod equation (eq 1). In evaluating this rate equation, we calculate the energy ΔG_C conserved by respiration,

$$\Delta G_C = \nu_{ATP} \cdot \Delta G_P, \tag{11}$$

as the product of the ATP yield ν_{ATP} – the number of ATPs produced per respiration reaction – and the phosphorylation energy ΔG_P – the energy required for ATP synthesis from ADP and phosphate [J·(mol ATP)$^{-1}$]. We calculate the chemical flux (mol·g^{-1}·s^{-1}) of respiration according to

$$F_{i,R} = \nu_{i,R} \cdot r'_R \ , \tag{12}$$

where $F_{i,R}$ is the flux of compound i, and its value is positive for reaction products and negative for reactants.

We estimate the ATP yield by applying FBA to the respiration pathways in genome-scale metabolic models. ATP is an intermediate metabolite produced by respiration pathways, and is consumed by biosynthesis pathways and maintenance reactions. The steady-state assumption of FBA dictates that the net flux of ATP is 0. In order to estimate ATP yields, we temporarily decouple ATP production and consumption by setting the rates of the biosynthesis and maintenance reactions to 0.

We add a hypothetical reaction of ATP output to the genome-scale metabolic models, and assume that respiration maximizes the ATP output flux. We carry out FBA using the uptake fluxes of electron donors D and/or acceptors A as the input. The FBA output includes the fluxes of ATP output and the production of oxidized electron donors D^+ and reduced electron acceptors A^-. The ratios of these fluxes give the stoichiometric equations of respiration,

$$\sum_{D} \nu_D D + \sum_{A} \nu_A A + \nu_{ATP} ADP + \nu_{ATP} P_i + \nu_{ATP} H^+_{cyt} \rightleftarrows \sum_{D^+} \nu_{D^+} D^+ + \sum_{A^-} \nu_{A^-} A^- + \nu_{ATP} ATP + \nu_{ATP} H_2O , \quad (13)$$

where ν_D and others are stoichiometric coefficients, and P_i is inorganic phosphate (HPO_4^{2-}).

2.3.3. Biosynthesis

We apply FBA to the biosynthesis pathways to predict the rates, chemical fluxes, and yields of biosynthesis. Biosynthesis pathways use ATP to make new biomass from growth nutrients. As discussed above, because ATP is an intermediate metabolite, we cannot analyze the effect of ATP supply on biosynthesis by applying FBA directly to genome-scale metabolic models. Instead, we stop the ATP-producing respiration pathways and the ATP-consuming maintenance reaction, and prescribe a supply flux of ATP as the input for biosynthesis. In order to analyze the relative importance of ATP and growth nutrients, we also prescribe nutrient uptake fluxes as the input.

We follow the standard practice of assuming that microbes maximize the rates of biosynthesis (Feist and Palsson, 2010). The FBA output includes biosynthesis rates r'_X and the chemical fluxes driven by the biosynthetic reactions, such as nutrient consumption and waste production. The ratios of these chemical fluxes can be applied to infer the stoichiometric equation for biosynthesis, and biosynthesis rates are applied to compute the rates of microbial growth (eq 3). The chemical fluxes are combined with those of respiration to compute the total chemical fluxes of cell metabolism. For example, the total flux $F_{i,T}$ of compound i is

$$F_{i,T} = F_{i,R} + F_{i,X}, \tag{14}$$

the sum of the fluxes driven by respiration ($F_{i,R}$) and by biosynthesis ($F_{i,X}$).

2.4. Application

We apply the hybrid method to the current genome-scale metabolic model of *M. barkeri* – model iMG746, a revised version of the original model iAF692 (Feist et al., 2006; Gonnerman et al., 2013). We update model iMG746 with the following revisions: the Na^+/H^+ antiporter translocates one proton per Na^+ (Jin, 2012) and the acetate transporter moves one acetate molecule, together with one proton, across the membrane (Welte et al., 2014). We set the growth-associated ATP consumption – the ATP requirement by the hypothetical biomass production reaction – to 109 $mmol \cdot g^{-1}$ (Kliphuis et al., 2012). We also follow the original model – iAF692 and remove the sulfite transporter activity, because no gene in *M. barkeri* genome codes for a sulfite transporter.

Microbial growth consumes sources of energy, carbon, nitrogen, phosphorous, and other elements. *M. barkeri* can fix N_2, but prefers ammonium as the source of nitrogen (Kenealy et al., 1982; Kessler et al., 2001), and both pathways are represented in model iMG746. Here we take ammonium uptake as an example (Boogerd et al., 2011),

248 $$NH_{3,env} + H^{+}_{env} \rightleftarrows NH_{3,cyt} + H^{+}_{cyt}, \quad (15)$$

249 to illustrate how explicitly accounting for nutrient availability and transport influences the
250 accuracy of microbial growth rate prediction via the hybrid method.

251 Before simulating the metabolism of *M. barkeri*, we apply FBA to the methanogenesis
252 pathway of model iMG746 to estimate the yield of ATP (fig 1B). We assume that cytoplasmic
253 ammonium concentrations are at optimum levels for biosynthesis, and solve the optimization
254 problem (eq 10) at different environmental ammonium concentrations using brute-force search.
255 We then use the estimated ATP yield and the optimized cytoplasmic ammonium concentrations
256 to simulate the overall metabolism of *M. barkeri*. At each time step, we first calculate the rate
257 and ATP flux of methanogenesis using the thermodynamically-consistent Monod equation (eqs.
258 1 and 12), and the flux of ammonium uptake using the thermodynamically-consistent Michaelis-
259 Menten equation (eqs. 7 and 8). We predict the rate and chemical fluxes of biosynthesis by
260 applying FBA to the biosynthesis pathway, using the fluxes of ATP synthesis and ammonium
261 uptake as the input. At the end of the time step, we update the concentrations of chemical
262 compounds and biomass in the environment (eqs. 3 and 14). Applying the hybrid method
263 requires a series of microbial and enzymatic parameters, which, for the present calculation, are
264 listed in table 1.

265 We implement the hybrid method by linking the COBRA toolbox and PHREEQC
266 software package and by using the Microsoft Component Object Model (COM) Server as a
267 control and data management source. COM is a Microsoft foundations technology for
268 exchanging information among software packages of different platforms. COBRA and
269 PHREEQC specialize in FBA and biogeochemical reaction modeling, respectively (Charlton and
270 Parkhurst, 2011; Schellenberger et al., 2011). We run COBRA using Gruobi version 6.5, a linear

271 solver that provides acceptable accuracy at small chemical fluxes. The input files of the
272 simulations are available in the Supplementary Material.

273 **3. Results**

274 We illustrate the hybrid method using the metabolism of *M. barkeri* growing on acetate.
275 We first estimate key parameters, including the ATP yield of methanogenesis, the stoichiometry
276 of biosynthesis reactions, and the flux of ammonium uptake. We then simulate the metabolism in
277 laboratory experiments, and predict the growth in aquifers under energy- vs. nutrient- limiting
278 conditions.

279 **3.1. Model predictions**

280 The hybrid method takes multiple steps to predict the rates of methanogenesis and
281 biosynthesis in *M. barkeri*. These steps include applying FBA independently to the
282 methanogenesis and biosynthesis pathways in model iMG746, as well as the optimization of
283 cytoplasmic nutrient concentration.

