

# Supplementary material 1

## Boltzmann statistics as founding principle of microbial growth

**Authors:** Elie Desmond-Le Quéméner et Théodore Bouchez\*

**Affiliation :** Irstea, UR HBAN, 1 rue Pierre-Gilles de Gennes, 92761 Antony cedex, France

\* corresponding author : [theodore.bouchez@irstea.fr](mailto:theodore.bouchez@irstea.fr)

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## 17 Boltzmann distribution of activated microbes

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

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

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

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

33 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.

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

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

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

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

37 then

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

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

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

40 Knowing that  $n = \frac{V_{tot}}{V_{harv}}$  and  $k = V_{tot} \cdot [S]^1$ , it leads:

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

41 The proportion of activated microbes ( $N^\ddagger/N$ ) is then given by the probability of finding occupied  
42 elementary volumes with more than  $\nu$  substrates molecules:

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

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

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

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

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

48 with

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

49

## 50 **Range of values commonly encountered in practical growth situations and validity of our** 51 **approximations**

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

- 54 • First, in order to use the canonical ensemble formalism and to derive the statistical  
55 distribution of molecules in elementary units, the volume not accessible to the microbes  
56 should constitute a reservoir of molecules. In practice, this situation is fulfilled when the  
57 volume of  $\Sigma'$  is a small fraction of the volume of  $\Sigma$  ( $N \cdot V_{harv} \ll V_{tot}$ ). In environmental  
58 conditions such as seawater, bacterial densities commonly encountered range around  
59  $10^6$  cells/ml (Porter and Feig 1980). In these conditions, one cell is, on the average,  
60 surrounded by  $10^6 \mu\text{m}^3$  of medium. In these cases, a harvesting volume ( $V_{harv}$ ) up to  $10^5 \mu\text{m}^3$   
61 assures a ratio less than 1/10 between occupied and non-occupied volumes. This harvesting  
62 volume is consistent with the volume estimated from Figure 2-A (fitted  $V_{harv} \approx 1 \cdot 10^5 \mu\text{m}^3$   
63 see sup. mat. 2). Our first hypothesis is therefore justified in many environmental conditions.

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<sup>1</sup> In this case  $[S]$  is expressed in molecules (not in mole) per volume unit.

64 However, in high density cultures, such as those obtained in laboratory conditions, *E. coli* can  
65 grow up to a density of  $10^9$  cells/ml. In these cases, the free volume around each cell is only  
66  $10^3 \mu\text{m}^3$  on the average and the harvesting volume should be less than  $100 \mu\text{m}^3$  to assure a  
67 ratio 1/10 between occupied and non-occupied volumes. Our model might therefore reach  
68 the limits of its validity domain under such high density conditions. However, as growth is  
69 exponential, it would still be applicable for most of the bacterial generations before reaching  
70 the saturation plateau, and thus for the major part of the growth curve. The development of  
71 an equation applicable to such high population densities would require further theoretical  
72 developments by considering a fully microcanonical ensemble without the canonical  
73 approximation, which is beyond the scope of the current paper.

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

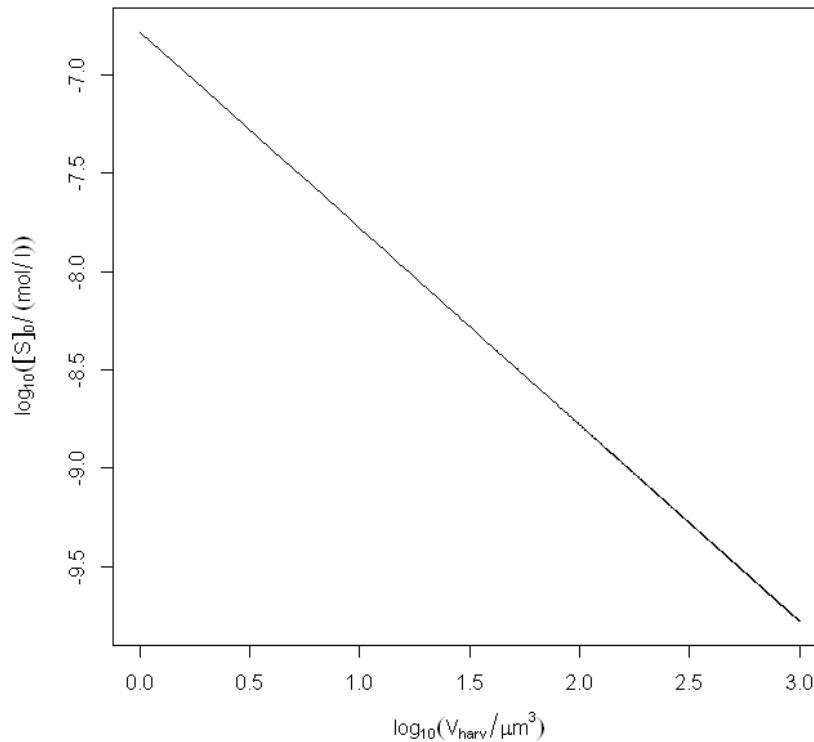
$$\frac{n}{k} = \frac{1}{V_{harv} \cdot [S] \cdot N_a}$$

