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A THERMODYNAMIC THEORY OF MICROBIAL GROWTH

SHORT COMMUNICATION

SUBJECT CATEGORY: Microbial engineering

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Running Title: Boltzmann statistics and microbial growth

ABSTRACT

Our ability to model the growth of microbes only relies on empirical laws, fundamentally restricting our understanding and predictive capacity in many environmental systems. In particular, the link between energy balances and growth dynamics is still not understood. Here we demonstrate a microbial growth equation relying on an explicit theoretical ground sustained by Boltzmann statistics, thus establishing a relationship between microbial growth rate and available energy. The validity of our equation was then questioned by analyzing the microbial isotopic fractionation phenomenon, which can be viewed as a kinetic consequence of the differences in energy contents of isotopic isomers used for growth. We illustrate how the associated theoretical predictions are actually consistent with recent experimental evidences. Our work links microbial population dynamics to the thermodynamic driving forces of the ecosystem, which opens the door to many biotechnological and ecological developments.

KEYWORDS: Exergy/ Gibbs energy/ growth rate/ microbial division/ microbial isotopic fractionation/ statistical physics

In his famous book “What is life?”, Erwin Schrödinger opened the debate on how life could be envisioned from the thermodynamic standpoint (Schrödinger 1944). Ilyia Prigogine (Prigogine 1955) then made an important contribution by pioneering the application of non-equilibrium thermodynamics to biology, underlying modern developments of biological flux-force models (Westerhoff et al 1982, Westerhoff et al 1983). Today, thermodynamic state functions are widely applied to living systems at different organization levels (Jørgensen and Svirezhev 2004). The study of microbes, the simplest form of life, however led to a deeper physical conceptualization of the problem (Heijnen and Vandijken 1992, Kleerebezem and Van Loosdrecht 2010, McCarty 1965, Rittmann and McCarty 2001, Roels 1980). In these contributions, microbial anabolism was linked to catabolism through energy dissipation, sometimes expressed as a universal efficiency factor. A relation between dissipated energy and growth stoichiometry was established, enabling the prediction and calculation of energy and matter balances of microbial growth. However, the key question of the link between microbial thermodynamics and growth kinetics remained unanswered.

At the beginning of the 20th century, chemistry was facing a similar problem which finally resulted in a thermochemical kinetic theory eighty years ago (Eyring 1935). The existence of a high energy transition state, resulting from the collision of reactants, was postulated. Statistical physics was invoked to estimate the probability for the colliding molecules to have enough energy to overcome the transition state energy. A link between reaction kinetics and thermodynamic state of the system was thus established. Lotka (Lotka 1922b) suggested that a similar approach could be applied to biological units: "The similarity of the [biological] units invites statistical treatment [...], the units in the new statistical mechanics will be energy transformers subject to irreversible collisions of peculiar type-collisions in which trigger action is a dominant feature…"

Let us treat statistically a clonal population of N microbes consuming substrates, transducing energy and dividing. For each individual, we propose that harvesting a threshold level of energy from the

environment fundamentally triggers the microbial division process. Each division can thus be described as a succession of two steps: (i) the reversible capture of energy by the microbe, (ii) its irreversible transduction leading to division. We indeed assume that the second irreversible step is a slow kinetically limiting process and that the first step can thus be considered as close to equilibrium. The elementary microbial division act can thus be symbolized as follows:

$$M \leftrightarrow X^{\ddagger} \rightarrow 2M \qquad \text{Equation 1}$$

where M represents a microbe and $X^{\ddagger}$ the intermediate microbial activated state in which the microbe is able to divide (Figure 1A).

During the first step, each microbe has access to a volume (V_{harv}) in which it can harvest the chemical energy in the form of substrate molecules. The part of the chemical energy that is available for growth depends on the thermodynamic state of the environment surrounding the microbe. It can thus be transcribed using the meaningful concept of chemical catabolic exergy, which represents the maximum work available when bringing the substrate molecules in thermodynamic equilibrium with the microbe's environment (see supplementary material). Consequently, exergy levels can be attributed to each elementary volume V_{harv} and thus to each microbe. Using statistical physics, we demonstrate that the occupancies of exergy levels by microbes follow Boltzmann statistics (see Supplementary Information for the detailed demonstration). Let us denote $E^{\ddagger}$ the threshold exergy level triggering division corresponding to both dissipated exergy E_{dis} and stored exergy E_M during growth (Figure 1A).