284 **3.1.1. ATP yield**

285 We apply FBA to the methanogenesis pathway of model iMG746 to estimate the ATP
286 yield of acetoclastic methanogenesis. The results show that *M. barkeri* makes ATP by
287 consuming acetate and proton and producing methane and CO_2. The ratios of the chemical fluxes
288 give a yield of 0.75 ATPs per methane, and the following stoichiometric equation of
289 acetcoclastic methanogenesis,

290 $$\text{Acetate} + \frac{3}{4}\text{ADP} + \frac{3}{4}\text{P}_\text{i} + 1\frac{3}{4}\text{H}^+ \rightleftarrows \text{CH}_4 + \text{CO}_2 + \frac{3}{4}\text{ATP} + \frac{3}{4}\text{H}_2\text{O}\,. \quad (16)$$

291 These ratios remain constant over acetate uptake fluxes of 10^{-5} mmol·g^{-1}·hr^{-1}, as in oligotrophic
292 environments (Hoehler and Jørgensen, 2013), to a maximum flux of 7.1 mmol·g^{-1}·hr^{-1} (table 1).
293 The FBA-derived ATP yield matches with the value estimated previously for *Methanosarcina*

294 species (Welte and Deppenmeier, 2013). The product of the ATP yield and the maximum acetate
295 uptake flux gives the maximum flux of ATP production at 5.3 $mmol \cdot g^{-1} \cdot hr^{-1}$ (eq 12).

296 **3.1.2. Biosynthesis reaction**

297 We apply FBA to the biosynthesis pathway to analyze the effect of microbial energy
298 conservation on biomass synthesis. Biosynthesis consumes both ATP and nutrients. In order to
299 focus on ATP supply, we use the flux of ATP as the only input for FBA, assuming that nutrient
300 uptake is not limiting with respect to biosynthesis, as would be typical in most laboratory
301 experiments (Whitman et al., 2006). Later we consider nutrient limitation by taking ammonium
302 as an example (section 3.3.1.).

303 The FBA results show that the biosynthesis pathway makes new cells by consuming
304 acetate, ammonium, proton, phosphate, and cysteine. The cysteine requirement is consistent with
305 the current practice of laboratory culturing: cysteine is an essential nutrient and serves as a
306 source of sulfur for *M. barkeri* (Mazumder et al., 1986). The biosynthesis pathway also produces
307 CO_2 and methylsulfide (methanethiol or methyl mercaptan, CH_3SH) as waste products. The
308 methylsulfide production complements previous laboratory observations that the methanogenesis
309 of *M. barkeri* can consume methylsulfide as a substrate or produce it as a product (Moran et al.,
310 2008; Zhang et al., 2008).

311 The rates and chemical fluxes of biosynthesis vary linearly with ATP fluxes. The overall
312 rate of biosynthesis is given by,

313 $$r'_X = Y_{X/ATP} \cdot F_{ATP}, \quad (17)$$

314 where $Y_{X/ATP}$ is the biomass yield per ATP, and has a value, 6.1 $g \cdot mol^{-1}$, that is close to the value
315 (6.2 $g \cdot mol^{-1}$) determined using laboratory bioreactors (Jin, 2012). When ATP is supplied at the
316 maximum production flux, biosynthesis reaches a maximum rate of 3.2×10^{-2} hr^{-1}, consuming

ammonium at a maximum flux of 0.35 mmol·g^{-1}·hr^{-1}. The predicted maximum biosynthesis rate is close to the maximum growth rate of *M. barkeri* in laboratory reactors (4.1×10^{-2} hr^{-1}) (Fukuzaki et al., 1990).

The linear variations of the chemical fluxes also give the following stoichiometric equation for biosynthesis,

$$1.73\text{Acetate} + 0.95\text{NH}_4^+ + 0.05\text{Cysteine} + 11.78\text{H}_2\text{O} + 14.17\text{ATP} \rightarrow \text{X} + 0.08\text{CO}_2 + 0.03\text{CH}_3\text{SH} + 14.17\text{ADP} + 14.09\text{P}_i + 13.29\text{H}^+ \quad (18)$$

Here X represents biomass with a chemical formula of $C_{3.52}H_{5.42}O_{1.33}NP_{0.08}S_{0.03}$ (molecular weight, 86.46). The chemical formula is arrived at on the basis of the cellular composition of macromolecules and metabolites (Gonnerman et al., 2013); following the common practice, we set the number of nitrogen atom in the biomass formula at 1. The FBA results show that the biosynthesis reaction also produces aminobenzoate and glycolaldehyde as waste products. However, as these compounds have stoichiometric coefficients of only 0.002, they are omitted from the reaction equation. Also note that ADP and phosphate do not have a 1:1 stoichiometry in the products, as would be the case if ATP hydrolysis was the sole reaction involving phosphate. Instead, the smaller quantity of phosphate relative to ADP is due to the consumption of 0.08 mol phosphate per mol biomass synthesized.

3.1.3. Ammonium transport

Optimizing cytoplasmic ammonium concentration requires a series of parameters, including the maximum ammonium uptake flux, the Michaelis constant of cytoplasmic ammonium, and the exponent *n* (see eqs 8, 9 and 10). We calculate the maximum ammonium uptake flux, V_{max}, of 0.35 mmol·g^{-1}·hr^{-1}, from the maximum biosynthesis rate and the stoichiometric equation of biosynthesis (eq 18). The maximum ammonium flux and biosynthesis

rate are close to those of *Geobacter sulfurreducens*: Scheibe et al. (2009) estimated that *G. sulfurreducens* has a maximum ammonium flux and biosynthesis rate of 0.5 $mmol \cdot g^{-1} \cdot hr^{-1}$ and 4.7×10^{-2} hr^{-1}, respectively.

The exponent *n* in the optimization function (eq 10) determines the magnitude of ammonium accumulation in the cytoplasm. A large *n* value favors the accumulation of cytoplasmic ammonium, while a small value lowers the accumulation. Kadam and Boone (1996) analyzed ammonium accumulation by *Methanolobus bombayensis*, *Methanolobus taylorii*, and *Methanohalophilus zhilinaeae* in laboratory reactors where ammonium concentrations are relatively large, > 10 mM (fig 2A). By trial and error, we found that by taking the exponent *n* as 2, the optimization solution matches the ammonium accumulation in those three methanogens (see fig 2A). The exponent *n* of 2 suggests that these methanogens likely favor ammonium accumulation in the cytoplasm, which comes at the expense of decreasing the thermodynamic factor and, hence, the ammonium transport flux (eq 8). This may reflect the need of a relatively large pool of cytoplasmic ammonium in order to speed up the ammonium-consuming enzymes with relatively large Michaelis constants. Specifically, in the cytoplasm of *M. barkeri*, ammonium-consuming enzymes include glutamine synthetase and glutamate dehydrogenase, and their Michaelis constants for ammonium can be as large as 2 mM (Boogerd et al., 2011).

Here we assume that for *M. barkeri*, the exponent *n* also takes a value of 2 and that the laboratory observations can be extrapolated to natural environments, where ammonium concentrations are generally much smaller than 10 mM (fig 2B). Figure 2B and C show the accumulation of cytoplasmic ammonium and the corresponding fluxes of ammonium uptake, given these assumptions. Both the accumulation and uptake respond strongly to the availability of ammonium. With an environmental ammonium concentration of 1 μM, *M. barkeri* can

362 accumulate ammonium in the cytoplasm by a factor of 33, and transport ammonium at a flux of
363 3.1×10^{-4} mmol·g^{-1}·hr^{-1}. At 10 mM ammonium, the accumulation reduces to four fold, but the
364 uptake flux increases to 0.31 mmol·g^{-1}·hr^{-1}, close to the maximum flux.