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

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

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

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



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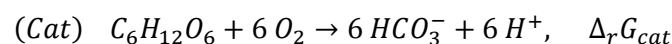
83 In the least favourable cases, corresponding to small harvesting volumes ( $1 \mu\text{m}^3$  to  $10^3 \mu\text{m}^3$ ),  
 84 threshold concentrations are still very low ( $[S]_0 < 1 \mu\text{mol/l}$ ). The second hypothesis is  
 85 therefore not limiting the applicability of the model.

86

### 87 Exergy balance for an elementary microbial division

88 Gibbs energy balance of growth can be estimated using different methods summed up in  
 89 (Kleerebezem and Van Loosdrecht 2010). The notations of this paper were slightly modified in order  
 90 to use the meaningful concept of exergy, which represents the maximum work available for a  
 91 microorganism during a chemical transformation. For more clarity, let us consider *E. coli* growing  
 92 aerobically on glucose as a case study. We use the Gibbs energy dissipation method initially proposed  
 93 by Heijnen to determine the growth stoichiometry.

94 The catabolism for aerobic growth on glucose is associated with a Gibbs energy variation, denoted  
 95  $\Delta_r G_{\text{cat}}$ :

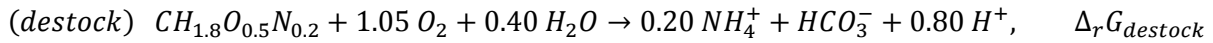


96 Thus, catabolic exergy (chemical energy available for the microbe) is:

$$97 \quad E_{\text{cat}} = -\Delta_r G_{\text{cat}} \text{ if } \Delta_r G_{\text{cat}} < 0$$

$$98 \quad E_{\text{cat}} = 0 \text{ otherwise}$$

99 Let us then define microbial exergy  $E_M$  as the chemical energy available in a C-mol of microbe (or in a  
 100 microbe), in other words the energy a microbe could virtually obtain by consuming its own biomass.  
 101 It should be noted that  $E_M$  depends on the type of microbial metabolism: for a given cell  
 102 composition, the exergy of cell constituents degraded through aerobic respiration is greater than the  
 103 exergy of the same constituents degraded into methane and carbon dioxide. For an aerobic microbe,  
 104 biomass oxidation can be written as follows:



105 Thus, microbial exergy is:

$$106 \quad E_M = -\Delta_r G_{destock} \text{ if } \Delta_r G_{destock} < 0$$

$$107 \quad E_M = 0 \text{ otherwise}$$

108  $CH_{1.8}O_{0.5}N_{0.2}$  represents a generic elemental composition of one C-mole of biomass. The standard  
 109 Gibbs energy of formation of all constituents is known, including the Gibbs energy of formation of  
 110 one C-mol of biomass (Kleerebezem and Van Loosdrecht 2010).  $E_{cat}$  and  $E_M$  can thus be calculated  
 111 from tables. The standard Gibbs energies for (*cat*) and (*destock*) corrected for pH=7, denoted  
 112  $\Delta_r G^{01}$ , are:

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

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

113 The metabolic equation of growth of *E. coli* on glucose is a linear combination of equations  
 114 (*destock*) and (*cat*).

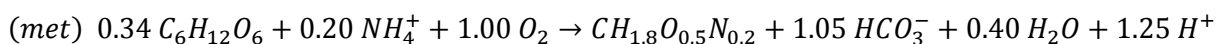
$$(met) = \nu(cat) - (destock), \quad \Delta_r G_{met}$$

115 The metabolic exergy dissipated during growth ( $E_{dis} = -\Delta_r G_{met}$ ) is equal to the exergy of the  $\nu$   
 116 substrates molecules consumed during the synthesis of one C-mol of biomass (or of one cell) minus  
 117 exergy stored in the biomass.