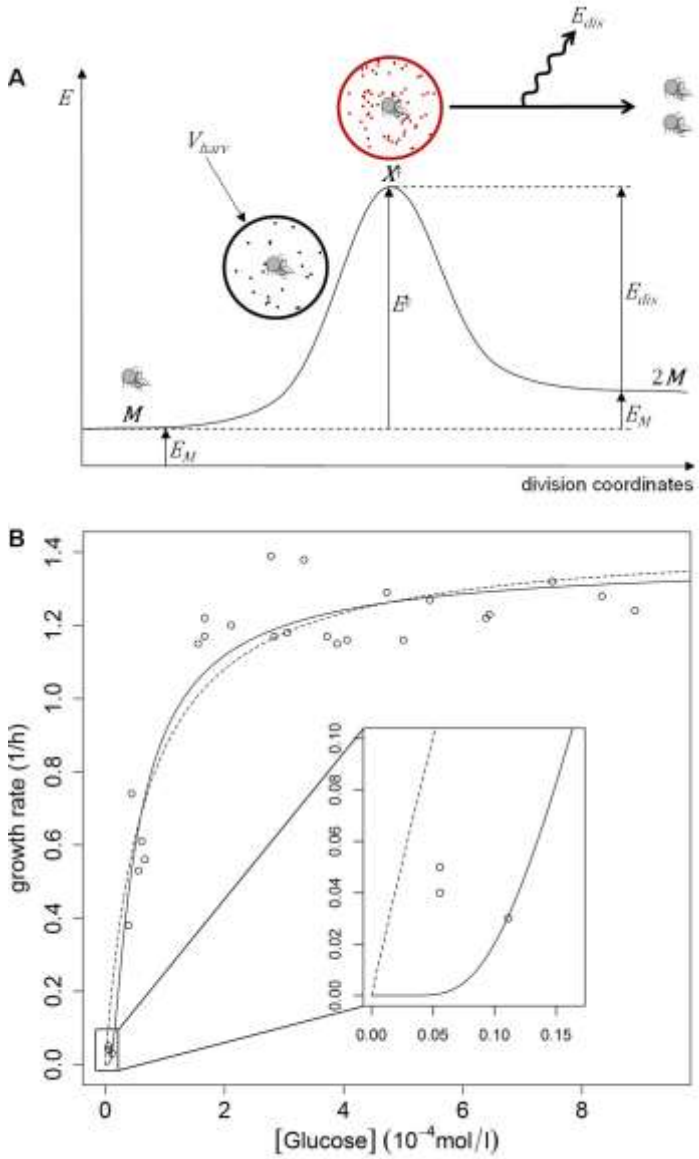

Figure 1: *(**A**) Graphical representation of microbial exergy levels along division coordinates. Microbial exergy (E_M) is augmented by the catabolic exergy within the harversting volume ($E_{cat} \cdot [S] \cdot V_{harv}$, symbolized as dots in a circle surrounding the microbe). Reaching the threshold catabolic exergy ($E_M + E^{\ddagger}$), the microbe is activated (a state denoted $X^{\ddagger}$), and an irreversible division process is triggered, associated with exergy dissipation ($E_{dis} = E^{\ddagger} - E_M$), resulting in two microbes. (**B**) Growth rate of E. coli as a function of glucose concentrations under aerobic conditions (Monod 1942). The plain curve shows the fit of Equation 2 on the data. The dashed curve shows the fit of a Monod equation. A detail of the growth rate/concentration dependency at low substrate concentration is shown, illustrating that the mathematical expression of our law naturally accounts for the existence of an apparent substrate threshold concentration for growth.*

These exergies can be evaluated using energy balances for growth established by different authors (see Supplementary Information and reference (Kleerebezem and Van Loosdrecht 2010) for a review). Let us now finally introduce the parameter μ_{max}, representing the division rate of an activated microbe (see Supplementary Information). Then comes the expression of microbial growth rate as a function of microbial exergy balance:

$$\mu = \mu_{max} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}}\right) \qquad \text{Equation 2}$$

S representing the energy limiting substrate, E_{cat} being the catabolic exergy of one molecule of energy limiting substrate.

Equation 2 introduces a flux-force relationship between microbial growth rate (μ) and catabolic exergy density ([S].E_{cat}). It correctly transcribes the well-known microbial growth rate dependence on substrate concentration enabling the modeling of any microbial experimental growth data as illustrated using Monod's historical experiments (Figure 1B) (Monod 1942). Additionally, our theory naturally accounts for the existence of an apparent threshold substrate concentration for growth (Figure 1B, detail), correcting a flaw of previous empirical equations (Kovarova-Kovar and Egli 1998). However, the correct transcription of microbial growth rate dependence on substrate concentration is not *per se* sufficient to support the validity of our theory. This can indeed be achieved with any empirical equation exhibiting a sigmoid shape as outlined by Monod himself (Monod 1942). Moreover, the simple process of fitting growth rate equations with measurements do not provide a sufficiently precise framework to compare different growth models as already demonstrated (Senn et al 1994).

Hopefully, in our theory, the growth rate more exactly depends on the spatial distribution of exergy around microbes. This implies that, additionally to substrate concentration, the intrinsic thermodynamic properties of molecules involved in the metabolism also determine the growth rate. We thus realized that the study of microbial isotopic fractionation related to pure culture experiments could provide us

with an adequate experimental opportunity to challenge the fundamental nature of the relationship between exergy and growth rate. Indeed, for a given microbe and in relation to a well-defined metabolic reaction (i.e. μ_{max} and V_{harv} are fixed), our theory predicts that the variation in catabolic exergy (E_{cat}) due to the differences in thermodynamic properties of isotopic isomers (isotopomers) would induce slight differences in substrate consumption rates between heavy or light molecules, thus giving a theoretical ground to the well-known microbial isotopic fractionation phenomenon.

Using our theory, we therefore derived a literal expression of the widely employed empirical kinetic fractionation factor $\alpha_{S/P}$ of substrates towards products (Mariotti et al 1981) (see Supplementary Information for details):

$$\alpha_{S/P} = \alpha_0 \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}^2} \cdot \Delta E_{cat}^{h-l}\right) \qquad \text{Equation 3}$$

α_0 being the residual biochemical fractionation factor of the catabolic reaction, ΔE_{cat}^{h-l} represents the difference between exergy of the catabolic reaction involving one molecule of the heavy isotopomer minus the exergy of its light counterpart.

