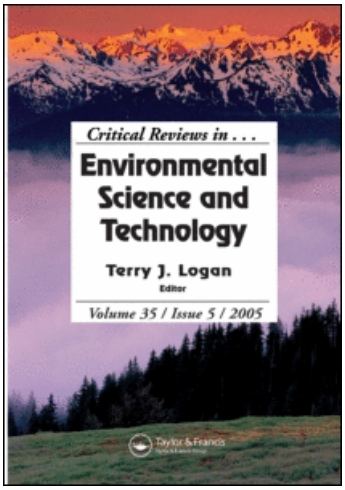

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A Generalized Method for Thermodynamic State Analysis of Environmental Systems

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A Generalized Method for Thermodynamic State Analysis of Environmental Systems

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Chemotrophic life depends on the regular supply of new material to maintain a thermodynamic non-equilibrium situation. A thermodynamic non-equilibrium situation is characterized by significant driving forces to drive phase and/or electron transfer in redox reactions. Microorganisms play an important role as catalysts of thermodynamically feasible chemical redox reactions, and are capable of utilizing the energy generated for microbial growth. An improved insight in the functioning of ecosystems and the microbial life encountered can be established by analyzing the thermodynamic state of the ecosystem. Still, conducting an extensive thermodynamic state analysis based on measured information of an ecosystem is generally considered a rather complicated and laborious task. In this paper, we describe a generalized and step-wise method for conducting an extensive thermodynamic state analysis of an environmental ecosystem. The method is based on the identification of the reactants in the system and the derivation of the stoichiometry of the catabolic and anabolic reaction. In a subsequent step, the thermodynamic system properties are calculated, and different methods to establish a full description of microbial metabolism are obtained. Several examples are presented to clarify the method proposed. We hope this relatively straightforward method encourages researchers of microbial ecosystems to include thermodynamic state analysis as an integral part of their research.

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and Technology for the following free supplemental resource: an elaboration in MS Excel of examples in the text regarding the fully generalized method described in this paper.]

KEY WORDS: thermodynamics, stoichiometry, Gibbs free energy, microbial growth

INTRODUCTION

Environmental ecosystems can be characterized as thermodynamically open systems with energy flowing through them. This is the reason why they can sustain life in a dissipative system at non-equilibrium conditions. The appearance of a high degree of organization in living systems can only be maintained as long as energy is supplied from an external source. According to the second law of thermodynamics, the high level of organization can only be maintained at the expense of a greater amount of disorganization in a series of reactions and transport process.

While dealing with the thermodynamics of environmental ecosystems, one should realize that process rates are independent of the thermodynamic properties of the system, and no kinetics will be treated here. Still, it is evident that most rate equations consist of one or more rate constants and a driving force that is related to the thermodynamic state of the system (see Table 1). Herewith, a direct relationship between the driving force and the overall rate exists, but the absolute value of the rate depends on the rate constant, which is independent of the properties of the system. The methods developed in this paper address stoichiometry and thermodynamics, not with process kinetics. The stoichiometric and thermodynamic concepts proposed enable straightforward coupling to microbial kinetic expressions to obtain a complete description of material and energy fluxes in microbial ecosystems. No specific kinetic expression for substrate uptake, substrate consumption for maintenance requirements, or other flux-related models will be discussed here. For a generalized description of microbial kinetics

TABLE 1. Examples of the description of environmental processes as a function of a driving force and one or more rate constant

Process	Overall rate equation	Driving force-related term	Rate-related term
Gas-liquid mass transfer	$R_{G/L} = k_L a \cdot (C^* - C)$	$C^* - C$	$k_L a$
Diffusion	$R_D = \frac{D}{\delta} \cdot (C^* - C)$	$C^* - C$	$\frac{D}{\delta}$
Microbial growth	$R_X = Y_{X/S} \cdot q_S \cdot X$	$Y_{X/S}$	$q_S \cdot X$
Liquid flow through a porous medium	$Q = -k \cdot A \cdot \frac{\Delta H}{L}$	$\frac{\Delta H}{L}$	$-k \cdot A$
Dissolution of solids	$R_{S/L} = k \cdot \frac{S}{\nu} \cdot \left(C_S^* - C_S\right)$	$C_S^* - C_S$	$k \cdot \frac{S}{\nu}$
Enzyme kinetics	$R_S = L \cdot \Delta G^1$	ΔG^1	L
	$V = V^{\max} \cdot (1 - e^{\Delta G^1})$	$(1 - e^{\Delta G^1})$	$V^{\max}$

in general and maintenance kinetics in particular, the reader is directed to previous reports (Heijnen, 1999; Russell and Cook, 1995; Tijhuis et al., 1993).

Quantitative analysis of the energy flowing through an ecosystem allows for an improved understanding and insight in its functioning. In this paper, a basic and generalized framework will be derived for analysis of environmental systems based on the laws of thermodynamics. To achieve this, first, a generalized approach for defining mass and electron balances and reaction stoichiometries will be established. Material balances form the backbone of the analysis of microbial ecosystems. In this paper, a vector-based calculation method is established for the derivation of the reaction stoichiometry of the redox reactions, upon which microbial metabolism is based on. The approach chosen enables easy implementation in any kind of spreadsheet program. The identification of the overall reaction stoichiometry allows for the identification of a number of important process factors, like nutrient requirements for microbial growth, the effect of microbial conversion reactions on the alkalinity and the pH, and the composition of biogas produced in anaerobic processes.

In the second part of the paper the application of Gibbs energy balances, or non-equilibrium thermodynamics, to microbial systems is introduced. The identification of the thermodynamic state allows for the prediction of redox reaction-induced temperature changes and to investigate if there are important driving forces in the system for phase transition (e.g., super-saturation of media with gases, or a tendency for precipitation if a solubility product is exceeded) or chemical or microbial electron transfer reactions. A second important application of thermodynamic balances is by coupling Gibbs energy production and consumption in microbial growth as reflected in the biomass yield. The application of non-equilibrium thermodynamics for estimation of biomass yield values provides a common framework that enables interpretation of measured growth data. Different methods to make a thermodynamic analysis of microbial growth are implemented and discussed.

The descriptions of specific examples of the broad spectrum of enzymatic microbial systems that are available in nature are avoided as much as possible in order to emphasize the common rather than the specific aspects. The approach presented can be employed to understand the multitude of microbial mediated redox reactions and the sequence in which they occur in time or space.

This paper describes methodologies to work with material balances and thermodynamic tools, with emphasis on the thermodynamics related methods. The paper deals with method development, and consequently no new experimental or calculation data will be presented. The aim is to describe a step-wise generalized calculation scheme that allows readers to conduct a thermodynamic state analysis of any microbial system under investigation. We have noticed that thermodynamic state analysis of environmental systems is only applied to a limited extent, and we think this may be due to the

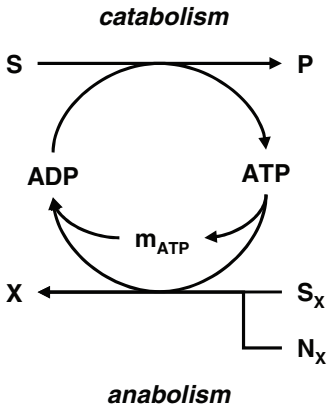

FIGURE 1. Schematic representation of microbial metabolism as a coupled network of catabolism and anabolism.

absence of a generalized calculation scheme that enables easy implementation of this kind of calculations in a computer program. The objective of this paper will be to provide such a fully generalized and relatively straightforward calculation scheme.

The methodology described here has been developed to be implemented in any kind of spreadsheet software package. The generalized calculation scheme enables the elaboration of very different kind of microbial systems, making the methods highly suitable for knowledge transfer purposes. All the examples presented have been worked out in MS Excel, and the file containing the examples can be downloaded from http://www.bt.tudelft.nl/ebt.

STOICHIOMETRY OF MICROBIAL METABOLISM

Microbial Metabolism

In a highly simplified view, microbial metabolism can be regarded as a network of catabolism and anabolism coupled by an energy carrier, as depicted in Figure 1. According to this scheme, a substrate (S) is converted to a product (P) in the catabolic reaction, and metabolic energy is generated and conserved in the form of *ATP*. In the anabolism, the numerous biomass components (X) are constructed from a carbon source (S_X) and a nitrogen source (N_X) under consumption of energy (*ATP*). Uncoupling between the catabolism and anabolism is established by the consumption of the energy carrier (*ATP*) for a non-growth-related maintenance process (m_{ATP}). The actual catabolic and anabolic fluxes are coupled by a stoichiometric relationship governed by the energetic housekeeping of the cell.

To define the overall stoichiometry of microbial metabolism, first the stoichiometry of the redox reactions describing the catabolism and anabolism need to be established. In a subsequent step, coupling between catabolism

and anabolism allows for the derivation of the stoichiometry of the overall metabolic growth equation. To establish this, one measured stoichiometric coefficient (i.e., the biomass yield on substrate) that links catabolism and anabolism needs to be identified. As shown in Figure 1, the coupling of catabolism and anabolism can be written in bioenergetic terms by realizing that in steady-state energy (*ATP*) production in the catabolism should equal energy consumption in the anabolism. The bioenergetic coupling of catabolism and anabolism in microbial metabolism will be the topic of the second part of this paper.

In this section, first, an introduction on electron transfer in microbial systems will be presented. In the subsequent paragraphs, a generalized method for derivation of the stoichiometry of the catabolic and anabolic reaction will be established. Based on the catabolic and anabolic reaction stoichiometry, the metabolic reaction equation can be derived based on a measured or estimated yield coefficient that links catabolism and anabolism.

The Redox Concept

In absence of light, microbial activity is energetically driven by electron transfer in redox reactions. In a redox reaction, an electron is transferred from a reduced electron donor to an oxidized electron acceptor. For stoichiometric balancing of electron transfer reactions, it is convenient to assign oxidation states to the redox labile elements involved.

By assigning the shared electrons, we can count the number of electrons belonging to each atom in the molecule. The resulting oxidation state is determined by the number of exchangeable electrons present in the outer orbital of the atom. In a covalent bond between two atoms, the shared electrons are assigned fully to the more electronegative atom. From Table 2, it can be seen that the order of electronegativity is O > N > C ~ S > H ~ P. It should be noted that in double bounds, the number of shared electrons in the outer orbital amounts two.

TABLE 2. Elements with a variable oxidation state that play an important role in microbiology and their minimum and maximum oxidation state (γ) with the elemental form as reference oxidation state

Name		Reference state state and phase	Electronegativity	Min γ		Max γ	
				γ mol-e	$mol^{-1}\gamma$	γ mol-e	$mol^{-1}\gamma$
Hydrogen	H	H_2 (g)	2.10	0	H_2	+I	H^{+1}
Carbon	C	C (s)	2.50	−IV	CH_4	IV	HCO_3^{-1}
Sulfur	S	S (s)	2.44	−II	HS^{-1}	VI	SO_4^{-2}
Nitrogen	N	N_2 (g)	3.07	−III	NH_4^{+1}	V	NO_3^{-1}
Iron	Fe	Fe (s)	1.64	0	Fe	III	Fe^{+3}
Manganese	Mn	Mn (s)	1.60	II	Mn^{+2}	IV	Mn^{+4}
Oxygen	O	O_2 (g)	3.50	−II	H_2O	0	O_2

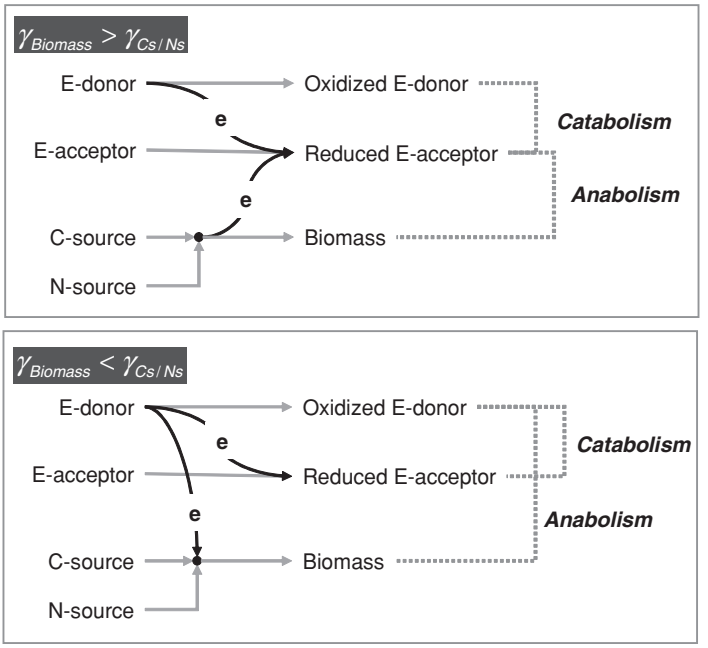

FIGURE 2. Schematic representation of electron flow in microbial metabolism. Symbols used are explained in the text.