365 **3.2. Laboratory experiments**

366 Previous laboratory studies have extensively examined the metabolism of *M. barkeri*
367 growing on acetate (e.g., Mah et al., 1978; Smith and Mah, 1978; Westermann et al., 1989, and
368 others). Here we apply the hybrid method to simulate the experiments of Fukuzaki et al. (1990).
369 They grew *M. barkeri* at 37 ºC in batch reactors of 50 mL of complex growth media and 75 mL
370 headspace. The media had pH 7.1 and contained 19.7 mM acetate and 9.3 mM ammonium. They
371 followed closely the progress of acetate consumption, even after acetate reached a constant
372 concentration of 2.0 mM at day 4 (fig 3A), which provides an opportunity to illustrate the
373 thermodynamic control on the progress of metabolism.

374 We simulated the progression of their experiments using an initial biomass concentration
375 of 30 mg·kg^{-1}. This value is estimated on the basis of the experimental results of the first two
376 days (fig 3A). The simulation results match the observations of Fukuzaki et al. (1990). As shown
377 in figure 3A, at the beginning of the experiments, acetate concentration decreases almost linearly
378 with time. After two days into the experiments, the decrease slows down. The simulation
379 predicts that methane and biomass build up in the reactors (fig 3A and B). At the end of the
380 simulation, acetate concentration decreases to 1.3 mM, and 16.9 mmol methane is produced per
381 liter of growth media. Note that our simulation partitions methane between the medium and
382 headspace, with respective volumes as given by Fukuzaki et al. (1990); figure 3 plots only the
383 dissolved methane concentration. These results give a stoichiometric ratio of methane production
384 to acetate consumption at 0.92, close to the value of 1 in the reaction equation (eq 16) (Smith and

385 Mah, 1978). Biomass concentration increases with time and reaches its maximum value at day 5.
386 The simulation also predicts notable ammonium consumption – during the experiments, a total of
387 0.8 mM ammonium is consumed (fig 3A).

388 We also simulate the experimental progress using dynamic FBA, and an initial biomass
389 concentration of 0.12 $g \cdot kg^{-1}$. Table 1 lists the required parameters and their values for the
390 simulation. As shown in figure 3A and B, dynamic FBA is only applicable to the first 2.5 days of
391 the experiments. It predicts well acetate consumption during this period. But after day 2.5,
392 dynamic FBA fails to return any solution, for reasons described in the discussion below.

393 The results of the hybrid method agree with previous characterizations of methanogen
394 metabolisms. For instance, previous studies describe the efficiency of cell metabolism in terms
395 of the biomass yield per carbon source, $Y_{X/C}$ (Roden and Jin, 2011). We can calculate the
396 biomass yield from the predicted biosynthesis rate r'_X and the total flux of acetate consumption
397 $F_{AC,T}$,

398 $$Y_{X/C} = \frac{r'_X}{F_{AC,T}}. \quad (19)$$

399 Substituting equation 14 and applying the simulation results give a $Y_{X/C}$ value of 4.0 $g \cdot mol^{-1}$,
400 near the upper end of the range of 1.2 to 4.2 $g \cdot mol^{-1}$ determined in laboratory bioreactors
401 (Scherer and Sahm, 1981; Sowers et al., 1984).

402 As a second example, acetate is used by *M. barkeri* in both methanogenesis (energy-
403 producing) and biosynthesis (carbon-utilizing) reactions. Here the fractions of acetate consumed
404 by methanogenesis and biosynthesis are denoted as f_R and f_X, respectively. The simulation
405 predicts that *M. barkeri* has f_R of 0.92 and f_X of 0.08 (fig 3C and D), close to the values of 0.95
406 and 0.05 suggested previously (Rittmann and McCarty, 2012).

The simulation results include the rates of individual biochemical reactions, which allow us to further track the cellular usage of acetate. The biosynthesis pathway of *M. barkeri* consumes acetate for two purposes. One is the production of acetyl-CoA and pyruvate – the central metabolites for the production of amino acids, nucleotides, and other biomass precursors. The other is the production of reducing power – reduced ferredoxins and cofactor F420, which are utilized in the synthesis of biomass precursors (Jablonski and Ferry, 1991; Krzycki et al., 1982). To produce the reducing power, acetate is first oxidized to methyl-tertahydrosarcinapterin, and then to CO_2. The first oxidation step is driven by the CO dehydrogenase/acetyl-CoA synthase enzyme complex and the second by the reversal of the CO_2 reduction portion of the hydrogenotrophic methanogenesis pathway.

The simulation results predict that, for *M. barkeri* growing on acetate in laboratory reactors, the biosynthesis pathway spends 72.7% of the acetate flux on the production of acetyl-CoA and pyruvate and the remaining 27.3% on the production of reducing power. Overall, of the total acetate consumed by *M. barkeri*, the fraction used to generate reducing power is 0.02, the fraction used for production of biomass precursors is 0.06, and the remaining fraction, 0.92, is consumed by the methanogenesis pathway for ATP production. These values fall into the range reported previously for *Methanosaeta harundinacea* strain 6Ac – another methanogen capable of acetoclastic methanogenesis. Zhou et al. (2015) analyzed the significance of reducing power production during the growth of strain 6Ac on acetate, and reported that the acetate fraction for reducing power production ranged from 0.005 to 0.037.

The simulation results indicate that, in typical laboratory culturing media, the growth of *M. barkeri* is limited primarily by energy sources (Hespell and Bryant, 1979; Tempest and Neijssel, 1984). In the simulation, *M. barkeri* lives in the growth media of about 9 mM

ammonium, and can transport ammonium at up to 0.31 $mmol \cdot g^{-1} \cdot hr^{-1}$, fast enough to support the biosynthesis pathway (fig 3D). In comparison, the energy available to ATP synthesis decreases with time, thereby decreasing the flux of ATP production and hence the rate of biosynthesis (fig 3C, E, and F).

3.3. Environmental application

Natural environments are often limited in growth nutrients and/or energy sources. But it has been challenging to experimentally analyze how nutrient availability impacts the kinetics of microbial metabolism, due to the technical difficulties in differentiating between metabolically active and dormant cells, in attributing bulk chemical measurements to specific microbial populations, and in untangling the overlapping effects of energy sources and growth nutrients. The hybrid method accounts for ATP production and nutrient consumption at the same time, which offers a computational approach to explore microbial growth under energy- vs nutrient-deprived conditions.

3.3.1. Ammonium limitation

We first take ammonium consumption as an example, and apply the hybrid method to predict how nutrient availability influences the kinetics and efficiency of biomass synthesis of *M. barkeri*. Ammonium is commonly limiting in natural environments (Knelman et al., 2014; LeBauer and Treseder, 2008). Its concentrations range from less than 1 μM in oligotrophic environments to over 1 mM in eutrophic settings (Buss et al., 2004). Figure 4A shows how the biosynthesis rate varies with acetate concentration in an environment containing 10 μM ammonium, 1 mM bicarbonate, and 1 to 10^3 μM methane. At 1 μM methane, where acetate concentration is less than 30 μM, the biosynthesis is limited by ATP supply, and the biosynthesis rate increases with acetate concentration. At acetate concentration greater than the threshold of

30 μM, the biosynthesis is limited by ammonium availability, and proceeds at a maximum rate of 1.9×10^{-4} hr^{-1}.

Increases in methane concentration increase the threshold acetate concentration for ammonium limitation. This is because methane accumulation slows down ATP production by methanogenesis (eqs 1 and 12) and therefore reduces the demand for ammonium in biosynthesis (eq 18). At 1 mM methane, the threshold acetate concentration increases to 475 μM, one order of magnitude larger than the acetate threshold at 1 μM methane.

Figure 4B illustrates the transition between energy-limited and N-limited regimes across ranges of environmentally-meaningful acetate, ammonium, and methane concentrations. Increases in ammonium concentrations raise maximum biosynthesis rates. Larger ammonium concentrations enable faster ammonium uptake (eq 7), which in turn supports faster biosynthesis (eq 18). At 1 mM ammonium, the biosynthesis can reach a maximum value of 1.2×10^{-2} hr^{-1}, about two orders of magnitude larger than the maximum rate at 10 μM ammonium.