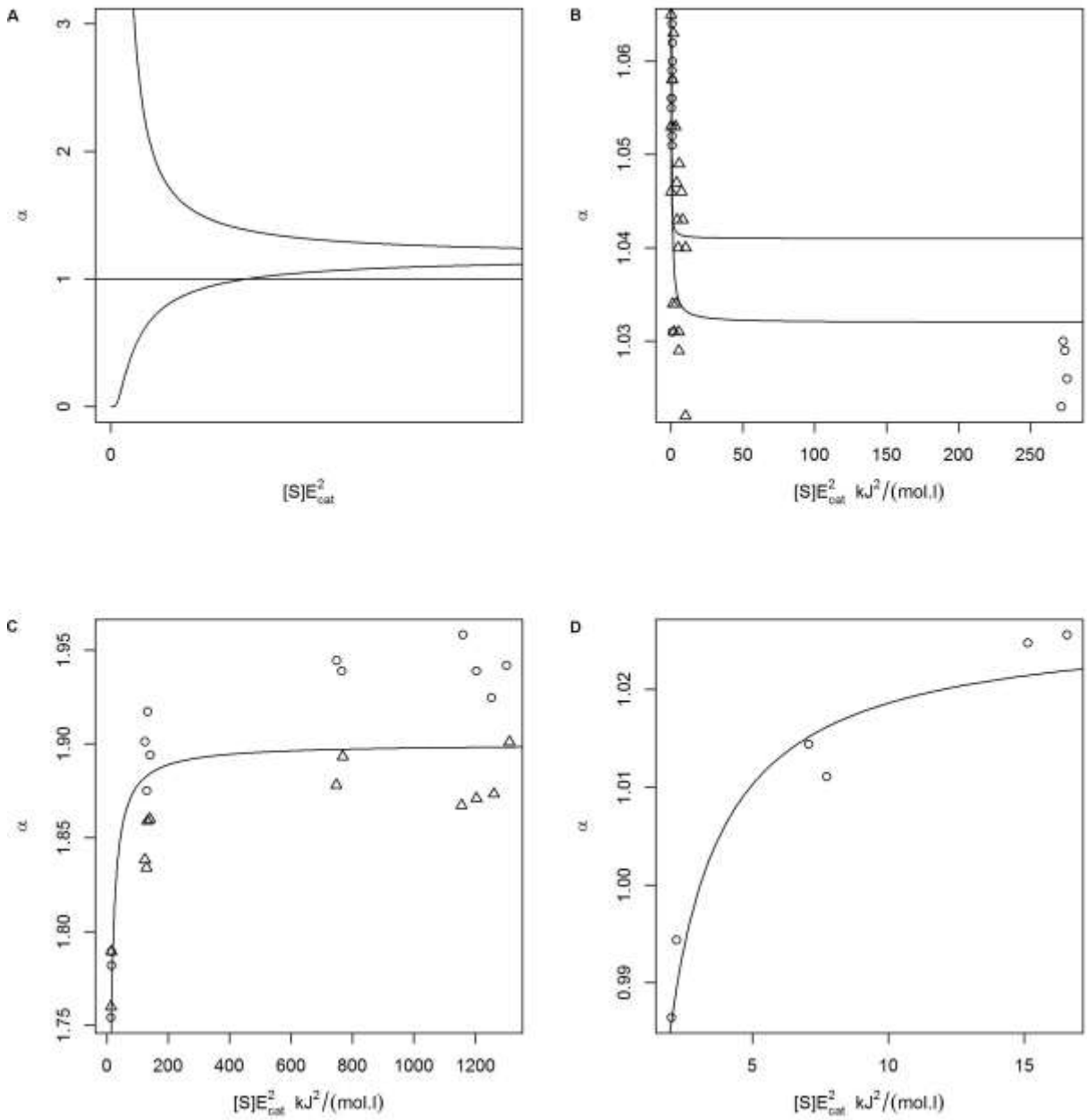

Figure 2: *Modeling the isotopic microbial fractionation phenomenon dependency on energy and substrate concentrations as derived from our theory. (**A**) Isotopic microbial fractionation factor (α) as a function of substrate concentration times the square of the catabolic exergy (X-axis) as predicted from our microbial growth theory (Equation 3). (**B**) Experimental data from (Penning et al 2005) (triangles) obtained with Methanobacterium bryantii; and (Valentine et al 2004) (circles) obtained with Methanothermobacter marburgensis; on* ^{13}C *isotopic fractionation associated with hydrogenotrophic methanogenesis confirming the existence of microbial overfractionation. (**C**) Experimental data from (Kampara et al 2008) including fractionation factor obtained after 50% (triangles) or 70% (circles) of toluene degradation by Pseudomonas putida under aerobic conditions*

*confirming the existence of microbial underfractionation. (**D**) Experimental data from (Goevert and Conrad 2009); on ^{13}C isotopic fractionation associated with aceticlastic methanogenesis by pure cultures of Methanosarcina barkeri and M. acetivorans. Interestingly, in this case, it illustrates a switch between depletion (α>1) and enrichment (α<1) of reaction products in heavy isotope depending on the value of* $[S] \cdot E_{cat}^2$ *as predicted by our model (see supplementary material).*

Unexpectedly, two classes of isotopic fractionation behaviours are predicted from Equation 3 depending on the sign of ΔE_{cat}^{h-l}, in relation to the thermodynamic properties of isotopomers (see supplementary material):

- if ΔE_{cat}^{h-l}<0, then α should decrease when ($[S] \cdot E_{cat}^2$) increases and this case could be named "microbial overfractionation" (Figure 2A, upper curve).
- Conversely, if ΔE_{cat}^{h-l}>0, then α should increase when ($[S] \cdot E_{cat}^2$) increases and this case could be named "microbial underfractionation" (Figure 2A, lower curve)

For both cases, α should vary with substrate concentration and catabolic exergy, increasing $[S].E_{cat}^2$ values leading to the convergence of microbial fractionation towards an asymptotical value (α_0). These theoretical predictions were then questioned with experimental microbial isotopic fractionation data from the literature.

Although it has long been considered that microbial isotopic fractionation was only dependent on the type of metabolism (Hayes 1993, Mariotti et al 1981), recent evidences have suggested that it could vary with environmental conditions (Conrad 1999, Conrad 2005). Strikingly, the dependency of microbial fractionation on substrate concentration (Goevert and Conrad 2009, Kampara et al 2008, Valentine et al 2004) and on Gibbs energy (Penning et al 2005) was recently evidenced. Figure 2-B/C/D illustrates how these experimental data are actually in agreement with the predictions obtained from our model and thus supports the dependency of microbial kinetics to the spatial distribution of exergy. Moreover, it demonstrates how the re-analysis of these data under a consistent theoretical framework (see supplementary information) show the existence of over- and under-fractionation as

predicted from our theory, which had never been explicitly claimed in the literature to our knowledge. The fact that these two fractionation classes are related to the thermodynamic properties of molecules (the sign of ΔE_{cat}^{h-l}) is a strong argument in favour of a threshold microbial exergy level triggering division, as considered in our theory. Without denying the need for additional data on purposely designed and carefully controlled isotopic experiments using pure cultures, the overall consistency of the different mathematical predictions of our model with microbial isotopic fractionation data already strongly supports our theory of microbial growth sustained by Boltzmann statistics of exergy distribution.

For clarity reasons, our approach was exposed for a single substrate in the case of energy limited microbial growth. However, we also demonstrate how it can easily be extended to multiple substrates or to the cases of stoichiometric growth limitation (see Supplementary Information). Equation 2 applied to mixed cultures links microbial population dynamics to the thermodynamic driving forces of the ecosystem, which has wide practical and fundamental implications. For example, implemented into engineering models for environmental bioprocesses such as ADM1 for anaerobic digestion (Batstone et al 2002), it could thus naturally transcribe the well-known dependence of microbial activity to thermodynamic conditions (Jin and Bethke 2007), correcting a major flaw of current kinetic equations (Kleerebezem and van Loosdrecht 2006, Rodriguez et al 2006). Moreover, our model offers the possibility to couple multiple biochemical reactions through the concept of exergy and to infer the fluxes generated by the “microbial engines that drive earth’s biogeochemical cycles” (Falkowski et al 2008). On a fundamental point of view, our theory could constitute a mathematical framework to evaluate how microbial communities would evolve considering various thermodynamic goal functions such as the maximum power (DeLong 2008, Lotka 1922a, Lotka 1922b), maximum exergy (Jørgensen and Svirezhev 2004) or minimum entropy production (Prigogine 1955). More generally, we also

believe that the study of microbes, the simplest form of life, constitutes a fertile thinking ground for a deeper interlinking between physical and biological concepts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

Supplementary information is available at ISMEJ's website

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A THERMODYNAMIC THEORY OF MICROBIAL GROWTH

SUPPLEMENTARY INFORMATION

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Demonstration of Boltzmann distribution of resource molecules among microbes

Let us first consider the system constituted by an isolated 3D physical space in which a clonal population of N microbes is consuming a total pool of k molecules of substrate S and dividing. Each individual microbe has access to a volume (V_{harv}) in which it can harvest the substrate. The n elementary volumes ($n = V_{tot}/V_{harv}$) constituting the 3D space define the statistical units of our model. Then, let us also consider a microstate consisting of a specific distribution of substrate molecules in the different statistical units. We use the framework of statistical physics, in which the ensemble constituted by all the microstates of an isolated system like ours is called the microcanonical ensemble. Let us assume that the total volume accessible to the microbes ($N \cdot V_{harv}$) is small compared to the rest of the system. Thus, three systems can be defined:

- Σ the system constituted by the 3D physical space, with $\Omega(n, k)$ its number of microstates.
- Σ' the subsystem constituted by the N statistical units containing microbes (representing the volume accessible to the microbes).
- R the reservoir, $R = \Sigma - \Sigma'$.