In traditional chemistry, the oxidation state zero, the reference state, is assigned to the elemental form of the atom (see Table 2). More reduced forms of the element have by definition a negative oxidation state, and more oxidized forms have a positive oxidation state. Herewith, formal oxidation states can be assigned to single atoms in a molecule. Some of the common elements in biological systems exhibit multiple oxidation states enabling electron transfer, as shown in Table 2. Phosphorous is an example of an element that is essential for life, but is generally considered redox conservative in natural environments with an oxidation state of $+V$ as in phosphate (PO_4^{-3}) (Morton and Edwards, 2005).

Figure 2 schematically shows the fate of electrons in microbial metabolism. In the energy-generating catabolism, electrons are transferred from a reduced electron donor to an oxidized electron acceptor. Electron transfer in the anabolism depends on the oxidation state of the substrates (carbon source and nitrogen source) compared to the product biomass. If the carbon and nitrogen source require electrons to be reduced to the oxidation state of biomass ($\gamma_{Biomass} > \gamma_{Cs/Ns}$), an electron donor needs to be defined that generates the electrons required. Normally, and as shown in Figure 2, this will be the same electron donor as in the catabolism. If electrons are produced upon conversion of the carbon and nitrogen source to biomass ($\gamma_{Biomass} < \gamma_{Cs/Ns}$), an electron acceptor is required. Also in this case, normally the same electron acceptor as in the catabolism will be assumed.

In the following paragraphs, a generalized calculation scheme is derived that allows for the identification of the catabolic and anabolic reaction stoichiometry. Based on these reaction stoichiometries and one parameter that links catabolism and anabolism, a generalized formulation of the stoichiometry of the overall metabolic reaction equation will be derived.

The calculation scheme developed is optimized for implementation in a calculation tool like a spreadsheet program and enables straightforward calculation of thermodynamic properties. It can be regarded as an alternative method for the electron-based stoichiometry derivation method as developed by McCarty (1975) or the degree of reduction-based balances and elemental balances-based methods described by Heijnen (1999). Evidently, the outcome of the different methods will be equal in most cases. The rare cases where different results are obtained with the methods developed by others, or when limitations are encountered in comparison with the method described here, will be elaborated in specific examples.

STOICHIOMETRY OF THE CATABOLISM

For definition of the stoichiometry of the catabolism or any other redox reaction, it is convenient to define the electron donor and electron acceptor reaction. In generalized terms, the electron donor and acceptor reaction equations (D and A) can be written as:

$$D: \quad -1 \cdot Ed + Y_e^D \cdot e^{-1} + \ldots = 0 \tag{1}$$

$$A: \quad -1 \cdot Ea + Y_e^A \cdot e^{-1} + \ldots = 0 \tag{2}$$

where Y_e^D and Y_e^A are the electron yield values in the electron donor and electron acceptor reaction respectively. Consequently, Y_e^D has a positive value, whereas Y_e^A is negative. The stoichiometric coefficients for the electron donor (Y_{Ed}^D) and acceptor (Y_{Ea}^A) are defined as -1. We have chosen to define the reactions D and A per mol electron donor/acceptor and not per electron, as proposed by McCarty (1975) because the number of electrons involved in the reaction is not known a priori. The electron donor and acceptor molecule have one or more atoms for which the oxidation state changes in the redox reaction. A method that allows for easy derivation of the stoichiometry of the electron donor and acceptor reaction is demonstrated in Appendix A. In a vector-based calculation scheme, this method requires only identification of the redox couple in the reaction. The O, H, and charge balance are subsequently solved by generalized equations for the stoichiometric coefficients for H_2O, H^{+1}, and e^{-1}.

For derivation of the overall stoichiometry of a chemical redox reaction (R), the reaction needs to be defined as a function of the electron donor and

acceptor reaction:

$$R = \lambda_D \cdot D + \lambda_A \cdot A \tag{3}$$

where λ_D and λ_A are multiplication factors for the electron donor and acceptor reactions. R, D, and A are vectors describing the stoichiometry of the catabolic, electron donor, and electron acceptor reaction. An advantage of writing *Cat* as a function of D and A is that it provides insight in the redox reaction by defining from which compound electrons are transferred.

After definition of the redox half reactions, the stoichiometry of the net redox reaction can be derived. A first generalization utilized is that no net production or consumption of electrons occurs and the electron neutrality equation can be written as:

$$\lambda_D \cdot Y_e^D + \lambda_A \cdot Y_e^A = 0 \tag{4}$$

We will furthermore assume that the reaction equation derived is calculated per mol of electron donor consumed:

$$\lambda_D \cdot Y_{Ed}^D + \lambda_A \cdot Y_{Ed}^A = -1 \tag{5}$$

Using Eqs. (4) and (5), λ_D and λ_A can be eliminated from Eq. (3), and the stoichiometric equation for the chemical redox reaction can be calculated as a function of the individual yield values:

$$R = \frac{Y_e^A \cdot D - Y_e^D \cdot A}{Y_e^D \cdot Y_{Ed}^A - Y_e^A \cdot Y_{Ed}^D} \tag{6}$$

This equation can be simplified by realizing that Y_{Ed}^D has by definition the value -1. Y_{Ed}^A is only unequal to 0 in case of reactions where the substrate is both electron donor and acceptor ($Ed = Ea$). In microbiology, these reactions that are based on internal electron transfer are called fermentations. In that case both Y_{Ed}^A and Y_{Ed}^D are -1. Herewith, the following two cases can be distinguished:

$$\text{External electron acceptor:} \quad R = D - \frac{Y_e^D}{Y_e^A} \cdot A \tag{7}$$

$$\text{Fermentation:} \quad R = \frac{Y_e^A \cdot D - Y_e^D \cdot A}{Y_e^A - Y_e^D} \tag{8}$$

The equations derived above are based on the assumption that single substrate product relationships can be identified for both the electron donor as well as the electron acceptor reaction. This is not the case for mixed product fermentations, for example, where different oxidized and reduced products are formed in a certain ratio. In that case, products need to be

lumped, either in the electron donor or acceptor reaction, in order to establish one electron donor and one electron acceptor reaction. The example of anaerobic glucose oxidation to acetate, bicarbonate, and molecular hydrogen is shown in Box 1.

BOX 1. Examples of redox reaction stoichiometries.

1. Partial ammonium oxidation to nitrite with molecular oxygen as electron acceptor.

$$D: \quad -1 \cdot NH_4^{+1} - 2 \cdot H_2O + NO_2^{-1} + 8 \cdot H^+ + 6e^{-1} = 0$$

$$A: \quad -1 \cdot O_2 - 4 \cdot H^{+1} - 4 \cdot e^{-1} + 2 \cdot H_2O = 0$$

Using Eq. (7), the stoichiometry of the overall redox reaction can be calculated:

$$Cat: -1 \cdot NH_4 + -1.5 \cdot O_2 + NO_2^{-1} + 1 \cdot H_2O + 2 \cdot H^+ = 0$$

2. Anaerobic glucose oxidation to acetate, bicarbonate, and molecular hydrogen.
Because there are three oxidation-reduction products formed, one product ratio has to be predefined in order to define the electron donor and acceptor reaction. In this example, we have chosen to define the acetate to bicarbonate ratio in the electron donor reaction (D) as 1:1:

$$D: -1 \cdot C_6H_{12}O_6 - 4 \cdot H_2O + 2 \cdot C_2H_3O_2^{-1} + 2 \cdot HCO_3^{-1} + 12 \cdot H^+ + 8 \cdot e^{-1} = 0$$

$$A: -1 \cdot H^+ - 1 \cdot e^- + 0.5 \cdot H_2 = 0$$

Herewith, the reaction is written as an anaerobic oxidation reaction with glucose as electron donor and protons as electron acceptor. As such, this reaction does not represent a true fermentation. The stoichiometry of the overall redox reaction can be calculated using Eq. (7):

$$Cat: -1 \cdot C_6H_{12}O_6 - 4 \cdot H_2O + 2 \cdot C_2H_3O_2^{-1} + 2 \cdot HCO_3^{-1} + 4 \cdot H_2 = 0$$

Other fixed ratios could have been defined as well (e.g., a constant acetate to hydrogen ratio in the electron acceptor reaction, and bicarbonate production from glucose as electron donor reaction). The choice of most suitable products to lump depends on biochemical knowledge and logic.

3. Anaerobic oxalate fermentation to methane and bicarbonate.
Anaerobic digestion of an organic substrate to methane- and carbon dioxide-containing biogas is a typical example of a fermentation reaction. In a fermentation reaction, the organic substrate by definition is both electron donor and acceptor. In this example, oxalate is oxidized to bicarbonate in the electron donor reaction, and reduced to methane in the electron acceptor reaction:

$$D: \quad -1 \cdot C_2O_4^{-2} - 2 \cdot H_2O + 2 \cdot HCO_3^{-1} + 2 \cdot H^+ + 2 \cdot e^{-1} = 0$$

$$A: \quad -1 \cdot C_2O_4^{-2} - 16 \cdot H^+ - 14 \cdot e^- + 2 \cdot CH_4 + 4 \cdot H_2O = 0$$

Using Eq. (8), the stoichiometry of the catabolic reaction equation can be established:

$$Cat: \quad -1 \cdot C_2O_4^{-2} - 1.25 \cdot H_2O - 0.25 \cdot H^{+1} + 0.25 \cdot CH_4 + 1.75 \cdot HCO_3^{-1}$$

Stoichiometry of the Anabolism

Microbial metabolism consists of catabolism and anabolism. The anabolic reaction is the overall reaction lumps the production biomass constituents from a carbon and a nitrogen source . For the definition of a simplified reaction equation for the anabolic reaction, the following variables need to be identified:

- the carbon source (*Cs*);
- the nitrogen source (*Ns*, and other nutrient sources when required); and
- the electron donor in case the carbon/nitrogen source is more oxidized than biomass, or the electron acceptor in case the carbon/nitrogen source is more reduced than biomass.

The stoichiometry of the anabolic reaction equation can be written as the half reaction for biomass production from a carbon and nitrogen source (An^*):

$$An^* = -Y_{Cs}^{An*} \cdot Cs - Y_{Ns}^{An*} \cdot Ns + 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + Y_{H2O}^{An*} \cdot H_2O + Y_H^{An*} \cdot H^{+1} + Y_e^{An*} \quad (9)$$

where $CH_{1.8}O_{0.5}N_{0.2}$ represents a simplified elemental composition of biomass. By definition, the stoichiometry of the anabolic reaction equation is defined per mol biomass (X) formed ($Y_X^{An*} = 1$). Depending on the oxidation state of *Cs* and *Ns*, an external electron donor (D) or acceptor reaction (A) needs to be defined to establish the overall anabolic reaction An. As for any other redox reaction, the anabolic reaction can be described as the sum of an electron donor and acceptor reaction:

$$An = An^* - \frac{Y_e^{An^*}}{Y_e^D} \cdot D, \quad \text{if} \quad Y_e^{An^*} < 0, \quad (10)$$

$$An = An^* - \frac{Y_e^{An^*}}{Y_e^A} \cdot A, \quad \text{if} \quad Y_e^{An^*} > 0. \quad (11)$$

Some examples for biomass producing anabolic reaction equations are derived in Box 2.

The approach described here for derivation of the stoichiometry of the anabolism is slightly different from the methods used by others (Heijnen and Vandijken, 1993; Heijnen et al., 1992; McCarty, 1975; Rittmann and McCarty, 2001). These authors proposed the use of electron equivalent half-reactions for derivation of the anabolic, catabolic, and metabolic reaction stoichiometries. One of the main limitations of these methods is the proposed use of inorganic carbon as substrate or product to close the electron balance in *An**.

BOX 2. Examples of anabolic reaction stoichiometries.