The efficiency of biosynthesis has been described using different parameters. As mentioned above, the efficiency of laboratory cultures is characterized using biomass yield $Y_{X/C}$ (eq 19). For natural communities, the efficiency is measured using carbon use efficiency (CUE) – the portion of total carbon consumed in cell metabolism that is assimilated into new biomass (Sinsabaugh et al., 2013; Stefano et al., 2012). The two parameters are exchangeable,

$$\mathrm{CUE} = f_{XC} \cdot \nu_{AC,X} \cdot Y_{X/C}, \tag{20}$$

where f_{XC} is the fraction of acetate flux assimilated into new biomass over the flux driven by biosynthesis, and $\nu_{AC,X}$ is the number of acetate molecules consumed per unit biomass synthesized. According to the simulation results, *M. barkeri* has a f_{XC} value of 0.73 and a $\nu_{AC,X}$ value of 0.02 $mol\cdot g^{-1}$ (see eq 18).

476 Figure 4C and D show according to the predictions (fig 4A and B), how the biomass
477 yield of *M. barkeri* varies with the concentrations of acetate, ammonium, and methane. Previous
478 studies emphasized the atomic ratios of nutrients, such as Redfield ratio, as a controlling factor
479 of metabolic efficiencies of natural communities (Cleveland and Liptzin, 2007; del Giorgio and
480 Cole, 1998). Figure 4E and F show how the CUE of *M. barkeri* changes with C:N ratios – the
481 atomic ratios of carbon in acetate to nitrogen in ammonium.
482 The predictions suggest that biosynthesis efficiencies depend on the availability of
483 acetate and ammonium. Acetate and ammonium concentrations modulate the fluxes of ATP
484 production and ammonium uptake, respectively (eqs 1 and 7). Where the ATP to ammonium flux
485 ratio is less than or equal to their stoichiometric ratio in the biosynthesis reaction (eq 18), i.e.,
486 20.9, biosynthesis is limited by the supply of ATP. Under this condition, biosynthesis rate varies
487 linearly with ATP production flux (or methanogenesis rate) (eq 17), and the metabolic
488 efficiencies reach their maximum values.
489 Substituting equation 12, 14, and 17 to 19, we can calculate maximum yield (Y_{max}),

490 $$Y_{max} = \frac{\nu_{ATP} \cdot Y_{X/ATP}}{\nu_{AC,R} + \nu_{ATP} \cdot \nu_{AC,X} \cdot Y_{X/ATP}}, \quad (21)$$

491 from the stoichiometric coefficients of acetate in methanogenesis and biosynthesis ($\nu_{AC,R}$ and
492 $\nu_{AC,X}$), the ATP yield ν_{ATP}, and the biomass yield per ATP, $Y_{X/ATP}$. Equation 20 and 21 suggest
493 that maximum metabolic efficiencies are intrinsic microbial parameters constrained by the
494 properties of metabolic pathways, and their values does not vary with environmental conditions.
495 For *M. barkeri*, by using the parameter values derived from model iMG746, the maximum yield
496 Y_{max} is 4.2 g·(mol acetate)$^{-1}$ and the maximum CUE (CUE_{max}) is 6.1% (figs 4C to F).
497 Where the ratio of ATP to ammonium fluxes surpasses their stoichiometric ratio in the
498 biosynthesis reaction (eq 18), the biosynthesis rate no longer responds to the changes in acetate

concentration or methanogenesis rate but, instead, is determined solely by the uptake flux of ammonium,

$$r'_{\mathrm{X}} = Y_{\mathrm{X/N}} \cdot F_{\mathrm{N}} \ , \tag{22}$$

where $Y_{\mathrm{X/N}}$ is the biomass yield per ammonium. The predictions (fig 4B) give a $Y_{\mathrm{X/N}}$ value of 91.5 g·(mol $\mathrm{NH_4^+}$)$^{-1}$ for *M. barkeri*, which is close to the value of 121 g·(mol $\mathrm{NH_4^+}$)$^{-1}$ determined in laboratory for *Methanothermobacter thermautotrophicus* (Kenealy et al., 1982). The decoupling of biosynthesis from methanogenesis has been observed for *M. barkeri* growing under ammonium-deprived conditions (Kenealy et al., 1982).

Under the conditions of ammonium limitation, metabolic efficiencies are not constant, but decrease nonlinearly with increasing acetate concentrations or increasing C:N ratios (figs 4C to F). These predictions agree with the CUE values reported for natural communities. In natural environments, ammonium concentrations tend to be small and thus likely limit the biosynthesis of natural communities. Accordingly, the CUE declines nonlinearly with C:N ratios (del Giorgio and Cole, 1998).

Where acetate concentrations are much larger than the half-saturation constant of acetate consumption (i.e., 5 mM for *M. barkeri*, table 1), the efficiencies reach their minimum values (figs 4C to F). Minimum efficiencies can be calculated from equation 19 and 22 by assuming that methanogenesis proceeds at its maximum rate. For example,

$$Y_{\mathrm{min}} = \frac{Y_{\mathrm{X/N}} \cdot F_{\mathrm{N}}}{\nu_{\mathrm{AC,R}} \cdot k + \nu_{\mathrm{AC,X}} \cdot Y_{\mathrm{X/N}} \cdot F_{\mathrm{N}}} . \tag{23}$$

According to this equation, the minimum efficiencies depend not only on the properties of metabolic networks but also on the uptake flux or the availability of ammonium. In natural environments with ammonium concentrations less than 10 μM, the minimum biomass yields and

CUEs can be as low as 0.027 g·(mol acetate)$^{-1}$ and 0.04%, respectively – two orders of magnitude smaller than the predicted maximum efficiencies (Y_{max} and CUE_{max}). On the other hand, in laboratory reactors, where ammonium is supplied at concentrations greater than 1 mM (Whitman et al., 2006), the minimum yields are greater than 1.45 g·(mol acetate)$^{-1}$, close to the predicted Y_{max} value. In other words, the biomass yields determined in laboratory bioreactor should be close to each other, but the CUEs of natural communities can vary over orders of magnitude. These predictions are consistent with the results of previous laboratory and field studies: the biomass yields of *M. barkeri* in laboratory reactors fall into a relatively narrow range of 1.2 to 4.2 g·(mol acetate)$^{-1}$ (Scherer and Sahm, 1981; Sowers et al., 1984), and the CUEs of natural communities vary more than an order of magnitude (del Giorgio and Cole, 1998).

Combining equation 12, 17, and 22, biosynthesis rates r'_X can be calculated according to

$$r'_X = \min\left(Y_{X/ATP} \cdot \nu_{ATP} \cdot r'_R, Y_{X/N} \cdot F_N\right). \quad (24)$$

This equation states that biosynthesis is subject to Liebig's Law of the Minimum (Bader, 1978; Droop, 1974; Egli, 2013; Zinn et al., 2004), and biosynthesis rate is determined either by ATP production flux F_{ATP} or by ammonium uptake flux F_N, depending on which one places a stronger limitation and whether or not biosynthesis is coupled to methanogenesis.

3.3.2. Growth kinetics

According to the above biosynthesis predictions (fig 4B), ammonium availability in the environment can place upper limits on the rates of biosynthesis (Kessler et al., 2001; LeBauer and Treseder, 2008; Ma and Thauer, 1990). These limits may account for the wide range of doubling time reported for microbes. For example, where the energy sources and growth nutrients are abundant, the hybrid method predicts that *M. barkeri* has a maximum biosynthesis rate of 3.2×10^{-2} hr^{-1}, which is equivalent to a doubling time of 22 hours. This prediction is close

544 to the doubling time of 24 hours determined for *M. barkeri* in laboratory bioreactors (Peinemann
545 et al., 1988; Smith and Mah, 1978).