Therefore, the microstates consisting of distributions of substrate molecules in the statistical units occupied by microbes (Σ') constitute a canonical ensemble. Consequently, the probability for a microbe to have i_0 molecules in its harvesting volume follows a Boltzmann distribution:

$$p(i_0) = \frac{1}{Z}\exp(-\beta \cdot i_0)$$

with $Z = \sum_{i=0}^{k} \exp(-\beta \cdot i)$ the partition function and $\beta = \frac{\partial \ln(\Omega(n,k))}{\partial k}$ by definition.

β can be inferred from the expression of $\Omega(n,k)$, which corresponds to the number of ways of distributing k indistinguishable objects in n boxes:

$$\Omega(n,k) = C_{n+k-1}^{k} = \frac{(n+k-1)!}{k!\,(n-1)!}$$

Using Stirling's formula, $\ln(\Omega(n,k))$ can be expressed as a function of n and k:

$$\ln(\Omega(n,k)) \approx n \cdot \ln\left(\frac{n+k}{n}\right) + k \cdot \ln\left(\frac{n+k}{k}\right)$$

then

$$\beta = \frac{\partial}{\partial k}\left(n \cdot \ln\left(\frac{n+k}{n}\right) + k \cdot \ln\left(\frac{n+k}{k}\right)\right) = \ln\left(1 + \frac{n}{k}\right)$$

In the case for which $k \gg n$ (the number of molecules is large compared to the number of statistical units, cf. discussion below) we obtain:

$$\beta \approx \frac{n}{k}$$

Knowing that $n = \frac{V_{tot}}{V_{harv}}$ and $k = V_{tot} \cdot [S]$, it leads:

$$\beta = \frac{1}{V_{harv} \cdot [S]}$$

The proportion of activated microbes ($N^{\ddagger}/N$) is then given by the probability of finding occupied elementary volumes with more than ν molecules, a threshold number of substrates molecules necessary to trigger an elementary division:

$$\frac{N^{\ddagger}}{N} = \sum_{i=\nu}^{k} p(i) = \exp(-\beta \cdot \nu) \cdot \frac{1}{Z} \sum_{i=0}^{k-\nu} \exp(-\beta \cdot i)$$

Finally, since $\nu \ll k$ (the number of molecules required for one division is small compared to the total number of molecules in the system):

$$\frac{N^{\ddagger}}{N} = \exp(-\beta \cdot \nu)$$

Energy limited growth - Calculating ν from the exergy balance of an elementary microbial division

In many cases, growth is limited by the amount of energy available. The threshold level ν of resources necessary to trigger division then corresponds to a threshold level of energy $E^{\ddagger}$ as presented in the main text. This threshold level can be estimated using different Gibbs energy balance methods summed up in (Kleerebezem and Van Loosdrecht 2010). The approaches presented in this paper were slightly modified in order to use the meaningful concept of exergy, which represents the maximum work available for a microorganism during a chemical transformation. We use the Gibbs energy dissipation method initially proposed by Heijnen to determine the growth stoichiometry. For more clarity, the approach is illustrated with the example of *E. coli* growing aerobically on glucose.

The catabolism is associated with a Gibbs energy variation, denoted $\Delta_r G_{cat}$:

$$(cat) \quad Electron\ donor + a \cdot Electron\ acceptor \ \rightarrow b \cdot Products, \quad \Delta_r G_{cat}$$

a and *b* being stoichiometric coefficients

In the case of *E. coli* growing aerobically on glucose:

$$(cat) \quad C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ HCO_3^- + 6\ H^+, \quad \Delta_r G_{cat}$$

Thus, catabolic exergy (chemical energy available for the microbe) is:

$E_{cat} = -\Delta_r G_{cat}$ if $\Delta_r G_{cat} < 0$

$E_{cat} = 0$ otherwise

Let us then define microbial exergy E_M as the chemical energy available in a unit of biomass, in other words the energy a microbe could virtually obtain by consuming its own biomass:

$$(destock) \quad Biomass\ unit + c \cdot Electron\ acceptor \rightarrow d \cdot Products, \qquad \Delta_r G_{destock}$$

c and *d* being stoichiometric coefficients

In the case of *E. coli* growing aerobically on glucose:

$$(destock) \quad CH_{1.8}O_{0.5}N_{0.2} + 1.05\ O_2 + 0.40\ H_2O \rightarrow 0.20\ NH_4^+ + HCO_3^- + 0.80\ H^+, \Delta_r G_{destock}$$

$CH_{1.8}O_{0.5}N_{0.2}$ represents a generic elemental composition of one C-mole of biomass.

The microbial exergy is:

$E_M = -\Delta_r G_{destock}$ if $\Delta_r G_{destock} < 0$

$E_M = 0$ otherwise

The metabolic equation of growth is a linear combination of equations (destock) and (cat).

$$(met) = \nu(cat) - (destock), \quad \Delta_r G_{met}$$

Resulting in:

$$(met) \quad \nu\ Electron\ donor + (\nu \cdot a - c) Electron\ acceptor$$
$$\rightarrow Biomass\ unit + (\nu \cdot b - d) Products, \quad \Delta_r G_{met}$$

The exergy dissipated during growth corresponds to the opposite of metabolic Gibbs energy ($E_{dis} = -\Delta_r G_{met}$). Using the Gibbs energy dissipation method, E_{dis} can be calculated for one unit of biomass (Kleerebezem and Van Loosdrecht 2010). In the case of energy limited growth, the factor ν representing the threshold number of molecules necessary to trigger an elementary division is thus determined by the growth energy balance:

$$\nu = \frac{E_M + E_{dis}}{E_{cat}}$$

And the threshold exergy level $E^{\ddagger}$ necessary for one division is:

$$E^{\ddagger} = \nu \cdot E_{cat} = E_M + E_{dis}$$

The standard Gibbs energy of formation of all constituents is known, including the Gibbs energy of formation of one C-mol of biomass (Kleerebezem and Van Loosdrecht 2010). E_{cat} and E_M can thus be calculated from tables. Thus, in the case of *E. coli* growing aerobically on glucose, the standard Gibbs energies for (cat) and $(destock)$ corrected for pH=7, denoted $\Delta_r G^{01}$, are:

$$\Delta_r G^{01}_{cat} = -2852 \text{ kJ/mol}$$

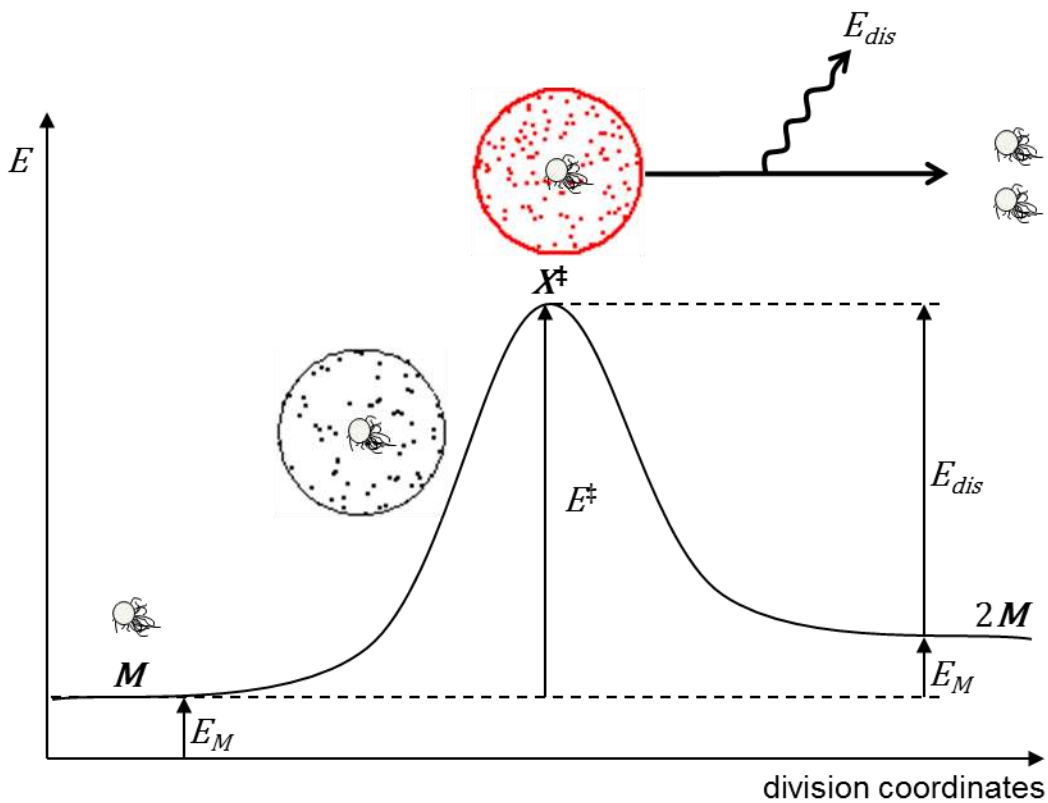
$$\Delta_r G^{01}_{destock} = -474 \text{ kJ/C} - \text{mol}$$

E_{dis} can be calculated for one C-mol of biomass knowing the number of carbon atoms in a substrate molecule and its oxidation degree (Kleerebezem and Van Loosdrecht 2010). The factor ν can then be obtained. For simplicity, it is possible to consider $E_{dis} = 500 \text{ kJ/C} - \text{mol}$ as a good approximation of dissipated exergy (von Stockar et al 2006). Thus in standard conditions corrected for pH=7, we obtain $\nu = 0.34$, leading to the following stoichiometric metabolic growth equation:

$$(met)\ \ 0.34\ C_6H_{12}O_6 + 0.20\ NH_4^+ + 1.00\ O_2 \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.05\ HCO_3^- + 0.40\ H_2O + 1.25\ H^+$$

Energy limited growth - From the distribution of exergy among microbes to growth rate

The variables associated to an elementary microbial division act are summarized in the following figure:



$$M \xleftrightarrow{K} X^{\ddagger} \xrightarrow{\mu_{max}} 2M$$

As explained in the main text, we assumed that the first step is reversible and can be considered as close to equilibrium. Thus the equilibrium constant K can be expressed:

$$K = \frac{[X^{\ddagger}]}{[M]} = \frac{N^{\ddagger}}{N} = \exp(-\beta \cdot \nu) = \exp\left(-\frac{\nu}{V_{harv} \cdot [S]}\right)$$

$N^{\ddagger}$ corresponding to the number of activated microbes and N to the total number of microbes.

In the case of energy limited growth, we previously established that:

$$\nu = \frac{E_M + E_{dis}}{E_{cat}}$$

Thus:

$$\frac{N^{\ddagger}}{N} = \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}}\right)$$

The kinetic of the elementary microbial division act is limited by the second irreversible step. The rate of this second step is denoted μ_{max}. It should be noted that μ_{max} is a parameter that depends on the type of microbe and substrate considered. At the level of the whole microbial population, the growth kinetic is then:

$$\frac{dN}{dt} = \mu_{max} \cdot N^{\ddagger}$$

Finally, the population growth rate μ can be expressed:

$$\mu = \frac{1}{N} \cdot \frac{dN}{dt} = \mu_{max} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}}\right)$$

Generalization of our law to multiple substrates and to the case of elemental growth limitation

We can now generalize this equation to multiple substrates $\{S_i\}$. If the different substrates distributions are independent, the joint probability is then the product of Boltzmann distributions for the different substrates and the proportion of activated microbes is given by:

$$\frac{N^{\ddagger}}{N} = \prod_i \exp(-\beta_i \cdot \nu_i)$$

with

$\beta_i = \frac{1}{V_{harv} \cdot [S_i]}$

In the case of elemental limitation of growth, the coefficient ν represents the stoichiometric coefficient of the molecule supplying the limiting element. ν can thus be directly obtained from the elemental composition of biomass ($CH_{1.8}O_{0.5}N_{0.2}$). Indeed if nitrogen is limiting and ammonium ions are the nitrogen source: $\nu = 0.2\ \frac{mol\ NH_4^+}{C\text{-}mol\ biomass}$.

A more detailed chemical composition of biomass (Battley 1998) can be used in order to model microbial growth under various types of elemental limitation (P, S, K, Mg, Ca,…) applying the same principle to obtain ν.