118 Exergy balance thus enable to calculate the factor  $\nu$  (Kleerebezem and Van Loosdrecht 2010). Using  
 119 the Gibbs energy dissipation method,  $\Delta_r G_{met}$  can be calculated for one C-mol of biomass knowing  
 120 the number of carbon atoms in a substrate molecule and its oxidation degree (Kleerebezem and Van  
 121 Loosdrecht 2010). For simplicity, it is possible to consider  $E_{dis} = 500 \text{ kJ/C - mol}$  as a good  
 122 approximation of dissipated exergy. The factor  $\nu$  can then be obtained:

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

123 Thus for *E. coli* growing on glucose in standard conditions corrected for pH=7,  $\nu = 0.34$ , leading to  
 124 the following stoichiometric metabolic growth equation:



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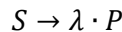
126 **Stoichiometric growth limitation and its dependence to elemental microbial composition**

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

131 A more detailed chemical composition of biomass can be used in order to model microbial growth  
 132 under various types of elemental limitation (P, S, K, Mg, Ca,...) applying the same principle to obtain  
 133  $\nu$ .

#### 134 **The microbial isotopic fractionation phenomenon as viewed from our theory**

135 Let us consider the biologic fraction due to the following reaction catalyzed by microbes:



136 with  $S$  the substrate,  $P$  the product and  $\lambda$  a stoichiometric coefficient. Substrate and product with a  
 137 heavy isotope will be denoted  $S^h$  and  $P^h$ , substrate and product with a light isotope will be denoted  
 138  $S^l$  and  $P^l$ .

139 The kinetic fractionation factor  $\alpha_{S/P}$  (sometimes written  $\alpha_{P/S} = 1/\alpha_{S/P}$ ) (Mariotti, Germon et al.  
 140 1981) of the reaction is:

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

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

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

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

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

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

145  $\mu_{max}^h$  and  $\mu_{max}^l$  are the maximal growth rates for heavy and light substrate respectively.

146  $E_{cat}^h$  and  $E_{cat}^l$  represents the catabolic exergy for the catabolic reaction with heavy or light substrates  
 147 respectively (see thermodynamic properties of isotopomers below).

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

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

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

151 Let us then calculate the kinetic fractionation factor:

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

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

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

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

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

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

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

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

159 Contrary to the classical derivation of a constant kinetic fractionation factor (Mariotti, Germon et al.  
 160 1981) this expression predicts the dependency of the fractionation to exergy and substrate  
 161 concentration as recently evidenced in various experimental reports (Valentine, Chidthaisong et al.  
 162 2004, Penning, Plugge et al. 2005, Kampara, Thullner et al. 2008, Govert and Conrad 2009).

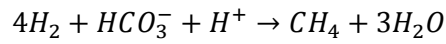
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## 164 Thermodynamic properties of isotopomers

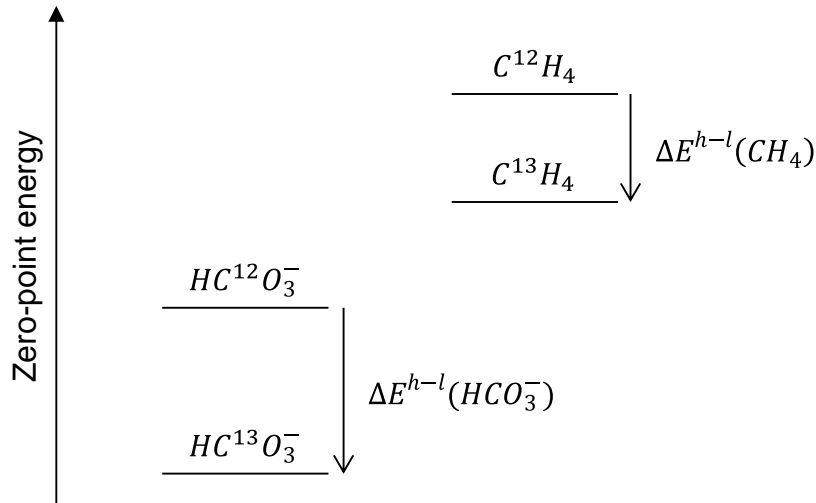
165 Different isotopomers have different energy levels (Zeebe and Wolf-Gladrow 2001). Indeed a  
 166 molecule containing a heavy isotope has stronger bonds than a molecule with a light isotope.

167 Let us consider hydrogenotrophic methanogenesis:





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



170

171 Covalent bonds are stabilized by heavy atoms thus exergies of heavy molecules are lower than  
 172 exergies of light molecules. In addition C-O bonds are relatively more stabilized by  $C^{13}$  than C-H  
 173 bonds, thus heavy carbonate is relatively more stabilized than heavy methane:

$$\Delta E_{cat}^{h-l} = \Delta E^{h-l}(CH_4) - \Delta E^{h-l}(HCO_3^-) < 0$$

174 This simple argument predicts overfractionation for hydrogenotroph methanogenesis as observed in  
 175 the experiments of (Penning, Plugge et al. 2005) and (Valentine, Chidthaisong et al. 2004) (cf. figure  
 176 2-B-2). The exact calculation is however more complicated and should take into account the real  
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