1. Anabolic reaction equation for growth of a denitrifying culture on formate. For anabolic reaction equations, we need to identify the N source and the C source:
   - C source: HCO_2^{-1},
   - N source: NO_3^{-1}

   $$An^* : -1 \cdot CHO_2^{-1} - 0.2 \cdot NO_3^{-1} + CH_{1.8}O_{0.5}N_{0.2} + 2.1 \cdot H_2O - 5 \cdot H^{+1} - 3.8 \cdot e^{-1} = 0$$

   Because the biomass formation half reaction is an electron-accepting reaction, electrons need to be supplied by an external electron donor. Besides its role as carbon source, we assume formate to be electron donor in the anabolism as well:

   $$D : \quad -1 \cdot CHO_2^{-1} + HCO_3^{-1} - H_2O + 2H^{+1} + 2 \cdot e^{-1} = 0$$

   And the anabolic reaction stoichiometry can be derived according to Eq. (10):

   $$An : \quad -2.9 \cdot CHO_2^{-1} - 0.2 \cdot NO_3^{-1} - 3.1 \cdot H^{+1} + CH_{1.8}O_{0.5}N_{0.2} + 1.9 \cdot HCO_3^{-1} + 2.1 \cdot H_2O = 0$$

   Herewith, it is assumed that in this system, no ammonium is present. If multiple inorganic *N*-sources are available, ammonium is preferred because it is already in the oxidation state required for assimilation into biomass.
2. Anabolic reaction equation for autotrophic growth of sulfide oxidizing bacteria.
   - C source: HCO_3^{-1},
   - N source: NH_4^{+1}.

   $$An^* : -1 \cdot HCO_3^{-1} - 0.2 \cdot NH_4^{+1} - 4H^{+1} - 4.2 \cdot e^{-1} + CH_{1.8}O_{0.5}N_{0.2} + 2.1 \cdot H_2O = 0$$

   And consequently, some sulfide oxidation to sulfate is needed to generate the electrons required for bicarbonate reduction:

   $$D : \quad -HS^{-1} - 4 \cdot H_2O + SO_4^{-2} + 9 \cdot H^{+1} + 8 \cdot e^{-1} = 0$$

   and the overall anabolic reaction equation becomes:

   $$An : \quad -HCO_3^{-1} - 0.525 \cdot HS^{-1} - 0.2 \cdot NH_4^{+1} - 0.6 \cdot H_2O + CH_{1.8}O_{0.5}N_{0.2} + 0.525 \cdot SO_4^{-2} + 0.725 \cdot H^{+1}$$

We changed this approach and enabled free definition of electron acceptors and donors in the anabolic reaction equation for the following reasons:

- The electron donor/acceptor in the catabolism and the anabolism are not equal in all cases, as can be seen later in the Anammox example.
- Redox balancing in the anabolism during heterotrophic growth with inorganic carbon as electron acceptor does in some cases result in unrealistic stoichiometries. The example of anaerobic ethanol oxidation to acetate, as shown in Box 3, demonstrates this.
- Another disadvantage of redox balancing with inorganic carbon in the anabolism is that it may result in confusing thermodynamic calculation results. This will be demonstrated later.

BOX 3. Definition of the electron acceptor in the anabolic reaction.

Both McCarty and Heijnen propose to define the anabolic reaction stoichiometry as a function of the half-reaction for oxidation of the carbon source to bicarbonate and biomass to bicarbonate. As defined by Heijnen, herewith the two anabolic reactions (An_1 and An_2) become:

$$An_1: \quad -1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 1 \cdot HCO_3^{-1} + Y_{Ns}^{An1} \cdot Ns + Y_e^{An1} \cdot e^{-1} + \ldots = 0,$$
$$An_2: \quad -1 \cdot Cs + 1 \cdot HCO_3^{-1} + Y_e^{An2} \cdot e^{-1} + \ldots = 0,$$

Closing the electron balance yields the anabolic reaction equation (An). Using a carbon source with an oxidation state different from biomass, this will result in the uptake or production of bicarbonate. In case of a carbon source that is more reduced than biomass ($Y_e^{An1} > Y_e^{An2}$), these equations imply the incorporation of bicarbonate into biomass. Biochemically, it is not realistic to assume that inorganic carbon is by definition the electron acceptor in the anabolism. In an aerobic system, for example, there is no reason to assume that the electrons generated upon substrate oxidation to the level of biomass will be reoxidized with a weak electron acceptor like inorganic carbon, when there is a strong electron acceptor (O_2) that enables the generation of metabolic energy.

Still, the assumption of bicarbonate incorporation into biomass will not give rise to an unrealistic stoichiometry of the metabolic reaction equation as long as bicarbonate is consumed or produced in the catabolism as well. It is just mechanistically unlikely to occur in the stoichiometric descriptions proposed by McCarty and Heijnen. More seriously, however, the limitation of this approach occurs when bicarbonate plays no role in the catabolism, as will be demonstrated below.

We will consider the acetogenic anaerobic oxidation of ethanol with protons as electron acceptor.

$$Cat: \; -1 \cdot C_2H_5OH - 1 \cdot H_2O + 1 \cdot C_2H_3O_2^{-1} + 1 \cdot H^{+1} + 2 \cdot H_2 = 0$$

Using the approach shown above, the anabolic reaction involving bicarbonate as electron acceptor becomes:

$$An: \; -0.35 \cdot C_2H_5OH - 0.3 \cdot HCO_3^{-1} - 0.2 \cdot NH_4^{+1}$$
$$+ 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 0.75 \cdot H_2O - 0.1 \cdot H^{+1} = 0.$$

At any biomass yield value, the metabolic reaction stoichiometry derived from these catabolic and anabolic reactions will involve the consumption of bicarbonate. Even though bicarbonate uptake cannot be excluded to occur during heterotrophic growth, the stoichiometric uptake of bicarbonate as electron acceptor in the anabolism is not realistic. A more plausible assumption is that ethanol is oxidized to an oxidation state comparable to biomass that is used as anabolic building block (e.g., acetate or acetyl-CoA), and the electrons generated in this reaction are transferred to an electron carrier (like *NAD/NADH*). The reduced electron carrier is subsequently reoxidized using the electron acceptor available. According to this approach, it is most likely that the same electron acceptor as in the catabolism is used, and protons will be reduced to molecular hydrogen, resulting in the following anabolic reaction:

$$An: \; -0.5 \cdot C_2H_5OH - 0.2 \cdot NH_4^{+1} + 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 0.9 \cdot H_2 + 0.2 \cdot H^{+1} = 0,$$

and biomass production is stoichiometrically coupled to elemental hydrogen production instead of bicarbonate reduction. In this way, a more realistic stoichiometry of the anabolism (and metabolism) is obtained because the unknown role of bicarbonate is excluded from the system description.

Stoichiometry of the Metabolism

Analogue to the redox reaction definitions, the metabolic reaction equation (*Met*) can be represented as a function of *Cat* and *An*:

$$Met = \lambda_{Cat} \cdot Cat + \lambda_{An} \cdot An \tag{12}$$

We will assume that like the anabolic reaction equation, the metabolism is defined per C-mol biomass formed ($\lambda_{An} = 1$). Thermodynamically, the multiplication factor of the catabolism (λ_{Cat}) can be regarded as the number of times the catabolic reaction needs to run to generate sufficient Gibbs energy to produce one C-mol of biomass.

One stoichiometric coefficient that links the catabolism with the anabolism has to be determined in order to derive the overall metabolic reaction stoichiometry. Typically, this stoichiometric coefficient is the biomass production per unit of substrate consumed, or product formed: $Y_{X/S}^{Met}$, where X represents biomass and S substrate. In a generalized form $Y_{X/S}^{Met}$ can be written as a function of the stoichiometric yield for both S and X in the catabolism and the anabolism:

$$Y_{X/S}^{Met} = \frac{\lambda_{Cat} \cdot Y_X^{Cat} + \lambda_{An} \cdot Y_X^{An}}{\lambda_{Cat} \cdot Y_S^{Cat} + \lambda_{An} \cdot Y_S^{An}} \tag{13}$$

This equation can be simplified by realizing that biomass (X) does not participate in the catabolic reaction ($Y_X^{Cat} = 0$), the anabolism is defined per C-mol biomass formed ($Y_X^{An} = 1$), and $\lambda_{An} = 1$:

$$Y_{X/S}^{Met} = \frac{1}{\lambda_{Cat} \cdot Y_S^{Cat} + Y_S^{An}} \tag{14}$$

It should be noted that if S is consumed in the metabolic reaction equation, $Y_{X/S}^{Met}$ has a negative value because the stoichiometric yield values for S are all smaller than 0. This makes perfect sense, because $Y_{X/S}^{Met}$ describes the amount of biomass *formed* per amount of substrate *consumed*; signing in the nominator and denominator is opposite resulting in a negative yield value.

Eq. (14) can be solved to derive the multiplication factor for the catabolic reaction equation (l_{Cat}):

$$\lambda_{Cat} = \frac{\frac{1}{Y_{X/S}^{Met}} - Y_S^{An}}{Y_S^{Cat}} \tag{15}$$

The values for λ_{Cat} and λ_{An} can be implemented in Eq. (12) to obtain the stoichiometry vector of the metabolic reaction equation. An example

BOX 4. Example of metabolic reaction stoichiometry.

As an example for the derivation of a metabolic reaction equation, chemolitoautotrophic growth on anaerobic ammonium oxidation (Anammox) will be considered:

$$Cat: \quad -NH_4^{+1} - NO_2^{-1} + N_2 + 2 \cdot H_2O = 0$$

$$An: \quad -1 \cdot HCO_3^{-1} - 0.2NH_4^{+1} - 2.1 \cdot NO_2^{-1} + 2.1 \cdot NO_3^{-1} - 0.6 \cdot H_2O + CH_{1.8}O_{0.5}N_{0.2} + 0.2 \cdot H^{+1} = 0$$

The reaction stoichiometries demonstrate that the electron donor in the catabolic reaction (NH_4^{+1}) and the anabolic reaction (NO_2^{-1}) are not necessarily the same.
The biomass yield for growth on ammonia has been characterized as follows (Hao et al., 2002; Strous et al., 1998):

$$Y_{X/NH4}^{Met} = -0.1\ g\ dw\ g\,NH_4\text{-}N^{-1}$$

where *dw* stands for dry weight. The unit of the measured biomass yield needs to be converted to a molar value by correcting for the inorganic fraction of biomass (*Asb* ~5% of the dry weight) and by correcting for the molecular weight (*MW*) of biomass and nitrogen:

$$Y_{X/NH4}^{Met}\left[mol\ mol^{-1}\right] = Y_{X/NH4}^{Met}\left[gX\,gN^{-1}\right] \cdot (1 - f_{Asb}) \cdot \frac{MW_N}{MW_X}$$

$$= -0.054\ X\text{-}mol\ NH_4 - mol^{-1}$$

where MW_X and MW_N are the molecular weight of biomass (24.6 g mol^{-1}) and nitrogen (14 g N-mol^{-1}), respectively, and f_{Asb} stands for the fraction of inorganic material in the biomass (~0.05 g g^{-1}). Using Eq. (15), the multiplication factor for the catabolic reaction can be calculated ($\lambda_{Cat} = 18.3$), and the overall metabolic reaction stoichiometry can be derived using Eq. (12):

$$Met = -18.5 \cdot NH_4^{+1} - 20.4 \cdot NO_2^{-1} - 1 \cdot HCO_3^{-1} + 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 18.3 \cdot N_2 + 2.1NO_3^{-1} + 36.0 \cdot H_2O + 0.2 \cdot H^{+}$$

It should be noted that the electron donor to acceptor ratio is strongly different in the catabolic and metabolic reaction (1.0 versus 0.91). Starting from an ammonium-based waste stream, this indicates that the degree of partial NH_4-oxidation cannot be determined straightforwardly from the catabolic reaction equation because both NH_4^{+1} and NO_2^{-1} play a double role in the Anammox metabolism: NH_4^{+1} serves as electron donor in the catabolism and *N* source in the anabolism, whereas NO_2^{-1} is the electron acceptor in the catabolism and electron donor in the anabolism.

derivation of a metabolic reaction equation stoichiometry based on a measured biomass yield is shown in Box 4.

In more general terms, the stoichiometry of the metabolism can be defined based on two (measured) rates of consumption/production of compounds participating in the catabolism and the anabolism, as will be described in the next section.

A More General Solution for Derivation of the Stoichiometry of the Metabolism

After defining the stoichiometry of the catabolism (*Cat*) and anabolism (*An*) of the microbial growth system, the overall stoichiometry of the metabolic system can be derived by the measurement of only two rates of consumption or production of compounds participating in the metabolic system. Determination of the biomass yield, as described in the text, can be regarded as a specific case where the biomass (X) production rate and a substrate (S) consumption rate were determined.