546 The doubling time of natural microbes is much longer than laboratory cultures. Phelps et
547 al. (1994a) estimated that, in the sediments of Lake Mendota, a eutrophic lake in Wisconsin,
548 USA, microbes have a doubling time of 5 to 10 days. At the observed sediment ammonium
549 concentration of approx. 100 μM (Austin and Lee, 1973), the hybrid method predicts a N-limited
550 growth rate for *M. barkeri* of 2.2×10^{-3} hr^{-1} (doubling time of 13 days), close to the estimate of
551 Phelps et al. (1994a).

552 In the Middendorf aquifer, South Carolina, USA, microbes have an average doubling
553 time of centuries (Phelps et al., 1994a). In this aquifer, the groundwater has about 1 μM
554 ammonium (Phelps et al., 1994b). According to the above predictions, *M. barkeri* growing at 1
555 μM ammonium has a biosynthesis rate up to 2×10^{-5} hr^{-1}, a value that is close to microbial
556 maintenance rate. In natural environments of limited resources, microbes may have maintenance
557 rates an order of magnitude smaller than laboratory cultures (Schmidt, 1992). Using a
558 maintenance rate of 1.8×10^{-5} hr^{-1} (table 1), *M. barkeri* would have a growth rate of 2×10^{-6} hr^{-1}
559 (eq 3), equivalent to a doubling time of 40 years. Other factors, such as non-optimal temperatures
560 and pHs and the scarcity of energy sources and other growth nutrients, can also slow down
561 growth, further extending the doubling time of aquifer microbes (Price and Sowers, 2004).

562 **3.3.3. Pristine aquifer**

563 The potential linkage between the long doubling time of aquifer microbes and the low
564 ammonium in groundwater suggests that the growth of aquifer microbes might be limited by the
565 availability of ammonium. Current biogeochemical reaction modeling calculates biosynthesis
566 rates as a linear function of methanogenesis rates (eq 2), assuming that the growth of natural

567 microbes is limited primarily by energy sources (Jin et al., 2013). Here we use *M. barkeri* as a
568 model microbe and test in pristine aquifers, whether the ammonium limitation on growth is
569 applicable to aquifer microbes.

570 We retrieved groundwater chemistry data from the U.S. Geological Survey (USGS)
571 National Water Information System (NWIS) database (https://nwis.waterdata.usgs.gov). We
572 searched the database for samples that were collected from wells (site type) and analyzed for
573 ammonium (USGS parameter 00608), methane (parameter 76994), and dissolved oxygen
574 (parameter 00300). We eliminated samples that contained 1 mg/L or more dissolved oxygen, as
575 likely being inhibitory for methanogenesis. Out of the 21 hydrologic regions in the database, the
576 Mid-Atlantic region returned the largest number (i.e., 170) of groundwater samples (see
577 Supplementary Material). These samples were from the siliciclastic aquifers in Pennsylvania,
578 USA – the aquifers of unconsolidated sediments, sandstones, siltstones, and shale (Ator et al.,
579 2005). They have an average temperature of 12 ºC, pH of 7.8, about 0.3 mM bicarbonate, and 4
580 μM methane. No acetate analysis is available for these samples. But in pristine aquifers, acetate
581 can reach a concentration of 20 μM (McMahon and Chapelle, 1991a; 1991b).

582 The hybrid method predicts that *M. barkeri* in the aquifers has a biosynthesis rate of
583 6.8×10^{-5} hr^{-1}. Taking the maintenance rate as 1.8×10^{-5} hr^{-1} (table 1), *M. barkeri* would have a
584 growth rate of 5.0×10^{-5} hr^{-1} (eq 3) or a doubling time of 578 days. If we neglected ammonium
585 limitation and calculated the biosynthesis rate using the linear biosynthesis equation (eq 2), we
586 would arrive at a growth rate of 8.5×10^{-5} hr^{-1} or 339 days. Considering that we assume a
587 relatively large acetate concentration (i.e., 20 μM), the difference between the two predictions
588 might indicate that at least at some locations of the aquifers, microbial growth is limited by
589 ammonium availability.

590 The potential ammonium limitation is consistent with the groundwater chemistry of the
591 aquifers. Figure 5 compares the concentrations of methane and ammonium in the groundwater.
592 Methane levels range from below 1 μM to over 100 μM, and correlate moderately with the
593 concentrations of ammonium (Spearman's coefficient, 0.495). If the ammonium availability
594 limits methanogen growth in the aquifers, it would also limit the production of methane gas.

595 **3.3.4. Bioremediation**

596 As a second example, we take *M. barkeri* as a model methanogen, and apply the hybrid
597 method to predict the growth of aquifer methanogens during *in situ* groundwater bioremediation.
598 Current *in situ* bioremediation practice removes or immobilizes groundwater contaminants by
599 injecting solutions of organic compounds into aquifers and by stimulating the metabolisms of
600 aquifer microbes (Majone et al., 2015).

601 We focus on a field bioremediation experiment in an alluvial aquifer, Colorado, USA
602 reported by Mouser et al. (2009). This experiment injected acetate solution into the aquifer
603 through a 10-meter-long array of injection wells, and monitored the chemistry and microbiology
604 in the groundwater from monitoring wells 2.5 meters (well D-02 and 04) to 5 meters (well D-05
605 and 08) down-gradient from the injection wells. The acetate injection stimulated the metabolisms
606 of aquifer microbes, including methanogens (Anderson et al., 2003; Komlos et al., 2008; Liang
607 et al., 2012). We assume that the biostimulation increased microbial metabolic rates, including
608 maintenance rates (van Bodegom, 2007). As a result, in the biostimulated aquifer, *M. barkeri*
609 may have a maintenance rate close to the value of 1.8×10^{-4} hr^{-1} determined in laboratory
610 bioreactors (Wandrey and Aivasidis, 1983).

611 Figures 6A to D show, during the experiment, how the ATP flux from acetoclastic
612 methanogenesis varied with time (eqs 1 and 12). The ATP fluxes are predicted from the reported

acetate concentrations during the experiment (Mouser et al., 2009, their figure 1B), and by taking pH of the groundwater at 7, bicarbonate at 3.4 mM, and methane at 0.1 μM (Druhan et al., 2014; Fang et al., 2009).

The variations in ATP fluxes reflected the acetate concentrations in the groundwater. The acetate injection occurred during the first 10 days and between day 17 and 30 of the experiment (Mouser et al., 2009). Accordingly, the ATP fluxes increased and then decreased during the first 20 days. Afterwards, the fluxes increased again. Also, the maximum ATP fluxes were larger in the wells close to the injection wells (well D-02 and 04) than in those away from the injection (well D-05 and 08).

Figure 6E to H show how the ammonium uptake fluxes varied with time. The ammonium fluxes reflected the ammonium concentrations in the groundwater, which were different at different locations. These differences have been attributed to the heterogeneous occurrence of sedimentary organic matter (Mouser et al., 2009).

Figure 6I to L show the predicted growth rates of *M. barkeri*. In well D-02, 04, and 05, the variations follow the trends of ammonium fluxes. In well D-02, the growth rate of *M. barkeri* increases with time to 2.3×10^{-3} hr^{-1}. In well D-04, the growth rate remains relatively constant at $1.1\pm0.3\times10^{-3}$ hr^{-1}. In well D-05, the growth rate remains close to 0. In contrast, in well D-08, the variation in growth rate is similar to that of ATP production flux. These results suggest that in well D-02, 04, and 05, the biosynthesis of *M. barkeri* is limited by ammonium availability and hence is decoupled from methanogenesis. On the other hand, in well D-08, the biosynthesis is limited by ATP production (or by acetate availability) and is coupled to methanogenesis.