Range of values commonly encountered in practical growth situations and validity of our approximations

The demonstration of Boltzmann distribution of activated microbes requires two main approximations:

- First, in order to use the canonical ensemble formalism and to derive the statistical distribution of molecules in elementary units, the volume not accessible to the microbes should constitute a reservoir of molecules. In practice, this situation is fulfilled when the volume of Σ' is a small fraction of the volume of Σ ($N \cdot V_{harv} \ll V_{tot}$). In environmental conditions such as seawater, bacterial densities commonly encountered range around 10^6 cells/ml (Porter and Feig 1980). In these conditions, one cell is, on the average, surrounded by 10^6 μm^3 of medium. In these cases, a harvesting volume (V_{harv}) up to 10^5 μm^3 assures a ratio less than 1/10 between occupied and non-occupied volumes. This harvesting volume is consistent with the volume estimated from Figure 1B (fitted $V_{harv} \approx 1 \cdot 10^5$ μm^3 see below). Our first hypothesis is therefore justified in many environmental conditions. However, in high density cultures, such as those obtained in laboratory conditions, *E. coli* can grow up to a density of 10^9 cells/ml. In these cases, the free volume around each cell is only 10^3 μm^3 on the average and the harvesting volume should be less than 100 μm^3 to assure a ratio 1/10 between occupied and non-occupied volumes. Our model might therefore reach the limits of its validity domain under such high density conditions. However, as growth is exponential, it would still be applicable for most of the bacterial generations before reaching the saturation plateau, and thus for the major part of the growth curve. More generally, the adaptation of our equation to real complex environmental

situations (high microbial density environments, presence of aggregates such as flocs, biofilms,…) requires further developments on our model , which are beyond the scope of the current paper.

- The second approximation stipulates that the total number of statistical units *n* is negligible with respect to the number k of molecules in the media *(*$k \gg n$). The fraction n/k can be expressed as a function of substrate concentration and harvesting volume (cf. above):

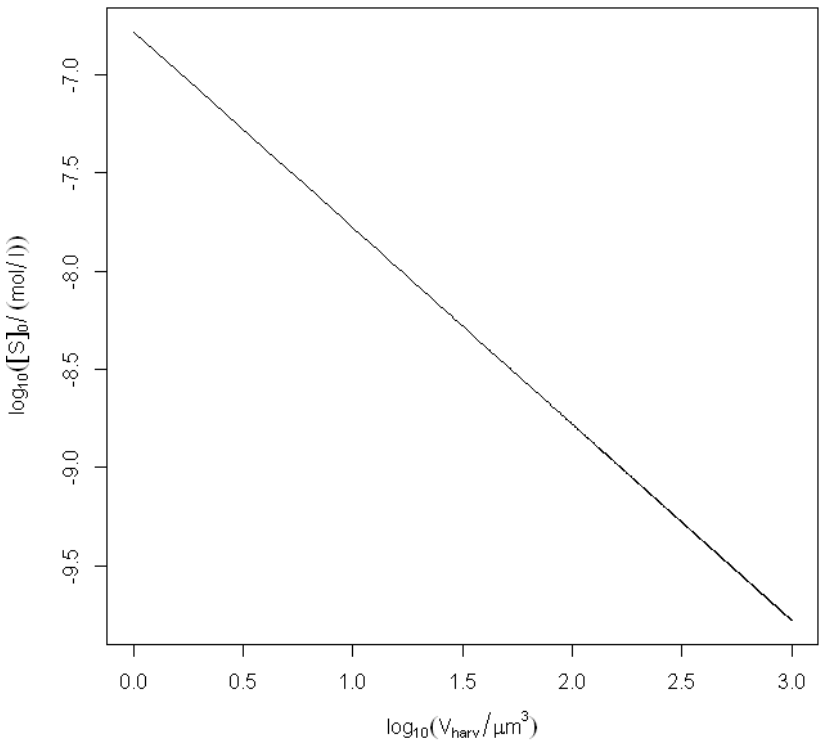
$$\frac{n}{k} = \frac{1}{V_{harv} \cdot [S] \cdot N_a}$$

In this case, to allow practical evaluations, $[S]$ is expressed in mol/l and N_a (Avogadro number) is therefore introduced in the expression.

If we take $\frac{k}{n} = 100$ as a threshold ratio, we can compute a corresponding limit concentration:

$$[S]_0 = \frac{100}{V_{harv} \cdot N_a}$$

The hypothesis ($k > 100 \cdot n$) is then fulfilled if $[S] > [S]_0$. In order to estimate the robustness of this hypothesis, let us plot $\log([S]_0)$ as a function of $\log(V_{harv})$:



In the least favourable cases, corresponding to small harvesting volumes (1 μm^3 to 10^3 μm^3), threshold concentrations are still very low ($[S]_0 < 1\ \mu mol/l$). The second hypothesis is therefore not limiting the applicability of the model.

The microbial isotopic fractionation phenomenon as viewed from our theory

Let us consider the isotope fractionation phenomena due to the following reaction catalyzed by microbes:

$$S \rightarrow \lambda \cdot P$$

with S the substrate, P the product and λ a stoichiometric coefficient. Substrate and product with a heavy isotope will be denoted S^h and P^h, substrate and product with a light isotope will be denoted S^l and P^l.

The kinetic fractionation factor $\alpha_{S/P}$ of substrate towards product (sometimes $\alpha_{P/S} = 1/\alpha_{S/P}$ is used instead) (Mariotti et al 1981) of the reaction is:

$$\alpha_{S/P} = \frac{\frac{[S^h]}{[S^l]}}{\frac{\partial[P^h]}{\partial[P^l]}}$$

With $\delta[P^l] = -\lambda \cdot \delta[S^l]$ and $\delta[P^h] = -\lambda \cdot \delta[S^h]$, let us rewrite $\alpha_{S/P}$:

$$\alpha_{S/P} = \frac{\frac{\partial[S^l]}{[S^l]}}{\frac{\partial[S^h]}{[S^h]}}$$

With our theory, kinetic isotopic fractionation can be explained by the difference between exergy of molecules containing light or heavy isotopes. Indeed the harvesting rates $r^h$and r^l for a microbe consuming only heavy or only light substrate are given by:

$$r^h = -\nu \cdot [M] \cdot \mu^h = -\nu \cdot [M] \cdot \mu^h_{max} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E^h_{cat}}\right)$$

$$r^l = -\nu \cdot [M] \cdot \mu^l = -\nu \cdot [M] \cdot \mu^l_{max} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E^l_{cat}}\right)$$

μ^h_{max} and μ^l_{max} are the maximal growth rates for heavy and light substrate respectively.

E^h_{cat} and E^l_{cat} represents the catabolic exergy for the catabolic reaction with heavy or light substrates respectively (see thermodynamic properties of isotopomers below).