In more general terms, two arbitrary compounds, $S1$ and $S2$, participating in the catabolic and/or anabolic reaction can be chosen. The rates at which these compounds are produced or consumed in the overall system (r_{S1} and r_{S2}) can be described as a function of the catabolic and anabolic reaction:

$$Y_{S1/S2}^{Met} = \frac{r_{S1}}{r_{S2}} = \frac{Y_{S1}^{Cat} \cdot \lambda_{Cat} + Y_{S1}^{An} \cdot \lambda_{An}}{Y_{S2}^{Cat} \cdot \lambda_{Cat} + Y_{S2}^{An} \cdot \lambda_{An}} \tag{16}$$

Using the assumption that the metabolic reaction stoichiometry is expressed per mol biomass formed ($\lambda_{An} = 1$), Eq. (16) can be solved to allow for the calculation of λ_{Cat} as a function of measured rates (r_{S1} and r_{S2}) and specific stoichiometry factors that can be derived from *Cat* and *An*:

$$\lambda_{Cat} = \frac{r_{S2} \cdot Y_{S1}^{An} - r_{S1} \cdot Y_{S2}^{An}}{r_{S1} \cdot Y_{S2}^{Cat} - r_{S2} \cdot Y_{S1}^{Cat}}, \tag{17}$$

or from a measured metabolic yield:

$$\lambda_{Cat} = \frac{Y_{S1}^{An} - Y_{S1/S2}^{Met} \cdot Y_{S2}^{An}}{Y_{S1/S2}^{Met} \cdot Y_{S2}^{Cat} - Y_{S1}^{Cat}} \tag{18}$$

Subsequently, the vector describing the overall stoichiometry of the metabolic system can be calculated:

$$Met = \lambda_{Cat} \cdot Cat + An \tag{19}$$

Due to the assumption that biomass only participates in the anabolic reaction, relatively simple equations have been derived for description of the metabolic rate equation. The importance of proper selection of variables to be measured for definition of the metabolic reaction stoichiometry is demonstrated with an example (Box 5).

BOX 5. Stoichiometry of anaerobic degradation in a Chemostat reactor.

The anaerobic degradation of propionate to a mixture of methane and carbon dioxide will be considered. In nature, this conversion is catalyzed by a mixed syntrophic culture via the intermediates acetate, carbon dioxide, and molecular hydrogen (Schink, 1997; Stams, 1994), but here we will assume direct conversion into a methane and carbon dioxide mixture. In this fermentation reaction, the oxidation of propionate to carbon dioxide is the electron donor reaction (D), and the reduction of propionate to methane is the electron acceptor reaction (A). Because propionate is more reduced than biomass and ammonium is assumed as N source, the anabolism consists of a biomass formation reaction accompanied by propionate reduction to methane. We will consider anaerobic degradation of propionate according to the following catabolic and anabolic reactions:

$$Cat: \quad -1 \cdot C_3H_5O_2^{-1} - 1 \cdot H^{+1} - 0.5 \cdot H_2O + 1.25 \cdot CO_2 + 1.75 \cdot CH_4 = 0$$

$$An: \quad -0.38 \cdot C_3H_5O_2^{-1} - 0.2 \cdot NH_4^{+1} - 0.18 \cdot H^{+1}$$
$$+ 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 0.14 \cdot CH_4 + 0.17 \cdot H_2O + 0.33 \cdot H^{+1} = 0$$

These two stoichiometric equations have been calculated from the half reactions using Eqs. (8) and (11), respectively. By measuring two substrate consumption or production rates, the overall stoichiometry of the conversion can be derived. If, for example, the measured molar ratio of methane and carbon dioxide produced ($Y^{Met}_{CH4/CO2}$) amounts 1.54, λ_{Cat} (Eq. (18)) and the stoichiometry of the metabolic reaction (Eq. (12)) can be calculated:

$$\lambda_{Cat} = 0.8$$

$$Met: -1.18 \cdot C_3H_5O_2^{-1} - 0.2 \cdot NH_4^{+1} - 0.14 \cdot H_2O - 0.98 \cdot H^{+1}$$
$$+ 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 1.00 \cdot CO_2 + 1.54 \cdot CH_4$$

It should be noted that the measured CO_2 produced represents the mass balance value of inorganic carbon produced, including bicarbonate and other species formed. Therefore, the measured $Y^{Met}_{CH4/CO2}$ does not correspond directly to the biogas composition. From the metabolic reaction stoichiometry, the biomass yield on substrate (propionate) can readily be calculated:

$$Y^{Met}_{X/Ed} = \frac{Y^{Met}_{X}}{Y^{Met}_{S}} = -0.841 \; mol \cdot mol^{-1}$$

In principle, the only criterion for the selection of variables to be identified for derivation of the metabolic reaction equation is that one or both have to participate in the catabolic and anabolic reaction equation. Still, accurate identification of dependent variables like the biomass yield from the measured compound concentrations requires careful selection of the proper measurable variables.

The importance of proper selection of suitable variables will be demonstrated by assuming a certain measurement error in one of the variables identified. The measurement of the uptake/production rates of CO_2 or NH_4^{+1} and propionate ($C_3H_5O_2^{-1}$) are assumed as variable measurement to which a measurement error ($\pm 10\%$) is introduced. The CH_4 production rate value is assumed constant, and the stoichiometry

(*Continued*)

BOX 5. (*Continued*)

derived above is used as the base case. The results of this basic sensitivity analysis are shown as the impact of the relative measurement error in CO_2, NH_4^{+1}, and propionate concentrations on the calculated biomass yield on propionate, as shown in Figure 3. It is evident from this example that when measuring CH_4 and CO_2 production in the system, a small measurement error in one of the two variable will lead to a highly erroneous biomass yield calculation. When measuring NH_4^{+1} besides CH_4, the biomass yield value calculated can be accurately predicted, and error propagation is limited. This is due to the fact that ammonium only participates in the anabolic reaction equation, enabling accurate identification of concentration of compounds that participate only in the anabolic reaction equation.

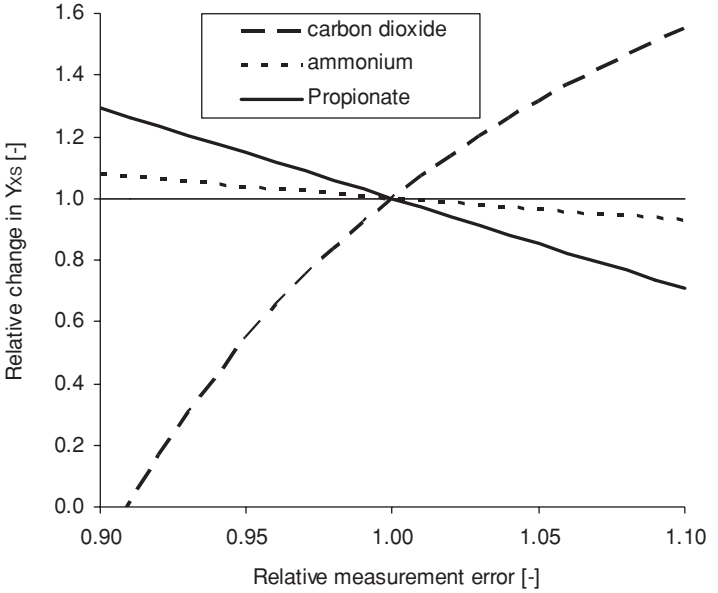

FIGURE 3. Sensitivity of the calculated biomass yield as a function of different measurement errors in a propionate degrading system. In all cases, methane was measured besides the variables indicated.

THERMODYNAMICS OF ECOSYSTEMS

Thermodynamic Basics

Thermodynamic system analysis allows for the identification of reactions that are possible in environmental ecosystems. The environment will select for microorganisms that are capable of catalyzing and growing on these thermodynamically feasible chemical reactions. Microbial activity can only be maintained as long as thermodynamic non-equilibrium conditions are

maintained. To achieve this, a regular flow of matter across the boundary of the system needs to be established.

In standard conditions, the Gibbs energy change of a chemical reaction (ΔG_R^1) describes the effects of enthalpy (ΔH_R^0) and entropy (ΔS_R^0) changes in the system:

$$\Delta G_R^0 = \Delta H_R^0 - T \cdot \Delta S^0 \tag{20}$$

The traditionally applied thermodynamic reference state of any compound is the elemental state ($\gamma = 0$) and standard phase. This means that the values for Gf^0 and Hf^0 in the standard state equal 0. The standard phase of the elemental state of the biologically important elements with a variable oxidation state is gaseous (N_2, O_2) at a pressure of 1 atm or solid (*Fe*, *Mn*, *S*, *P*, *C*). Table A3 identifies the reference compounds for *C*, *N*, *S*, *Fe*, and *Mn* compounds.

The standard state for an aqueous solution of a dissolved solute is defined for a hypothetical ideal solution containing 1 mol dm^{-3} of solute. The standard value is extrapolated to infinite dilution (ionic strength I = 0) to correct for thermodynamic effects of dilution. Herewith, the contradicting definition of the standard state of a dissolved solute is based on a concentration of 1 molal at infinite dilution.

The standard Gibbs energy change (ΔG^0) of an arbitrary reaction can be calculated from the Gf^0 values of the compounds participating in the reaction and the stoichiometry of the reaction. The calculation is fully equivalent to the previously presented calculation of mass and electron balances from chemical reactions:

$$\Delta G_R^0 = \sum_{i=1}^{n} Y_i^R \cdot Gf_i^0 \tag{21}$$

As described for the mass balances, the Gibbs energy balance can conveniently be derived by identifying the reactants involved in the reaction in a vector. Subsequent vector notation for the stoichiometric coefficients and the Gf^0 values allows for direct derivation of ΔG_R^0 using Eq. (21). An extensive list of Gf^0 and Hf^0 values of environmentally important species can be found in Table A3.

Non-Ideality Corrections

The standard Gibbs energy change of a reaction is defined at the activities of dissolved substrates of 1 molar and of gaseous substrates of 1 atmosphere. For most environmental ecosystems, the standard state is refined to compensate for the environmentally more realistic proton activity (pH) of 1E-7

molar:

$$\Delta G_R^{01} = \Delta G_R^0 + R \cdot T \cdot Y_{H+}^R \cdot \ln\left(1 \cdot 10^{-7}\right) \tag{22}$$

The actual Gibbs energy change (ΔG^1) of a reaction is defined as the driving force at the actual environmental activities of substrates and products. For the reaction:

$$S1 + S2 \longrightarrow P1 + P2 \tag{23}$$

the actual Gibbs energy change is typically written as:

$$\Delta G_R^1 = \Delta G_R^0 + R \cdot T \cdot \ln \frac{a_{P1}^{Y_{P1}^R} \cdot a_{P2}^{Y_{P2}^R}}{a_{S1}^{Y_{S1}^R} \cdot a_{S2}^{Y_{S2}^R}} \tag{24}$$

For calculation of the ΔG^1 based on a vector with substrate activities and a vector describing the stoichiometry of the reaction, the following equivalent equation should be applied:

$$\Delta G_R^1 = \Delta G_R^0 + R \cdot T \cdot \sum_{i=1}^{n} Y_{Si}^R \cdot \ln\left(a_{Si}\right) \tag{25}$$

The sign of the ΔG^1 value determines the direction in which chemical reactions may occur. A negative value indicates that the reaction is thermodynamically favorable, whereas a positive value for ΔG^1 indicates that the reaction cannot occur without external energy input. A ΔG^1 value of exactly 0 suggests that the reaction is in thermodynamic equilibrium. Reactions with a negative Gibbs energy change are named exergonic, and those with a positive Gibbs energy change are named endergonic. For the purposes described here, the calculation of ΔG^{01} values for chemical redox reactions is generally sufficiently accurate for thermodynamic system analysis. In fermentation systems, redox systems that consist of a strong electron donor and a weak acceptor, or vice versa, the ΔG^{01} generally is close to equilibrium. Only in these cases, the calculation of ΔG^1 will be required to get a proper thermodynamic system analysis. Even though the absolute change in ΔG^1 as a result of changes in the actual activities of the participating species is the independent of the value for ΔG^{01}, the relative change will be much bigger when the ΔG^{01} is close to terminal equilibrium. In this paper, we will use the formulation ΔG when it depends on the actual system at hand and the environmental conditions if it is required to calculate accurate ΔG^1 values or that ΔG^{01} values are adequate.

Enthalpy changes (ΔH^1 values) are calculated in a fully equivalent manner to Gibbs energy changes. A negative value for ΔH^1 indicates that heat

is released (i.e., the reaction is exothermic). A positive value indicates that heat is taken up and the reaction is endothermic.