The metabolic decoupling is also evident from the predicted CUEs (figs 6M to P). In well D-02, 04, and 05, at most sampling time points, the CUEs remain smaller than the maximum

636 value of 6.1%, reaching as low as 0.06% in well D-05. However, in well D-08, the CUEs remain
637 constant at the maximum value throughout the experiments.
638 The growth rate predictions suggest that methanogens would not be able to flourish
639 around well D-05, but they could live and develop around well D-02, 04, and 08. In the aquifer,
640 the main source of ammonium was from the degradation of sedimentary organic matter (Mouser
641 et al., 2009). This natural nitrogen source was insufficient to support the metabolisms of aquifer
642 microbes during the biostimulation (fig 6I to P). Thus the addition of acetate, without any source
643 of nitrogen, might have hindered the stimulation of aquifer microbes. In addition, the
644 heterogeneous distribution of ammonium can account, at least in part, for the uneven spatial
645 distribution of microbes in the aquifer and their metabolic activities during biostimulation tests
646 (Liang et al., 2012).
647 If we neglected the impact of ammonium and assumed a linear relationship between
648 biosynthesis and methanogenesis (eq2), as in current practice of biogeochemical reaction
649 modeling (Li et al., 2009; Yabusaki et al., 2011), we would arrive at growth rates up to an order
650 of magnitude larger than the values predicted by the hybrid method (see figs 7I to L). These
651 differences reflect the limiting effect of ammonium in aquifers, and resonate with the previous
652 notion that current modeling frameworks (eqs 1 to 3) may not capture accurately the rates of
653 microbial metabolisms *in situ* (Brown et al., 2000; Murphy and Schramke, 1998; Phelps et al.,
654 1994a).
655 We can test the occurrence of ammonium limitation on the basis of gene expression.
656 Specifically, *M. barkeri* and other prokaryotes accumulate ammonium in the cytoplasm using
657 ammonium transporter enzyme. Mouser et al. (2009) analyzed the expression of the enzyme by
658 the aquifer microbes during the field experiment. They enumerated the transcripts of an

ammonium transporter gene, *amtB*, using reverse-transcription polymerase chain reaction (RT-PCR), and compared the transcript numbers to those of a housekeeping gene *recA* (fig 7). A BLAST search against currently available genome sequences found that their primer sets for *amtB* retrieved sequences from diverse microbes, including *M. barkeri*. Their results thus might have reflected the response of aquifer microbes in general.

We quantify the significance of ammonium limitation using the ratio of ammonium concentration m_{env} in groundwater to the concentration m_{req} required to support the stoichiometrically-balanced biosynthesis of *M. barkeri.* Figures 6Q to T compare the ammonium concentration in the groundwater to the concentrations required by the stoichiometrically-balanced growth. Figure 7 shows that during the field experiments, the expression of *amtB* gene correlated with the significance of ammonium limitation (Spearman's coefficient of –0.852). The strong negative correlation is consistent with our assessment about the ammonium limitation around well D-02, 04, and 05.

4. Discussion

We illustrated in this paper how to predict the kinetics of microbial metabolisms by combining genome-scale metabolic models with thermodynamically-consistent rate laws. We took methanogenesis as an example and simulated the metabolism of *M. barkeri* in both laboratory bioreactors and aquifers. The results show that the new method expands and improves the predictions of microbial kinetics, and can be applied to diverse environments, from laboratory bioreactors of optimal growth conditions to natural environments of limited resources.

4.1. Method development

The hybrid method follows dynamic FBA, and uses genome-scale metabolic models to account for the pathways of cell metabolisms (fig 1). But the two methods differ from each other

682 in the treatment of metabolic pathways (fig 1). Dynamic FBA uses standard genome-scale
683 metabolic models, which lump together the biochemical reactions of catabolic and biosynthetic
684 pathways. Additionally, they use a hypothetical ATP-consuming reaction to account for the
685 energy demand by maintenance (Thiele and Palsson, 2010). For this reason, direct applications
686 of genome-scale metabolic models require that ATP production fluxes from catabolism surpass
687 ATP hydrolysis rates of maintenance. As a consequence, dynamic FBA always predicts that
688 microbes are actively growing. But in most subsurface environments, active growth might be
689 sporadic because of limited energy sources and growth nutrients, and stationary and death phases
690 can be the common metabolic states (Morita, 1997; Price and Sowers, 2004; Roszak and
691 Colwell, 1987).

692 In comparison, the hybrid method applies FBA separately to the catabolic and
693 biosynthetic pathways in genome-scale metabolic models and, following standard practice in
694 biogeochemical modeling, accounts for cellular maintenance using negative rates of biosynthesis
695 (Jin and Roden, 2011; Jin et al., 2013). The separate treatment of catabolic, biosynthetic, and
696 maintenance pathways enables the application of the hybrid approach to a wider range of
697 metabolic states, from active growth to stationary and to death phase. For example, in the
698 simulation of the laboratory experiments, dynamic FBA fails to find a solution after 2.5 days into
699 the experiments. After this point, the ATP flux from methanogenesis is no longer able to match
700 the ATP consumption flux by maintenance assumed in model iMG746 (fig 3A, B, and E). On the
701 other hand, the hybrid method predicts that the metabolism of *M. barkeri* follows the typical
702 sequence of exponential and stationary phases (fig 3B).

703 The hybrid method predicts microbial kinetics by combining genome-scale metabolic
704 models with rate laws. There are different microbial rate laws on which to potentially build this

705 application (Simkins and Alexander, 1984). The choice of the thermodynamically-consistent
706 Monod equation (eq 1) reflects the need to consider thermodynamics in predicting the kinetics of
707 microbes under the conditions that frequently characterize natural systems (Jin and Bethke,
708 2005). As demonstrated in the example application (figs 3C, D, and F), the hybrid method
709 captured the response of acetate consumption flux to the changes in the chemical energy of the
710 environment: the acetate fluxes are larger at larger available energies, and vice versa. If we
711 applied dynamic FBA without accounting for the thermodynamics of microbial catabolism, we
712 would not be able to capture the response of acetate fluxes to the thermodynamic conditions of
713 the environment (fig 3A and F).

714 Applying the hybrid method requires the evaluation of the thermodynamically-consistent
715 rate laws and the simulation of biosynthesis using FBA. In the example application to laboratory
716 experiments (fig 3), we coupled FBA directly to the evaluation of the rate laws. At each iteration,
717 we use the rate laws to compute the fluxes of ATP synthesis and nutrient uptake, and feed the
718 fluxes to FBA to obtain biosynthesis rates (fig 1B). The direct coupling ensures a rigorous and
719 real-time exchange of microbial rates and fluxes between the rate laws and FBA, but running
720 FBA at every iteration can be computationally expensive – especially in field-scale applications
721 that repeat FBA at every grid cell and time step.

722 Alternatively, we can couple FBA and microbial rate laws indirectly by replacing FBA in
723 the hybrid method with Liebig's Law of the Minimum (eq 24). According to the FBA
724 predictions, biosynthesis rates are determined by the most limiting fluxes of ATP production and
725 nutrient uptake, and the relationship can be described according to Liebig's Law of the
726 Minimum. Thus rather than running the time-consuming FBA at each iteration, we can arrive at
727 the same results by the simple evaluation of Liebig's Law of the Minimum. Specifically, we first

apply FBA to biosynthesis pathways to estimate the biomass yields for ATP and for relevant growth nutrients and to build Liebig's Law of the Minimum for biosynthesis (eq 24). We then compute metabolic rates by combining the thermodynamically consistent rate laws for respiration and nutrient uptake and Liebig's Law of the Minimum for growth.