In practice, the microbe is always consuming a mixture of light and heavy molecules. Consumption rates of heavy and light substrates can be expressed in respect to the relative proportion of light ($[S^l]/[S]$) and heavy ($[S^h]/[S]$) molecules:

$$\frac{\partial[S^h]}{\partial t} = \frac{[S^h]}{[S]} \cdot r^h$$

$$\frac{\partial[S^l]}{\partial t} = \frac{[S^l]}{[S]} \cdot r^l$$

Let us then calculate the kinetic fractionation factor:

$$\alpha_{S/P} = \frac{\frac{\partial[S^l]}{[S^l]}}{\frac{\partial[S^h]}{[S^h]}} = \frac{r^l}{r^h} = \frac{\mu_{max}^l}{\mu_{max}^h} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S]}\left(\frac{1}{E_{cat}^l} - \frac{1}{E_{cat}^h}\right)\right)$$

$$=> \ \alpha_{S/P} = \frac{\mu_{max}^l}{\mu_{max}^h} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot \left(E_{cat}^h \cdot E_{cat}^l\right)}\left(E_{cat}^h - E_{cat}^l\right)\right)$$

Considering that $E_{cat}^h \approx E_{cat}^l$ we have $E_{cat}^h \cdot E_{cat}^l \approx E_{cat}^2$

Let us denote $\Delta E_{cat}^{h-l} = E_{cat}^h - E_{cat}^l$

$$\Delta E_{cat}^{h-l} = \left(\Delta G^{l0} + RT \cdot \log(\Pi)\right) - \left(\Delta G^{h0} + RT \cdot \log(\Pi)\right) = \Delta G^{l0} - \Delta G^{h0}$$

This difference is independent from chemical concentrations. It only depends on intrinsic characteristics of the molecules considered, i.e. the stabilization of the electronic bonds by the isotopes within the molecules as predicted from quantum theory (Zeebe and Wolf-Gladrow 2001). Then comes the expression:

$$\alpha_{S/P} = \alpha_0 \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S_{lim}] \cdot E_{cat}^2}\Delta E_{cat}^{h-l}\right)$$

With S_{lim} the limiting substrate and $\alpha_0 = \frac{\mu_{max}^h}{\mu_{max}^l}$.

Contrary to the classical derivation of a constant kinetic fractionation factor (Mariotti et al 1981) this expression predicts the dependency of the fractionation to exergy and substrate concentration as recently evidenced in various experimental reports (Goevert and Conrad 2009, Kampara et al 2008, Penning et al 2005, Valentine et al 2004).

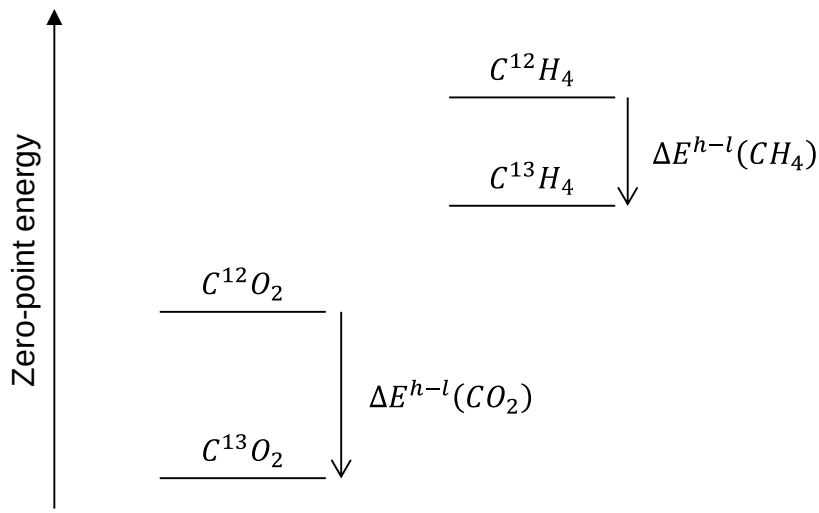
Predicting different classes of microbial isotopic fractionation from the thermodynamic properties of isotopomers

Different isotopomers have different energy levels (Zeebe and Wolf-Gladrow 2001). Indeed a molecule containing a heavy isotope has stronger bonds than a molecule with a light isotope.

Let us consider <u>hydrogenotrophic methanogenesis</u>:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

Putative zero point energies for light and heavy carbonate and methane are shown on the graph below:



Covalent bonds are stabilized by heavy atoms thus exergies of heavy molecules are lower than exergies of light molecules. In addition C-O bonds are relatively more stabilized by C^{13} than C-H bonds (Zeebe and Wolf-Gladrow 2001), thus heavy carbonate is relatively more stabilized than heavy methane:

$$\Delta E_{cat}^{h-l} = \Delta E^{h-l}(CH_4) - \Delta E^{h-l}(CO_2) < 0$$

This calculation is consistent with known carbon equilibrium fractionation for the system CO_2-CH_4 over a wide range of temperature (Horita 2001). Together with Equation 3, this simple argument thus predicts overfractionation for hydrogenotrophic methanogenesis.

If we now consider <u>aerobic degradation of toluene</u>:

$$C_7H_8 + 9O_2 \rightarrow 7CO_2 + 4H_2O$$

Bigeleisen reports that equilibrium fractionation factor of protium-deuterium between toluene and water is smaller than one (Bigeleisen 1965). Thus water is relatively more stabilized by deuterium than

toluene. It is consistent with a simple rule established by Bigeleisen: *"The heavy isotope goes preferentially to the chemical compound in which the element is bound most strongly."* (Bigeleisen 1965). Thus:

$$\Delta E_{cat}^{h-l} = \Delta E^{h-l}(H_2O) - \Delta E^{h-l}(C_7H_8) > 0$$

Again, the sign of this inequality together with Equation 3 implies the existence of microbial underfractionation.

For <u>acetoclastic methanogenesis</u>:

$$H_3C - COOH \rightarrow CH_4 + CO_2$$

Evaluating the isotope stabilization effect on a polyatomic molecule such as acetate is a complicated task because of the existence of multiple vibrational and rotational modes. Moreover, experimental data on equilibrium fractionation of ^{13}C between acetate and methane/carbon dioxide had never been reported to our knowledge, precluding any theoretical prediction about the nature of the aceticlastic microbial fractionation phenomenon. However, if our theory is correct, the microbial underfractionation phenomenon deduced from (Goevert and Conrad 2009) (cf. figure 2D) conversely implies that ΔE_{cat}^{h-l} of the catabolic microbial reaction is positive. It thus suggests that ^{13}C stabilizes more methane than acetate.

Confronting predictions to experimental data

*Illustrating microbial growth rate dependence on substrate concentration using Monod's historical experiment (*Figure 1B)

We estimate two parameters (μ_{max} and K_S) for our model and Monod model on Monod's data for the growth of *E. coli* on glucose ((Monod 1942) table XVII p 72.):

Our model:

$$\mu = \mu_{max} \cdot \exp\left(-\frac{K_S}{[S]}\right)$$

With $K_S = \frac{E_M + E_{dis}}{V_{harv} \cdot E_{cat}}$

Monod model:

$$\mu = \mu_{max} \cdot \frac{[S]}{K_S + [S]}$$

Estimation is made minimizing the residual sum of squares (RSS) on growth rates with a nonlinear regression.

$$RSS(\mu) = \sum \left(\mu_{measured} - \mu_{predicted}\right)^2$$

RSS values show that our model fits slightly better on the data than the Monod model.