In the first section, we have defined that the stoichiometries of substrates and products correspond to mass balance values. For thermodynamic calculations, the stoichiometric coefficients correspond to the actual activities of the species in the reaction. The Gibbs energy of formation of the individual compounds depends on the form in which they can be found, as shown below:

$$C_{+IV} : CO_2(g),\ CO_2,\ H_2CO_3,\ HCO_3^{-1},\ CO_3^{-2}$$
$$P_{+V} : PO_4^{-3}(g),\ HPO_4^{-2},\ H_2PO_4^{-1},\ H_3PO_4$$
$$Acetate : C_2H_3O_2^{-1},\ C_2H_4O_2$$

If no actual Gibbs energy changes are calculated, the reaction equation should be represented using the dominant species in the given conditions. Herewith, the error made by the calculation of the Gibbs energy change under standard conditions is minimized. An example that demonstrates the differences in is shown in Box 6.

Correction for Salt Concentration

In dilute aqueous systems, the actual activities of dissolved species are equal to the molar concentrations, the gaseous species are represented by the partial pressure expressed as atmosphere, and water and solids have an

BOX 6. Gibbs energy change dependency on actual activities.

This example demonstrates that the Gibbs energy change depends on the actual activities of the species involved in the reaction. Table 3 shows the stoichiometry of acetate fermentation to a mixture of methane and carbon dioxide. This reaction can be written in several ways, involving protonated or ionic reactants and gaseous or aqueous compounds (*R*1 and *R*2). Table 3 shows that *R*1 and *R*2 are equivalent from a mass balance point of view except for the consumption of water in *R*2. However, in diluted systems, consumption and production of water are generally neglected. From a thermodynamic point of view, R1 and R2 are different, as can be seen from the different ΔG^0- and ΔG^{01} values.

When we calculate the ΔG^1, however, the values of both reactions are equal, as long as we assume a thermodynamic equilibrium between the equivalent compounds in Table 3: acetate and acetic acid, and carbon dioxide and bicarbonate. In Table 3, the assumption of thermodynamic equilibrium was implemented by calculating the concentration (activity) acetic acid from acetate:

$$C_2H_4O_2 \rightarrow C_2H_3O_2^{-1} + H^{+1},$$
$$\Delta G^0 = +27.2\ kJ \cdot reaction^{-1}$$

(*Continued*)

BOX 6. *(Continued)*

Thermodynamic equilibrium is now defined as the concentration op substrates and products where $\Delta G^1 = 0$. The actual concentration acetic acid can be calculated from the given concentration of acetate using (from Eq. (24)):

$$Ka = \exp\left(-\Delta G^0/_{R \cdot T}\right) = \frac{C_2H_3O_2^{-1} \cdot H^{+1}}{C_2H_4O_2}$$

In exactly the same way, the bicarbonate concentration in Table 3 was calculated from the given partial pressure carbon dioxide. By definition, this should result in the same values for ΔG^1 for $R1$ and $R2$.

It should be noted that from a mass balance point of view, neither the use of acetate or acetic acid is fully correct. The mass balance value for acetate equals the sum of both concentrations, but can in this case be approximated by the concentration of acetate, because acetic acid is a negligible fraction (see Table 3). At the given pH value of 7 in this example, the CO_2 partial pressure and the concentration of bicarbonate are comparable, suggesting that the calculated ΔG^1 value will be independent of the inorganic carbon specie defined at around a neutral pH.

Evidently, this implies that the species used in stoichiometric equations for calculation of the widely applied ΔG^{01} value of a reaction should be based on the specie for which activity is closest to the reference activity (1 mol dm^{-3} for aqueous species, 1 atm for gases). In the example in Table 3, the use of acetate and carbon dioxide as in $R1$ is preferred, as can be seen from the ΔG^{01} value for $R1$, which is significantly closer to ΔG^1 than the value for $R2$.

Table 3. Thermodynamic properties of acetate fermentation to a methane and inorganic carbon mixture according to two different stoichiometries (R1 and R2)

Name	Structural f.	G_f^0	a	R1	R2
Acetate	$C_2H_3O_2^{-1}$	−369.4	1.0E-03	−1.0	0.0
Acetic acid	$C_2H_4O_2$	−396.6	5.8E-06	0.0	−1.0
Methane	$CH_4(g)$	−50.8	7.0E-01	1.0	1.0
Carbon dioxide	$CO_2(g)$	−394.4	3.0E-01	1.0	0.0
Bicarbonate	HCO_3^{-1}	−586.9	4.4E-02	0.0	1.0
Water	H_2O	−237.2	1.0E+00	0.0	−1.0
Proton	H^{+1}	0.0	1.0E-07	−1.0	1.0
ΔG^0	kJ reaction^{-1}			−75.7	−3.8
ΔG^{01}	kJ reaction^{-1}			−35.8	−43.8
ΔG^1	kJ reaction^{-1}			−22.5	−22.5

The concentrations bicarbonate and acetic acid have been calculated using thermodynamic equilibrium equations described in the text.

activity of one. The implementation of activity correction factors to convert molar concentrations to dimensionless activities is generally only required at ionic strength values higher than 0.01. Correlations as established by Debeye-Huckel and Davis are available to calculate the activity correction factors as a function of the ionic strength of the solution and the charge of the molecule (Stumm and Morgan, 1996).

Temperature Correction

In addition to the compensation of the actual Gibbs energy change of a reaction for the actual activities of the compounds involved in the conversion, correction can be established for the actual temperature as well. The standard Gibbs energy change of a reaction has been defined at a temperature of 298.15 K (T_S). The calculation of the Gibbs energy change at a temperature different (T) from the standard temperature can be done using the Gibbs-Helmholz equation:

$$\Delta G^0_T = \Delta G^0_{T_S} \cdot \frac{T}{T_S} + \Delta H^0_{T_S} \cdot \frac{T_S - T}{T_S} \tag{26}$$

An example demonstrating the influence of the temperature on the actual Gibbs energy change is given in Box 7.

Strong and Weak Electron Donors and Acceptors

Calculation of the Gibbs energy change per electron allows for analysis of the driving force of a compound to donate or accept electrons. To establish such an analysis, an inventory was made of the average Gibbs energy change per electron for compounds with a variable oxidation state, as shown in Table 2 and Figure 5. The values were calculated based on full oxidation to the most oxidized state of all compounds identified in Table A3. For ammonium oxidation, for example, the Gibbs energy change was calculated from the following reaction equation:

$$-0.125 \cdot NH_4^{+1} - 0.375 \cdot H_2O + 0.125 \cdot NO_3^{-1} + 1.25 \cdot H^{+1} + 1 \cdot e^{-1} = 0, \tag{27}$$

Values for atoms that are found in numerous oxidation states (C, S, N) were averaged, and the standard deviation is indicated in Figure 5. For the other compounds, values were calculated from the main oxidation state transformation that occurs in nature.

The negative ΔG^{01} values found for the organic compounds, sulfur compounds, and molecular hydrogen (H_2) indicate that these compounds can generally be regarded as *strong* electron donors. The negative ΔG^{01} values furthermore suggest that the reverse reactions are thermodynamically unfavorable, and that $C-$, $S-$, and H-based compounds are *weak* electron acceptors. The opposite is true for N, Fe, O_2, and Mn compounds: they can be regarded as *strong* electron acceptors but *weak* electron donors.

Even though the standard deviations in the Gibbs energy content per electron can be rather big, some general conclusions can be drawn from Figure 5. By definition, combination of a strong electron donor and a strong electron acceptor reaction will always result in a thermodynamically favorable reaction, whereas a weak electron donor and weak electron acceptor

BOX 7. Influence of the temperature on ΔG^1.

For demonstration of the influence of the temperature on ΔG^1, the reduction of bicarbonate with molecular hydrogen (homoacetogenesis) is considered:

$$Cat: \; -1 \cdot H_2 - 0.5 \cdot HCO_3^{-1} - 0.25 \cdot H^{+1} + 0.25 \cdot C_2H_3O_2^{-1} + 1 \cdot H_2O$$

$$\Delta G^0 = -36.1 \; kJ \cdot reaction^{-1},$$

$$\Delta G^{01} = -26.1 \; kJ \cdot reaction^{-1}$$

In methanogenic environments, the actual concentrations of the different compounds participating in the reaction are as follows:

H_2 1.10^{-4} Atm

HCO_3^{-1} 0.05 M

$C_2H_3O_2^{-1}$ 0.001 M

resulting in the following actual Gibbs energy change at 298 K:

$$\Delta G^1 = -3.9 \; kJ \cdot reaction^{-1}$$

The ΔG^1 values as a function of the temperature can now be calculated using Eq. (26). The resulting Figure 4 clearly demonstrates that the *DG* values depend strongly on the actual temperature. Whereas homoacetogenesis is an energetically favorable process at temperatures lower than 318 K, the reverse reaction is favorable at higher temperatures. Due to the proximity to thermodynamic equilibrium of the reaction catalyzed, either the forward or backward reaction may occur depending on the actual activities of the species involved and the actual temperature. It has even been demonstrated that one type of microorganism is capable of both catalyzing the forward and backward reaction, demonstrating the fully reversible nature of the catabolic enzyme system (Hattori et al., 2000; Schnurer et al., 1997).

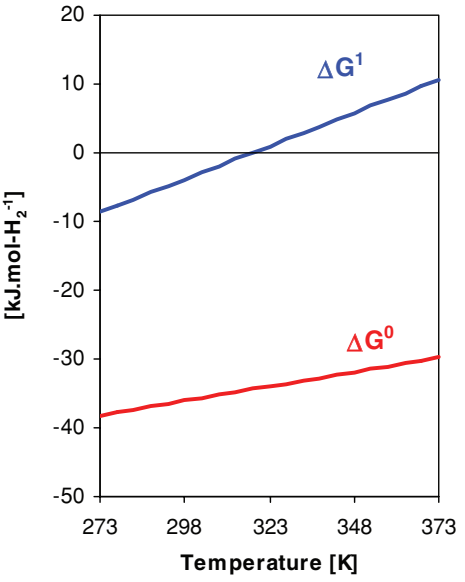

FIGURE 4. Temperature dependency of the standard and actual Gibbs energy change of anaerobic bicarbonate reduction with molecular hydrogen to acetate.

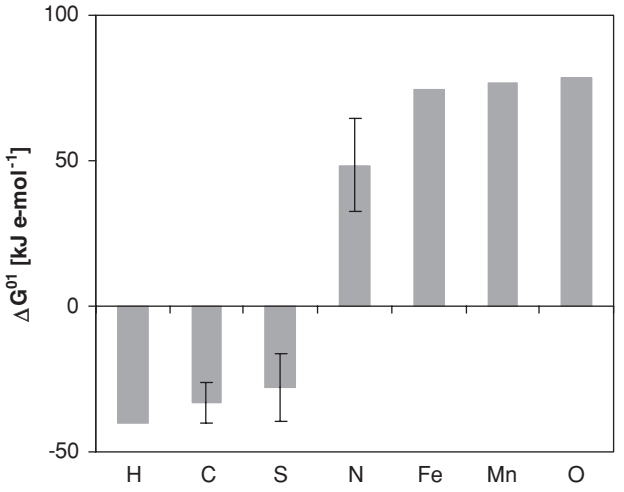

FIGURE 5. Average Gibbs energy change per electron for full oxidation of the central atom of the compounds in Table A3.

will result in a thermodynamically unfavorable reaction. The combination of a strong electron donor and a weak electron acceptor or vice versa can either be favorable or unfavorable depending on the actual compounds and the environmental conditions.

With regard to the standard deviation values in the $C-$, $N-$, and S-based compounds, some remarks need to made:

- The standard deviation found for the S-species is primarily the result of two species that have a significantly higher Gibbs energy content per electron upon oxidation to sulphate: sulphite (-50.4 kJ e-mol^{-1}) and trithionate (-41.5 kJ e-mol^{-1}), whereas the other reduced sulphur species generally release about -20 kJ e-mol^{-1}.
- The main N compound with a distinctly different Gibbs energy content per electron (ΔG_e^{01}) is dinitrogen gas (N_2). Atmospheric nitrogen is the dominant form in the environment and is the thermodynamically most stable form of nitrogen. Gibbs energy is required to convert N_2 to the biologically most important oxidation state of *–III* as in ammonium (NH_4^{+1}) and amino acids.
- Fermentation of organic compounds depends fully on the standard deviation in the amount of Gibbs energy available per electron upon full oxidation, as shown in Figure 5. The environmentally important fermentation of glucose to methane and carbon dioxide via acetate according to:

$$C_6H_{12}O_6 \rightarrow 3 \cdot C_2H_4O_2 \rightarrow 3 \cdot CH_4 + 3 \cdot CO_2$$

is thermodynamically favorable because the ΔG^{01} values of the organic compounds decrease in the reaction sequence according to:

$$-40.9 \rightarrow -28.1 \rightarrow -23.6\ kJ\ e-mol^{-1}$$

THERMODYNAMICS OF GROWTH

Introduction

Based on the simplified network shown in Figure 1, several researchers have formulated methods for bioenergetic coupling of catabolism and anabolism for the description of microbial growth. All methods are based on the fundamental idea that a certain amount of energy is required for biomass production, and this energy should be generated in the energetically favorable catabolic reaction. Three main methods will be described briefly here:

1) *ATP*-balancing method based on generalized biochemical considerations;
2) the Gibbs energy dissipation method; and
3) the thermodynamic Electron Equivalent Method (*TEEM*).