4.2. Methanogenesis

Methanogenesis is a final step of organic matter degradation, and methane fluxes are a key parameter in predicting future environmental changes (Nisbet et al., 2016; Thauer et al., 2008). Previous studies predicted the metabolic activities of methanogens using dynamic FBA (Stolyar et al., 2007) and microbial rate laws (Jin and Roden, 2011; Yang and Okos, 1987). Here we applied the hybrid method and predicted the metabolic rates and chemical fluxes of *M. barkeri* in both laboratory reactors and aquifers.

The hybrid method enhances the predictions of microbial carbon fluxes by accounting for biochemical mechanisms at the enzyme level. By applying FBA to genome-scale metabolic models, the hybrid method tracks not only the total fluxes of carbon, but also the contribution of different biochemical pathways. In the application to the laboratory experiments (fig 3), in addition to acetoclastic methanogenesis, the growth of *M. barkeri* also consumes acetate using two different pathways – the reversal of CO_2 reduction pathway that generates reducing power by oxidizing acetate to CO_2 and the biosynthesis pathway that assimilates acetate into new biomass. Specifically, the pathways of methanogenesis, acetate assimilation, and reducing power production consume 92%, 6%, and 2% of the total acetate fluxes, respectively. These fractions reflect how *M. barkeri* allocates acetate to the three pathways in order to meet its needs of ATP, carbon, and reducing power for biosynthesis.

The hybrid method is organism-specific – its predictions are based on genome-scale

751 metabolic models of individual organisms and their kinetic parameters of respiration,
752 maintenance, and nutrient uptake. But *M. barkeri* and other methanogens rarely live alone in
753 natural environments. Instead, they mingle with each other, and partner with diverse fermenting
754 microbes, sulfate reducers, acetogens, and others of different metabolic functions to build
755 microbial communities and to carry out organic matter degradation, nutrient cycling, and other
756 ecological functions (Nielsen et al., 2011; Schimel and Schaeffer, 2012).

757 Applying the hybrid method to microbial communities is feasible, provided that genome-
758 scale metabolic models of key community members are available. The application assumes that
759 the metabolisms of community members are at quasi steady state, and applies iterative
760 procedures to track the progress of community metabolisms (Zhuang et al., 2010). At each time
761 step of simulation, the hybrid method is applied to individual community members, and the
762 results are combined to compute the total fluxes of microbial communities, and to update the
763 abundances of community members and the concentrations of chemical compounds in the
764 environment. Such applications may hold promise for probing the ecological functions of
765 microbial communities and their dependence on environmental conditions – for example, the
766 contribution of different methanogens to methane fluxes and how environmental conditions
767 dictate the development and function of microbial communities (Keller and Bridgham, 2007; Ye
768 et al., 2012).

769 **4.3. Microbial growth**

770 The hybrid method can be applied to microbial metabolism under energy- vs. nutrient-
771 limiting conditions. We illustrated this capability by analyzing the growth of *M. barkeri* across
772 wide spectra of acetate and ammonium availabilities (fig 4). The results confirm that linear
773 growth equations (e.g., eqs 2 and 3) are best applied to laboratory bioreactors and eutrophic

774 environments, where nutrients are abundant. Under this condition, biosynthesis is limited by
775 ATP synthesis, and is coupled to respiration (Russell and Cook, 1995; Tempest and Neijssel,
776 1984). The results also show that in natural environments, biosynthesis rates are determined by
777 the fluxes of either ATP production or ammonium uptake, depending on which one places a
778 stronger limitation (eq 24).

779 The hybrid method is universal, and can also be applied to other growth nutrients by
780 explicitly accounting for the uptake and consumption of the nutrients. For example, if we expand
781 the application and consider the availability of phosphate – another limiting nutrient for natural
782 microbes (Elser et al., 2007), we would arrive at similar results. Specifically, biosynthesis rates
783 are subject to Liebig's Law of the Minimum, and are determined by the factors of most
784 significant limitation,

785 $$r'_{\mathrm{X}} = \min\left(Y_{\mathrm{X/ATP}} \cdot \nu_{\mathrm{ATP}} \cdot r'_{\mathrm{R}}, Y_{\mathrm{X/N}} \cdot F_{\mathrm{N}}, Y_{\mathrm{X/P}} \cdot F_{\mathrm{P}}\right), \quad (25)$$

786 where $Y_{\mathrm{X/P}}$ is the biomass yield per phosphate, and F_{P} is the flux of phosphate uptake. The $Y_{\mathrm{X/P}}$
787 value can be determined from the variations in biosynthesis rate with phosphate uptake flux, and
788 the value is 1.1×10^3 g·(mol phosphate)$^{-1}$. Similar to the above predictions of ammonium
789 limitation, where phosphorus is limiting, biosynthesis is also decoupled from methanogenesis – a
790 prediction consistent with laboratory observations (Archer, 1985).

791 The agreement between the hybrid method and Liebig's Law of the Minimum arises from
792 the similar underlying assumptions of the two methods. Liebig's Law of the Minimum assumes
793 that the pathways of respiration and nutrient utilization do not interact with each other, and
794 biosynthesis rates are determined by the more limiting of ATP supply or nutrient uptake fluxes
795 (Zinn et al., 2004). The hybrid method takes the fluxes as inputs. These fluxes are independent of
796 each other and meet the energy and element needs of biosynthesis pathways. As a result, the

most limiting flux determines the rate of biosynthesis (Edwards et al., 1999; Orth et al., 2010).

In addition to the linear growth equation (eqs 2 and 3) and Liebig's Law of the Minimum (eqs 3 and 25), other equations have also been applied to predict microbial growth. For instance, previous studies accounted for growth nutrient limitation using the multiplicative Monod equation (Bader, 1982; Jin et al., 2013; MeGee et al., 1972). This model quantifies the effect of nutrient availability using a Monod-type factor, and computes biosynthesis rates according to

$$r'_{\mathrm{X}} = Y_{\mathrm{X/i}} \cdot \nu_{\mathrm{i,R}} \cdot r'_{\mathrm{R}} \cdot \frac{m_{\mathrm{N,env}}}{m_{\mathrm{N,env}} + K_{\mathrm{N,env}}} \ . \tag{26}$$

This model assumes that the pathways of respiration interact with those of growth nutrient consumption, and should be accounted for at the same time (Zinn et al., 2004).

Previous studies also predicted microbial growth using sigmoidal functions, such as the logistic equation and the Gompertz equation (Zwietering et al., 1990). These functions focus on biomass concentrations, and assume *a priori* that microbial growth follows a sigmoid function. They calculate biomass concentrations from lag time, maximum growth rate, and maximum biomass concentration of the environment, and hence offer limited link to respiration pathways (Mitchell et al., 2004). For this reason, they may not be the best option for biogeochemical reaction modeling – a modeling discipline that emphasizes the chemical interactions between microbial metabolisms and natural environments.

In summary, we proposed a hybrid method that combines FBA with rate laws to predict the kinetics of microbial metabolism in natural environments. This method accounts for the availability of energy sources and growth nutrients, and applies FBA independently to the respiration and the biosynthesis pathways of genome-scale metabolic models. The application to *M. barkeri* in laboratory experiments shows that the method explicitly tracks cellular fluxes of energy and carbon, thereby bringing unprecedented metabolic detail to biogeochemical reaction

modeling. The applications to aquifers show that the growth of natural methanogens is limited either by energy sources or by the most limiting nutrient, and can be described according to Liebig's Law of the Minimum. These predictions are consistent with the decoupling of biosynthesis from methanogenesis and the slow growth of natural microbes under nutrient-deprived conditions, the correlation between dissolved methane and ammonium in the pristine aquifers of Pennsylvania, USA, and the expression of ammonium transporter gene in the biostimulated alluvial aquifer of Colorado, USA.