Parameters values estimated for both model are shown in the table below:

	Our model	**Monod model**
$\boldsymbol{\mu_{max}}$ (1/h)	1.38	1.44
$\boldsymbol{K_S}$ (10^{-4} mol/l)	0.42	0.67

The K_s value estimated for our model enable the calculation of the harvesting volume

$$V_{harv} = \frac{E_M + E_{dis}}{K_S \cdot E_{cat}}$$

For *E. coli* growing on glucose (cf. sup mat 1):

$E_{cat} = 2.9 \cdot 10^3 \text{ kJ/mol}$

$E_{dis} = 0.5 \cdot 10^3 \text{ kJ/C} - \text{mol}$

$E_M = 0.5 \cdot 10^3 \text{ kJ/C} - \text{mol}$

$$=> V_{harv} = 8.2 \cdot 10^3 \text{ l/C} - \text{mol}$$

A dry cell of E. coli weight 3e-13 g with 50% carbon and thus contains 1.2e-14 C-mol/cell.

$$=> V_{harv} = 9.9 \cdot 10^{-10} \text{ l/cell}$$

$$=> V_{harv} \approx 1 \cdot 10^5 \text{ µm}^3\text{/cell}$$

<u>Challenging prediction about microbial isotopic fractionation with experimental data (Figure 2)</u>

Data for isotopic fractionation were taken from (Goevert and Conrad 2009, Kampara et al 2008, Penning et al 2005, Valentine et al 2004).

Values from (Kampara et al 2008) (Figure 2C) and (Penning et al 2005) (Figure 2B triangles) were interpolated using ImageJ (Abramoff et al 2004). Values from (Valentine et al 2004) were picked from Table 6 (Figure 2B circles) and values from *Methanosarcina barkeri* and *M. acetivorans* experiments (Goevert and Conrad 2009) (for Figure 2D) were picked from Table 1.

- Data interpolated from (Penning et al 2005) figures 2 and 3:

P(H_2) (kPa)	**α**	**ΔG** (kJ/mol)
0,142	1,046	-71,748
1,326	1,031	-91,385
2,036	1,034	-95,105
2,964	1,029	-98,229
2,775	1,04	-98,08
2,424	1,043	-96,89
2,301	1,047	-96,295
1,856	1,053	-93,617
1,108	1,063	-87,815
0,199	1,079	-69,516
0,143	1,053	-70,409
0,143	1,065	-69,814
0,914	1,034	-87,964
2,9	1,031	-98,973
4,957	1,022	-103,882
4,971	1,04	-104,477
4,1	1,043	-102,394
3,757	1,046	-101,353
3,057	1,049	-98,973
1,957	1,053	-93,766
0,686	1,058	-82,459

- Data interpolated from (Kampara et al 2008) figure 2:

Enrichement factors for 50% degradation of substrate (triangles in Figure 2C):

$\log_{10}$(C/1µM)	**ε**
1,961	-958,118
2,011	-941,932
1,977	-938,897
1,994	-924,532
1,78	-938,897
1,77	-944,36
1,02	-917,248
0,991	-901,265
1,044	-894,183
1,011	-874,962
0,035	-754,173
0,058	-781,892
0,08	-789,378

Enrichement factors for 70% degradation of substrate (circles in Figure 2c):

$\log_{10}$(C/1µM)	**ε**
2,014	-901,062
1,997	-873,343
1,977	-870,916
1,959	-867,274
1,782	-893,171
1,77	-877,997
1,042	-859,939
1,017	-858,877
0,991	-838,392
1,009	-833,991
0,032	-760,04
0,056	-743,045
0,079	-707,84

As shown above, our theory directly implies the dependence of $\alpha_{S/P}$ to $[S].E_{cat}^2$ (see Equation 3). This theoretical relationship is depicted on Figure 2A, where increasing $[S].E_{cat}^2$ leads to the convergence of microbial fractionation towards an asymptotical value (α_0) whereas the behavior is divergent at low values of $[S].E_{cat}^2$. In the literature, it has long been claimed that microbial isotopic fractionation was only dependent on the nature of the metabolism involved (Hayes 1993, Mariotti et al 1981). Recent evidences however increasingly testify that, for a given metabolism, it also varies with environmental conditions (Conrad 1999, Conrad 2005). Reported isotopic fractionation data confirms the predicted

dependency on Gibbs energy (Penning et al 2005) and substrate concentration (Goevert and Conrad 2009, Kampara et al 2008, Valentine et al 2004). Moreover, from our theory, two classes of fractionation behaviours are expected (Figure 2A, upper and lower curves) depending on the thermodynamic properties of isotopomers considered (see above). The upper curve corresponds to the case ΔE_{cat}^{h-l}<0 for which α decreases when ($[S] \cdot E_{cat}^{2}$) increases and could be named "microbial overfractionation". The lower curve (i.e. "microbial underfractionation") corresponds to the case ΔE_{cat}^{h-l}>0 for which α increases with ($[S] \cdot E_{cat}^{2}$). Figure 2B/C/D illustrates the fitting of Equation 3 on experimental data. Two sets of independently obtained data on ^{13}C isotopic fractionation associated with hydrogenotrophic methanogenesis confirm the existence of microbial overfractionation as shown on Figure 2B (Penning et al 2005) (Valentine et al 2004). Conversely, experimental data on $^{2}D/^{1}H$ fractionation obtained on aerobic toluene degradation (Kampara et al 2008) attest the existence of the microbial underfractionation phenomena (Figure 2C). Strikingly, in the case of microbial underfractionation, Equation 3 predicts that microbial isotopic fractionation can either enrich (α<1) or deplete (α>1) reaction products in heavy isotope depending on the value of ($[S] \cdot E_{cat}^{2}$). The existence of such a phenomenon has never been clearly stated in the literature and would require further confirmation. Nevertheless, several sets of experimental data appear to support this prediction although alternative hypotheses were sometimes raised to account for these observations (Fry et al 1986, Fry et al 1988, Goevert and Conrad 2008, Goevert and Conrad 2009). An illustration of how Equation 3 could give a theoretical ground to such kind of observations is illustrated using data from (Goevert and Conrad 2009); on ^{13}C isotopic fractionation associated with aceticlastic methanogenesis, for which experimental values of α shows an apparent inversion of the isotopic fractionation phenomena (Figure 2D).

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