To implement the different biomass yield estimation methods, only the stoichiometry and the (actual) Gibbs energy changes of both the catabolic and anabolic reaction need to be established first according the methods described above. Both catabolism and anabolism are assumed to be represented as vector *Cat* and *An,* respectively.

After a brief description of the three yield estimation methods, the different methods are compared and discussed, and possible improvements are presented. The main objective of this section, however, is to demonstrate how easily the biomass yield estimation concepts can be implemented in the vector-based calculation procedure developed.

The *ATP*-Balancing Method

According to the scheme for microbial growth shown in Figure 1, microbial metabolism can be described as a balance of catabolism and anabolism coupled by the cofactor *ATP/ADP*. In the thermodynamically favorable catabolic redox reaction, substrate (*S*) conversion is accompanied by energy conservation by formation of *ATP* from *ADP* and inorganic phosphate (*Pi*):

$$ADP + Pi \rightarrow ATP : \Delta G^{01} = 32.6\ kJ\ reaction^{-1}$$

The thermodynamically unfavorable *ATP* production reaction is driven by the favorable catabolic redox reaction. *ATP* production can either be directly coupled to a redox reaction by substrate level phosphorylation, or

indirectly by utilization of the electrochemical gradient across the microbial membrane or by oxidative phosphorylation related to oxidation of electron carriers like *NADH*.

In the anabolic reactions, a carbon source (S_X) and a nitrogen source (N_X) are converted to biomass (X) at the expense of *ATP*. Stouthamer (1979) made a theoretical inventory of the *ATP*-based energy requirements for the anabolic reaction system. Based on generalized microbial principles, the *ATP* requirements for synthesis of the different cell components from different carbon was estimated. Additional growth-related energy sinks were furthermore identified, such as the transport of specific nutrients and the turnover of mRNA and the energy requirements for conversion of nitrogen to the ammonium form required for incorporation in biomass components. When the full *ATP*-based energy requirement is established, the *ATP*-consumption per C-mol of biomass produced is known.

Knowing the stoichiometry of *ATP* production in the catabolic reaction per substrate (S) converted ($Y^{Cat}_{ATP/S}$), plus the stoichiometry of *ATP* consumption per C-mol biomass formed ($Y^{An}_{ATP/X}$), the stoichiometry of the net metabolic reaction equation can be derived. Assuming cellular steady state, there is no net *ATP* production or consumption, and consequently:

$$\lambda_{Cat} \cdot Y^{Cat}_{ATP} + \lambda_{An} \cdot Y^{An}_{ATP} = 0$$

Taking into account that we want to derive the metabolic reaction equation per mol biomass formed, the stoichiometry of the metabolic rate equation equals:

$$Met = An - \frac{Y^{An}_{Atp}}{Y^{Cat}_{ATP}} \cdot Cat \tag{28}$$

and the biomass yield can be calculated according to:

$$Y^{Met}_{X/S} = \frac{1}{Y^{An}_{S} - Y^{Cat}_{S} \cdot \frac{Y^{An}_{ATP}}{Y^{Cat}_{ATP}}} \tag{29}$$

By balancing *ATP* production in the catabolism and *ATP* consumption in the anabolism, the stoichiometry of the overall metabolism is defined. An example demonstrating this approach based on glucose fermentation is described in Box 8.

As there is assumed to be a flux through the metabolic network shown in Figure 1, all catabolic and anabolic reactions catalyzed have to be exergonic ($\Delta G^1 < 0$), and, consequently, the overall metabolic equation needs to be exergonic as well. Whether this boundary condition is fulfilled can readily be calculated by calculating the Gibbs energy change of the metabolic reaction ($\Delta G^{)}_{Met}$. The calculated value for the Gibbs energy change of the metabolic

BOX 8. Derivation of metabolic equation for glucose fermentation.

During glucose fermentation to ethanol, two *ATP* is produced per glucose converted in the catabolic reaction equation:

$$Cat: \ -1 \cdot C_6H_{12}O_6 - 2 \cdot ADP - 2 \cdot Pi - 2 \cdot H_2O + 2 \cdot C_2H_5OH + 2 \cdot ATP + 2 \cdot HCO_3^{-1} + 2 \cdot H^{+1}$$

The *ATP* requirement for biomass formation on different carbon sources has been elaborated extensively by Stouthamer (1979). For growth on glucose in a minimal medium, this author proposed a $Y_{ATP/X}$ of -1.26 mol *ATP* mol X^{-1}:

$$An: \ -0.175 \cdot C_6H_{12}O_6 - 0.2 \cdot NH_4^{+1} - 1.243 \cdot ATP + 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 0.05 \cdot HCO_3^{-1} + 1.243 \cdot ADP + 1.243 \cdot Pi + 0.4 \cdot H_2O + 0.25 \cdot H^{+1}$$

A minor fraction of the glucose is oxidized to inorganic carbon in order to generate the reduction equivalents required to drive the reduction of glucose to biomass. From *Cat* and *An*, *Met* can be established using Eq. (28):

$$Met: \ -0.797 \cdot C_6H_{12}O_6 - 0.2 \cdot NH_4^{+1} - 0.843 \cdot H_2O + 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 1.243 \cdot C_2H_5OH + 1.293 \cdot HCO_3^{-1} + 1.493 \cdot H^{+1}$$

and the biomass yield ($Y_{X/Glu}^{Met}$) equals -1.26 mol $\times$ mol Glu^{-1}.
To check whether the metabolic reaction equation derived obeys the second law of thermodynamics, the standard Gibbs energy change of the reaction can be calculated from the Gibbs energies of formation of the individual components as outlined above. After correction for a pH value of 7, as typically encountered in biological systems, the Gibbs energy change equals:

$$\Delta G_{Met}^{01} = -163.1 \ kJ \ mol - X^{-1}$$

Evidently, the reaction equation derived obeys thermodynamic laws, and the Gibbs energy change value found can be regarded as the amount of energy dissipated per mol biomass formed.

reaction equation actually equals the Gibbs energy dissipated per C-mol biomass formed, which is the basis of the yield estimation method elaborated by Heijnen.

The Gibbs Energy Dissipation Method

Bioenergetic coupling of catabolism and anabolism can be elaborated in terms of *ATP* production and consumption, as described in previous the paragraph. Taking into account that *ATP* production and consumption are equivalent to Gibbs energy consumption and production, a fully equivalent thermodynamic description of microbial growth based on Gibbs energy balancing can be elaborated. First, a theoretical maximum for the biomass yield can be defined based on the assumption that all Gibbs energy generated in

the catabolic reaction is conserved for biomass production in the metabolic reaction:

$$\Delta G_{Met} = \lambda_{Cat} \cdot \Delta G_{Cat} + \lambda_{An} \cdot \Delta G_{An} = 0 \tag{30}$$

Knowing the Gibbs energy change of the catabolic and anabolic reaction, and by definition that ΔG_{Met} is calculated per C-mol biomass formed ($\lambda_{An} = 1$), the multiplication factor for the catabolic reaction (λ_{Cat}) can be calculated. Knowing λ_{Cat}, the biomass yield can be calculated using Eq. (14).

However, the theoretical maximum biomass yield has no practical significance as it assumes thermodynamic equilibrium. This approach is irrelevant for open systems that exchange material with their environment like actively growing microorganisms. Furthermore, numerous bioenergetic inefficiencies in both the catabolic and anabolic pathways imply that significant amounts of Gibbs energy are dissipated during microbial growth.

The Gibbs energy dissipation concept as proposed by Heijnen (Heijnen and Vandijken, 1993; Heijnen et al., 1992) is based on the hypothesis that microbial growth can be characterized in terms of the Gibbs energy dissipated per C-mol biomass formed (ΔG_{Dis}). Given the definition of the metabolic reaction equation (Eq. (12)) and taking into account that Gibbs energy balances can be written analogously to mass balances, the Gibbs energy balance for a metabolic system becomes:

$$\lambda_{Cat} \cdot \Delta G_{Cat} + \lambda_{An} \cdot (\Delta G_{An} + \Delta G_{Dis}) = 0 \tag{31}$$

This equation can be solved to for λ_{Cat} by taking into account that λ_{An} equals one by definition:

$$\lambda_{Cat} = \frac{\Delta G_{An} + \Delta G_{Dis}}{-\Delta G_{Cat}} \tag{32}$$

This equation reflects the number of times the catabolic reaction needs to run (λ_{Cat}) to generate the Gibbs energy required to form one C-mol biomass in the anabolic reaction.

Solving this equation for ΔG_{Dis} and combining with Eq. (14) give an equation that enables the calculation of the Gibbs energy dissipation as a function of a measured or otherwise identified biomass yield for any given metabolic system:

$$\Delta G_{Dis} = -\Delta G_{Cat} \cdot \frac{\frac{1}{Y_{X/S}^{Met}} - Y_S^{An}}{Y_S^{Cat}} - \Delta G_{An} = 0 \tag{33}$$

The authors that proposed this concept made an extensive inventory of ΔG_{Dis} data using data from aerobic, denitrifying, sulphate reducing, fermenting, and methanogenic microbial growth systems. They concluded from

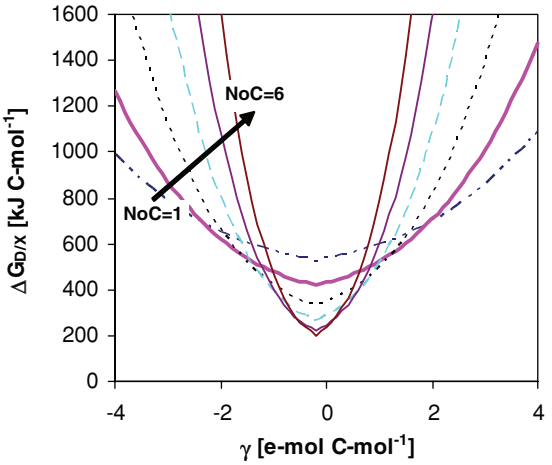

FIGURE 6. Estimated Gibbs energy dissipation for the formation of 1 C-mol biomass as calculated using Eq. (34) from carbon sources with γ values ranging from -4 to 4 e-mol C-mol^{-1} and a carbon chain length (NoC) ranging from 1 to 6.

the data obtained that in a metabolic growth system, the amount of Gibbs energy dissipated per C-mol biomass formed (ΔG_{Dis}) depends only on the carbon source utilized for growth. The following equation was derived that allows for the estimation of ΔG_{Dis} for microbial growth on an organic carbon source (heterotrophic growth):

$$\Delta G_{Dis} = 200 + 18(6 - NoC)^{1.8} + \exp\left[\{(-0.2 - \gamma)^2\}^{0.16} \cdot (3.6 + 0.4 \cdot NoC)\right] \quad (34)$$

where γ and NoC stand for the oxidation state and the carbon chain length of the carbon source. The value for γ uses elemental carbon as reference oxidation state, as shown in Table 2, and can vary from -4 (CH_4) to $+4$ (CO_2). Therefore, Eq. (34) is slightly different from the equation proposed originally by Heijnen and Van Dijken, who defined γ as the number of electrons per C-mol carbon source that are liberated upon full oxidation to carbon dioxide. The method has been calibrated for NoC values up to 6 (glucose). The unit of ΔG_{Dis} is $kJ\ C\ mol^{-1}$, and the equation was found to allow for estimation of heterotrophic biomass yield values with an average estimation error of no more than 13%. The dependency of the ΔG_{Dis} values as a function of the degree of reduction of the carbon source and the carbon chain length according to Eq. (34) is shown in Figure 6.

Eq. (34) suggests that the minimum value for ΔG_{Dis} is established with glucose as carbon source ($\Delta G_{Dis} = 236\ kJ\ C\ mol^{-1}$), which is close to the value calculated with the *ATP*-based method described in Box 8.