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Supplementary Information

1. Mendeley Data: A MATLAB code for simulating the metabolism of *Methanosarcina barkeri* by linking COBRA toolbox with PHREEQC.
2. Groundwater chemistry data from the Mid Atlantic hydrologic region.

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1172 Bacterial Growth Curve. Applied and Environmental Microbiology 56, 1875-1881.
1173

1174 Table 1. Parameters and their values for applying different modeling methods to *Methanosarcina barkeri* growing on acetate.

Parameter[a]	Value	Rate law	dynamic FBA	Hybrid method
Respiration parameter				
Rate constant k ($mmol \cdot g^{-1} \cdot hr^{-1}$)	7.1[a,b]	✓		✓
half-saturation constant K_D (mM)	5.0[b,c]	✓		✓
ATP yield ν_P	0 ~ 1[d]	✓		
Phosphorylation energy ΔG_P ($kJ \cdot mol^{-1}$)	40[d]	✓		✓
Average stoichiometric number χ	2[a,e]	✓		✓
Proton motive force (V)	0.1[d]			✓
Growth parameters				
Growth yield $Y_{X/R}$ ($g \cdot mol^{-1}$)	2.9[d]	✓		
Maintenance rate m (hr^{-1})	1.8×10^{-4}[f]	✓	✓[g]	✓
Acetate uptake				
Maximum rate V_{max} ($mmol \cdot g^{-1} \cdot hr^{-1}$)	2.2[h]		✓	
Michaelis constant $K_{ac,out}$	0.3[h]		✓	
Ammonium uptake				
Maximum rate V_{max} ($mmol \cdot g^{-1} \cdot hr^{-1}$)	0.25[i]		✓	

Michaelis constant for ammonium in the environment $K_{N,env}$ (μM)	200[j]		✓	✓
Michaelis constant for cytoplasmic ammonium $K_{N,cyt}$ (μM)	2000[k]			✓
Exponent *n*	2[i]			✓
Total parameters		7	5	9

Note:

(a). Values expressed for reaction equation 16.

(b). Smith and Mah (1978).

(c). Acetate is accounted for as the electron donor in the modified Monod equation (eq 1).

(d). Jin (2012).

(e). Jin and Bethke (2009).

(f). Wandrey and Aivasidis (1983).

(g). In model iMG746, the hypothetical ATP hydrolysis reaction of maintenance has a rate of 2.0 $mmol \cdot g^{-1} \cdot hr^{-1}$ (Gonnerman et al., 2013).

(h). Button (1998)

(i). This study.

(j). Boogerd et al. (2011).

(k). Chang et al. (2014).

Figure Caption

Figure 1. Comparison of dynamic FBA (A) and the hybrid method of FBA and microbial rate law (B). Dynamic FBA calculates uptake fluxes of electron donors, acceptors, and nutrients using the Michaelis-Menten equation (eq 5), and applies FBA to entire genome-scale metabolic models. The hybrid method applies FBA to the respiration pathways to estimate ATP yields, and computes respiration rates and ATP fluxes using the thermodynamically-consistent Monod equation (eqs 1 and 12); it estimates nutrient uptake fluxes by optimizing cytoplasmic nutrient concentration (eq 10) and by using the thermodynamically-consistent Michaelis-Menten equation (eqs 7 and 8), and then applies FBA to the biosynthesis pathways using the fluxes of ATP production and nutrient uptake as the input.

Figure 2. Variations in cytoplasmic ammonium accumulation (A and B) and uptake (C) with ammonium concentrations in the environment. Panel A and B show ammonium accumulation in laboratory reactors and in natural environments, respectively. Data points in panel A are the laboratory observations of *Methanohalophilus zhilinaeae* (◇), *Methanolobus bombay* (○), and *Methanolobus taylorii* (□) by Kadam and Boone (1996). The solid lines in panel A and B are the optimization results (eq 10). The solid line in panel C is computed according to the thermodynamically-consistent Michaelis-Menten equation (eqs 7 and 8) using the parameters in table 1, while the dashed line indicates the maximum ammonium uptake flux.

Figure 3. Variations in the concentrations of acetate, methane, and ammonium (A), and biomass (B), the fluxes of ATP production and acetate consumption by methanogenesis (C), the fluxes of acetate and ammonium consumption by biosynthesis (D), growth rate (E), and the energy ΔG_A

1212 available from methanogenesis (F) during the growth of *M. barkeri* on acetate. Data points are
1213 the experimental results of Fukuzaki et al. (1990); solid lines are the simulation results of the
1214 hybrid method; the dashed lines in panel A and B are the result of dynamic FBA. The dotted line
1215 in panel D shows the maximum possible uptake flux of ammonium, and the dotted line in panel
1216 F shows the energy ΔG_C conserved by methanogenesis (eq 11). The lines labeled "FBA
1217 infeasible" in panel A and B indicate the time point beyond which ATP synthesis rate by
1218 methanogenesis is smaller than ATP consumption rate by maintenance assumed in model
1219 iMG746, and dynamic FBA fails to find a solution.
1220
1221 Figure 4. Variations with acetate concentration in biosynthesis rate and generation time (A) and
1222 biomass yield (C) at 10 μM and at other ammonium concentrations (B and D), and changes in
1223 carbon use efficiency (CUE) with the atomic ratios of C in acetate and N in ammonium at 10 μM
1224 (E) and at other ammonium concentrations (F). The solid lines are the predictions of the hybrid
1225 method by taking temperature at 25 ºC, pH at 7, and bicarbonate concentration at 1 mM; the
1226 dashed lines in panel A and B are the maximum biosynthesis rates at given ammonium
1227 concentrations; the dashed lines in C and D are the maximum and minimum biomass yields; the
1228 dashed lines in E and F are the maximum and minimum CUEs; labels in panel A, C, and E show
1229 the methane concentrations in μM; labels in panel B, D, and F show ammonium concentrations
1230 in μM. The generation time is computed as the ratio of ln(2) to specific biosynthesis rate.
1231 Biomass yields and CUEs are calculated according to equation 19 and 20, respectively.
1232
1233 Figure 5. The correlation between ammonium and methane concentrations in the siliciclastic
1234 aquifers of Pennsylvania, USA.

Figure 6. Variations with time in the ATP fluxes (○) from methanogenesis (A to D), the ammonium uptake fluxes (●, E to H), the growth rates predicted by the hybrid method (▲) and by the linear equation (eqs 2 and 3, Δ, I to L), the carbon use efficiencies (CUEs, M to P), groundwater ammonium concentrations (■), and the ammonium concentrations (□) required to support the stoichiometrically-balanced biosynthesis of methanogens (Q to T) in the groundwater from wells D-02, D-04, D-05, and D-08 during the field bioremediation experiments (Mouser et al., 2009). The dotted lines in panel I to L indicate no growth.

Figure 7. Variations in the expression of ammonium transporters with the ratio of ammonium concentration m_{env} in the environment to the concentration m_{req} required to support the stoichiometrically-balanced biosynthesis of *M. barkeri*. The expression is measured as the ratio in mRNA transcript copy of an ammonium transporter gene *amtB* to a housekeeping gene *recA* gene in groundwater samples from well D-02 (○), 04 (□), 05 (◇), and 08 (▲) by Mouser et al. (2009, their figure S4); the shaded area indicates that ammonium in the environment cannot meet the requirement of biosynthesis, and hence is limiting growth.