For autotrophic growth with carbon dioxide as carbon source, the ΔG_{Dis} value depends on the electron donor utilized in the anabolic reaction equation. If the ΔG_{An} value is negative, Eq. (34) can be utilized. A positive ΔG_{An} suggests that the electrons donated by the electron donor do not have sufficient energy to drive the formation of the building blocks for biomass formation from inorganic carbon. In this case, membrane gradients need to be utilized to increase the energy content of the electrons donated and to drive the formation of central biomass building blocks. This energetically expensive mechanism of reversed electron transfer (*RET*) results in a significant increase of ΔG_{Dis}:

$$\Delta G_{Dis}(CO_2) = 986\ kJ\ C\ mol^{-1}\ (-RET) \tag{35}$$

$$\Delta G_{Dis}(CO_2) = 3500\ kJ\ C\ mol^{-1}\ (+RET) \tag{36}$$

From Figure 5, it can be derived that the *strong* electron donors (H_2, organic C, reduced S compounds) generally require no *RET*, whereas *weak* inorganic $N-$ and *Fe*-based electron donors do require reversed electron transfer for inorganic carbon reduction.

Estimation of the biomass yield value based on the ΔG_{Dis} value and the catabolic and anabolic reaction stoichiometry can be established by rewriting Eq. (33):

$$Y_{X/S}^{Met} = \frac{1}{Y_S^{An} + \lambda_{Cat} \cdot Y_S^{Cat}} \tag{37}$$

The stoichiometry of the metabolic reaction equation can subsequently be calculated using Eq. (12).

The Thermodynamic Electron Equivalents Model (TEEM)

Already in the early 1960s, McCarty developed a method that enabled estimation of biomass yields based on thermodynamic grounds (McCarty, 1965, 1972). Recently, the same author described an update of this method (McCarty, 2007). Based on catabolic and anabolic Gibbs energy balancing and an energetic efficiency assumption, auto- and heterotrophic growth yields can be estimated.

McCarty derived the biomass yield estimation method based on a representation of the catabolic and anabolic reaction in electron equivalent-based units. Here, we have converted this approach to a mol-based method for easy comparison with yield estimation methods described above. The anabolic reaction system as schematically shown in Figure 7 consists of three reactions. First, a carbon source is assumed to be converted to an intermediate compound that serves as building block for the different organic cell components. McCarty assumed this compound to be pyruvate, but for sake

of comparability with the previously described methods, we use an imaginary biomass building block with the same elemental composition as used in the dissipation method: $CH_{1.8}O_{0.5}N_{0.2}$. Thermodynamically, both approaches are largely equivalent because the Gibbs energy content per electron of biomass and pyruvate upon oxidation to inorganic carbon (and ammonium) are almost equal (−34.2 and −33.9 kJ e-mol^{-1}, respectively). Both the stoichiometry and the Gibbs energy change of this step (C and ΔG_C in Figure 7) herewith are the same as proposed in the Gibbs energy dissipation method described before. In comparison with the original method described by McCarty, we have made a principal modification. McCarty assumes that the stoichiometry and Gibbs energy of the anabolic reaction are independent of the electron acceptor utilized. For carbon sources that are more reduced than biomass (or pyruvate in the original TEEM method), inorganic carbon is assumed as an electron acceptor in the anabolic reaction. We propose that when a carbon source is more reduced than biomass, it is more realistic to consider the electron acceptor of the catabolic reaction as electron acceptor in the anabolic reaction, according to Figure 7 and Eq. (11). An example that illustrates the difference between both approaches is shown in Box 9.

The second step in the TEEM procedure concerns the conversion of the inorganic nitrogen source to ammonium, which has the same oxidation state as nitrogen in cellular material. Because ammonium is the most reduced state of nitrogen, this conversion requires an external electron donor for all nitrogen sources except ammonium. The bioenergetic implications of this process, referred to as *assimilative denitrification* (ΔG_N), can readily be included in the stoichiometry and Gibbs energy change of the anabolism (An and ΔG_{An}).

The third step in the anabolic reaction scheme is the conversion of the biomass building block to the different biomass components under expense of Gibbs energy (ΔG_{Syn}). McCarty proposed a constant value of 132 kJ mol X^{-1} for this term (McCarty, 1972).

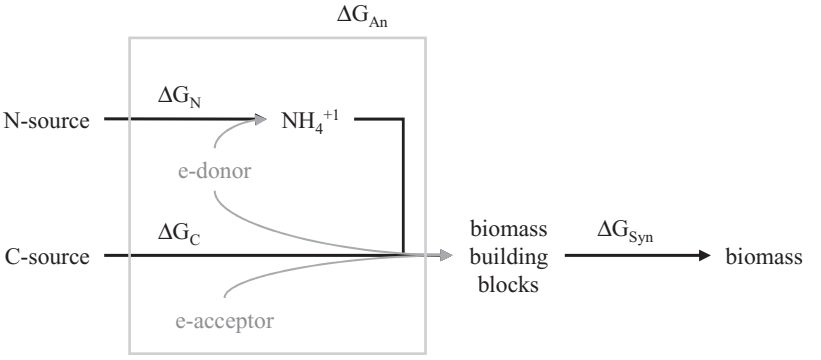

FIGURE 7. Schematic representation of the anabolism as proposed by McCarty.

BOX 9. Influence of the anabolic reaction definition on the TEEM method.

In this example, the impact of the way the anabolic reaction is defined on the outcome of the estimated biomass yield value according to the TEEM method is described based on the example of aerobic ethanol oxidation. Electron donor and acceptor in the catabolic reaction are ethanol (*EOH*) and oxygen (O_2), respectively:

$$Cat: \ -1 \cdot C_2H_5OH - 3 \cdot O_2 + 2 \cdot HCO_3^{-1} + 1 \cdot H_2O + 2 \cdot H^{+1} = 0,$$
$$\Delta G^{01} = -1309.1 \ kJ \ reaction^{-1}$$

Realizing that the degree of reduction of the carbon source ($\gamma_{EOH} = -2$ *e*-mol mol^{-1}) is bigger than biomass ($\gamma_X = -0.2$ *e*-mol mol^{-1}), the anabolic reaction should be defined according to Eq. (11) using oxygen as electron acceptor and becomes:

$$An: \ -0.5 \cdot C_2H_5OH - 1.3 \cdot O_2 - 0.2 \cdot NH_4^{+1} + 1 \cdot CH_{1.8}O_{0.5}N_{0.2}$$
$$+ 0.9 \cdot H_2O + 0.2 \cdot H^{+1} = 0,$$
$$\Delta G^{01} = -181.7 \ kJ \ reaction^{-1}$$

According to McCarty, the anabolic reaction equation is written with bicarbonate as electron acceptor:

$$An_{McC}: \ -0.35 \cdot C_2H_5OH - 0.3 \cdot HCO_3^{-1} - 0.2 \cdot NH_4^{+1} - 0.1 \cdot H^{+1},$$
$$+ 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + 0.75 \cdot H_2O = 0,$$
$$\Delta G^{01} = +14.7 \ kJ \ reaction^{-1}$$

Opposed to the weak electron acceptor bicarbonate, oxygen is a strong electron acceptor, and the formation of biomass building blocks from a reduced carbon source by partial oxidation with molecular oxygen is an exergonic process. Biomass building block production by bicarbonate reduction is energetically unfavorable. In the yield estimation method derived by McCarty, this results in a different value for parameter *m*: with oxygen, $m = +1$, and with bicarbonate $m = -1$. This different sign for *m* results in different values for the biomass yield that are calculated (assuming $\Delta G_{Syn} = 200 \ kJ \ molX^{-1}$, $\eta = 0.43$, $Y_{X/EOH} = -1.40 \ molX \ mol^{-1}$ with O_2, and $Y_{X/EOH} = -1.31 \ molX \ mol^{-1}$ with HCO_3^{-1}).

It is very unlikely that electrons generated upon carbon source oxidation to the level of biomass will be used for bicarbonate reduction. In presence of a strong electron acceptor like molecular oxygen, the oxidation of the carbon source to the oxidation state of biomass will normally be associated with metabolic energy production and the reduction of oxygen. Consequently, the first way to write the anabolic reaction equation is preferred.

It should be noted that both ways of writing the anabolic reaction result in the same metabolic reaction equation and biomass yield values when using the dissipation method. The dissipation method is fully linear, suggesting that the outcome of the calculations is independent of the way the individual reactions are written, as long as the stoichiometry of the overall metabolic reaction equation remains the same.

In order to derive an overall bioenergetic balance of the metabolic system, McCarty assumed that a constant fraction (η) of energy is dissipated in all enzymatic steps of the process. Gibbs energy dissipation implies that thermodynamically favorable reactions are closer to thermodynamic equilibrium (ΔG closer to zero), whereas unfavorable reactions require more investment of energy and are consequently further from equilibrium. McCarty implemented this assumption by using a powered efficiency expression: $\Delta G \cdot \eta^m$. The power factor (m) of the efficiency parameter (η) is equal to $m = +1$ if it concerns a thermodynamically favorable reaction and $m = -1$ in case of a positive Gibbs energy change.

Now the overall Gibbs energy balance for microbial metabolism can be established:

$$\lambda_{Cat} \cdot \Delta G_{Cat} \cdot \eta + \lambda_{An} \cdot \left(\Delta G_C \cdot \eta^m + \Delta G_N \cdot \eta^n + \Delta G_{Syn}\right) = 0 \tag{38}$$

where m and n either have the value of $+1$ or -1 depending on the values for ΔG_C and ΔG_N respectively. As before, we can express the metabolic equation per mol biomass formed, resulting in the following equation that allows for calculation of λ_{Cat}:

$$\lambda_{Cat} = \frac{\Delta G_C \cdot \eta^m + \Delta G_N \cdot \eta^n + \Delta G_{Syn}}{-\Delta G_{Cat} \cdot \eta} \tag{39}$$

The biomass yield can now be calculated from λ_{Cat} using Eq. (37), and the overall stoichiometry of the metabolic equation can be derived using Eq. (12).

Comparing the Yield Estimation Methods

The main advantage of the *ATP*-based method is that it includes biochemical knowledge in the estimated biomass yield value obtained. Herewith, it can be regarded as a grey box method, as opposed to the black box dissipation and TEEM methods. The main advantage is also a clear limitation: the *ATP* method requires rather detailed biochemical knowledge and includes important generalizations and assumptions concerning the biochemical pathways considered. In many experimental systems, this information is not available, mainly when dealing with mixed substrates and mixed microbial populations as generally encountered in natural ecosystems. This generally implies that the *ATP*-based method can only be used for pure culture fermentation systems that rely on substrate level phosphorylation for *ATP* production. Still, also in these systems, membrane processes may play an important in the generation of primary and secondary metabolic energy (Konings et al., 1995; Rodriguez et al., 2006). The stoichiometry of *ATP* production resulting from reoxidation of electron carriers using an external electron acceptor is basically variable and ranges from 1 to 3 *ATP* per mol *NADH* when reoxidized

with a strong electron acceptor like nitrate or oxygen. Still, we have repeatedly applied successfully the *ATP*-balancing method using the P/O ratio (*ATP* per oxygen ratio for aerobic systems) for ployhydroxybutyrate-producing mixed cultures of bacteria, as well as for polyphosphate- and glycogen-accumulating microorganisms (Filipe et al., 2001; vanAalastvanLeeuwen et al., 1997; Zeng et al., 2003). In these systems, the P/O ratio is used as a principle variable to validate the system description. However, it should be noted that independent identification of the P/O ratio from an experimental system is troublesome because the P/O value obtained is linearly dependent on the *ATP* requirements in the anabolism (Y_{ATP}^{An}).

Both the TEEM and the dissipation methods are based on black box models. The models allow for prediction of the biomass yield, requiring only the identification of the anabolic carbon and nitrogen source, and the electron donor-acceptor couple in the energy generating catabolic reaction equation. At first sight, the TEEM method and the dissipation method seem rather different, but the actual differences are in many cases negligible. In the specific case that both m and n are equal to $+1$ (or $m = +1$ and $\Delta G_N = 0$ which applies when NH_4^{+1} is the N source), and when taking into account that the sum of assimilative denitrification and conversion of the carbon source to biomass building blocks equals the anabolism ($\Delta G_{An} = \Delta G_C + \Delta G_N$; see Figure 7), Eq. (39) reduces to:

$$\lambda_{Cat} = \frac{\Delta G_{An} + \Delta G_{Syn}/\eta}{-\Delta G_{Cat}}$$

which is equal to Eq. (32), assuming that $\Delta G_{Dis} = \Delta G_{Syn}/\eta$. Because both η and ΔG_{Syn} in the TEEM method and ΔG_{Dis} in the dissipation method are assumed constant, both equations are in the given conditions the same. Herewith, the major difference between both methods is the dependency of the biomass yield on the degree of reduction (γ) and the carbon chain length (*NoC*) of the carbon source when applying the dissipation method. The TEEM method assumes that the Gibbs energy dissipation per mol biomass formed is constant for all growth systems, and independent of the carbon source.

We have used the measured biomass yield and Gibbs energy dissipation values presented in the paper from Heijnen to compare both biomass yield estimation methods. The measured yield values include measurements in aerobic systems, nitrate reducing systems, and methanogenic systems. The calculation methods utilized and the results obtained for all carbon sources are shown in Table A5. Electron acceptors considered are oxygen (O_2), nitrate (NO_3^{-1}), sulphate (SO_4^{-2}), and carbon dioxide (CO_2, producing methane, CH_4). Using the generalized approach here, the estimation of the biomass yield using either the dissipation or the TEEM method becomes a simple task. The results for both methods are presented in Figure 8, showing the measured biomass yield as a function of the estimated yield.

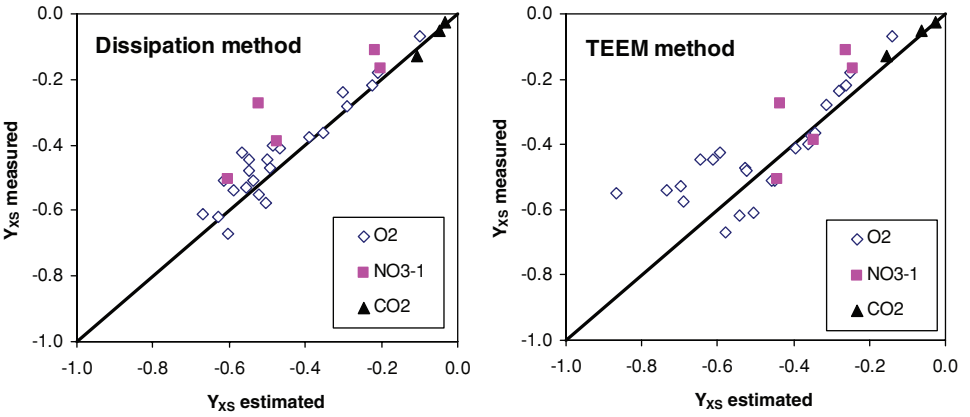

FIGURE 8. Calculated versus measured biomass yield value according to the TEEM method (right graph) and the dissipation method (left graph).

In the description of the TEEM-based yield estimation method, McCarty proposed the following values for the constants: $\eta = 0.6$ and $\Delta G_{Syn} = 132$ kJ mol X^{-1}. Using the measured data presented by Heijnen, these values resulted in a consistent overestimation of the biomass yield. We therefore assumed the value for ΔG_{Syn} to be equal to the minimum value for ΔG_{Dis} suggested by the dissipation method (200 kJ mol X^{-1}, Eq. (34)). Subsequently, the efficiency value (η) was estimated by minimization of the difference between the measured and estimated biomass yield values using h as variable. Using this method, a value of 0.43 for η was estimated and allowed for estimation of the biomass yield with an average error of 20%. The values for ΔG_{Syn} and η correspond to a ΔG_{Dis} value of 465 kJ mol X^{-1}. It should be noted that ΔG_{Syn} and η are linearly related variables that cannot be identified independently. The efficiency value proposed here corresponds well to the recently proposed values of 0.37 by McCarty (2007) and 0.43 by Xiao and VanBriesen (2006). The dissipation method proposed by Heijnen enabled the estimation of the biomass yield with an average error of no more than 15%.

Evidently, both methods provide an adequate estimation of the biomass yield. The slightly superior results of the dissipation method can be explained by the improved description of the dependency of the Gibbs energy dissipation on the nature of the carbon source. The establishment of $C - C$ bonds, as required for the production of biomass components, is an energetically expensive process that is accounted for in the dissipation method by making ΔG_{Dis} C-length dependent. Energetically expensive reduction processes as required for biomass production from oxidized C-sources are included as well in the dissipation method. Effectively, the TEEM method suggests that the biomass yield per electron is constant for all organic carbon sources

when we assume that the differences in energy content per electron for all organic compounds is limited.

Model Extensions

In recent years, a number of limitations have been identified in the yield estimation methods when the degradation of a number of specific substrates is considered. When substrate degradation involves oxygenation reactions, for example, estimated yield values generally are consequently higher than the measured biomass yield values. This is due to the fact that oxygenation reactions are uncoupled from energy conservation and can consequently be regarded as additional sources of Gibbs energy dissipation. Oxygenation reactions by dioxygenase or monooxygenase enzymes play a role in the initial aerobic activation of some aromatic compounds like benzene (Yuan and VanBriesen, 2002), chlorinated phenol (Dahlen and Rittmann, 2000), and phenanthrene (Woo and Rittmann, 2000), as well as some chemically highly stable compounds like methane and ammonium.

Vanbriesen and coworkers and McCarty have proposed methods to extend the TEEM method to include oxygenation reactions. The extensions are based on the identification of the number of electrons involved in the oxygenation reactions. Only the electrons that are not involved in the oxygenation reactions are assumed to be available for metabolic energy generation. Also, the dissipation method can readily be extended to account for Gibbs energy dissipation in oxygenation reactions, as is shown in the example of EDTA degradation in Box 10.

The main disadvantage of the model extensions proposed is that they require the opening of at least a part of the black box of substrate conversion and microbial growth. Consequently, specific biochemical knowledge is required on the number and type of oxygenation steps in the catabolic substrate degradation pathway before the methods can be applied. Herewith, these methods become largely comparable to the *ATP*-based method. In Box 11, we demonstrate the use of the *ATP* method for estimating the biomass yield for aerobic growth on EDTA. The *ATP*-based method uses the biochemical information available to identify the P/O ratio, which in turn provides information on the biochemistry. This is why we prefer to use the *ATP*-based method for the establishment of growth models if adequate biochemical information is available.

CONCLUDING REMARKS

Mass and energy balances provide the backbone for the development of mathematical models for quantitative description of environmental

BOX 10. Modification of the dissipation method for growth on EDTA.

Aerobic degradation of ethylenediaminetetraacetic acid (EDTA) involves four oxygenation reactions (Yuan and VanBriesen, 2002). The sixteen electrons involved in the four oxygenation reactions are not coupled to energy conservation and result in the production of 3 glyoxylate ($C_2HO_3^{-1}$) and ethylenediamine ($C_2H_4(NH_2)_2$). These substrates are subsequently available for catabolic bioenergy generation and as carbon source for microbial growth.
Catabolic respiration of EDTA involves transfer of 38 electrons to 9.5 oxygen:

$$Cat: \ -1 \cdot C_{10}H_{16}N_2O_8 - 9.5 \cdot O_2 - 5 \cdot H_2O + 10 \cdot HCO_3^{-1} + 2 \cdot NH_4^{+1} + 8 \cdot H^{+1} = 0,$$

$$\Delta G_{Cat} = -3952.1\, kJ\ reaction^{-1}$$

Due to the involvement of the oxygenation reactions, 16 electrons are not available for energy generation. Assuming that the Gibbs energy content per electron is basically constant for organic compounds, this suggests that the actual Gibbs energy available for energy generation (ΔG^*_{Cat}) should be corrected for the involvement of only 38–16=22 electrons:

$$\Delta G^*_{Cat} = \frac{22}{38} \cdot -3952.1 = -2288.1\, kJ\ reaction^{-1}$$

The dissipation method requires definition of the degree of reduction (γ) and the carbon chain length (*NoC*) of the carbon source. Still, the method is only calibrated for carbon chain length values up to six, and therefore we need to define an alternative C source. An obvious choice is the main intermediate in the EDTA degradation pathway, glyoxylate ($\gamma = 2$, $NoC = 2$), and ΔG_{Dis} can be calculated (Eq. (37)):

$$\Delta G_{Dis} = 706.2\, kJ\ molX^{-1}$$

The stoichiometry of the metabolism can now be derived, and the estimated biomass yield ($Y^{Met}_{X/EDTA}$) amounts −2.3 mol X mol EDTA^{-1}, instead of −3.4 mol X mol EDTA^{-1} when all electrons are considered. The measured biomass yield is between both predicted values: −2.7 mol × mol EDTA^{-1}.

ecosystems. Dynamic modeling of environmental systems is based on an adequate description of the stoichiometry of the reactions that occur, combined with a number of rate equations. The vector-based description of the reaction stoichiometries as proposed in this paper provide directly the building blocks required for defining a full stoichiometry matrix as basis of environmental mathematical models involving electron transfer reactions.

In many cases, the outcome of the calculation methods described in this paper are equivalent to those described by McCarty (1965, 2007), Heijnen (Heijnen and Vandijken, 1993; Heijnen et al., 1992), and later modified by VanBriesen (VanBriesen, 2001, 2002; VanBriesen and Rittmann, 2000; Xiao and VanBriesen, 2006; Yuan and VanBriesen, 2002). In some cases, the methods developed by McCarty and Heijnen generate erroneous results, as demonstrated in the examples described in Boxes 3 and 9. The use of a specific reference oxidation state of carbon (i.e., as carbon dioxide)

BOX 11. *ATP* method for growth on EDTA.

Using the *ATP*-based method, we will assume that all *ATP* production is based on reoxidation of reduced electron carriers (e.g., *NADH*) in the electron transfer chain. In Box 10, it was outlined that only 22 out of 38 electrons per mol EDTA are available for energy generation upon oxidation to carbon dioxide. This can most straightforwardly be implemented in the *ATP*-based method by assuming that the electron donor reaction includes uptake of four oxygen molecules to account for the oxygenation reactions:

$$D: \ -1 \cdot C_{10}H_{16}N_2O_8 - 4 \cdot O_2 - 16 \cdot H_2O + 10 \cdot HCO_3^{-1} + 2 \cdot NH_4^{+1} + 30 \cdot H^{+1} + 22 \cdot e^{-1} = 0,$$

The electron acceptor reaction is subsequently associated with *ATP* production assuming a specific value for the P/O-ratio:

$$A: \ -1 \cdot O_2 - 4 \cdot H^{+1} - 4 \cdot e^{-1} + 2 \cdot H_2O + 2 \cdot P/O \cdot ATP = 0,$$

The catabolic reaction stoichiometry can now readily be established by solving the electron balance according to Eq. (7):

$$Cat: \ -1 \cdot C_{10}H_{16}N_2O_8 - 9.5 \cdot O_2 - 5 \cdot H_2O + 10 \cdot HCO_3^{-1} + 2 \cdot NH_4^{+1} + 11 \cdot P/O \cdot ATP + 8 \cdot H^{+1} = 0,$$

The *ATP* requirement for biomass production from glyoxylate (as proposed as C source in Box10) was estimated from data presented by Stouthamer (1979):

$$Y_{ATP/X}^{An} = -3 \ mol ATP \ mol X^{-1}$$

The catabolic and anabolic reaction equation can be combined to close the *ATP* balance and to establish the metabolic stoichiometric equation. Using a P/O-ratio of 1, a biomass yield equal to the measured biomass yield of -2.7 mol-X mol-EDTA^{-1} is found. This value for the P/O-ratio is at the lower limit of the values found in literature, which suggests that the net catabolic *ATP*-yield is low. A potential reason for the low energy production efficiency can be the energy requirement for transport processes.

by Heijnen and McCarty is the fundamental difference with the method described in this paper. This results in the use of carbon dioxide as electron acceptor in cases where this is not realistic, as in the de Heijnen and MacCarty method. In the methods described in this paper, we do not use predefined reference states for any of the elements involved in the redox reactions, increasing the general applicability of the method compared to previously described methods. Furthermore, whereas the methods described by Heijnen and McCarty are optimized for solution on a sheet of paper, the method we describe aims for straightforward implementation in a calculation procedure.

The fully generalized step-wise approach described here for derivation of reaction stoichiometries and thermodynamic calculations uses a limited

number of specific terms and very simple equations, and allows for straightforward implementation in a spreadsheet program like MS Excel. The individual steps provide furthermore insight in the processes as outlined on several places in the text.

The methodology described in this paper includes a number of steps:

- definition of the electron donor and acceptor in the catabolic and anabolic reactions and their stoichiometry;
- derivation of the catabolic and anabolic reaction stoichiometry by closing the electron balance;
- calculation of the thermodynamic properties of the reactions catalyzed; and
- derivation of the stoichiometry metabolic reaction equation by using either a measured biomass yield, an estimated biomass yield (according to the *ATP*, dissipation, or the TEEM method), or by measuring the consumption/production rate of two compounds participating in the reaction system.

The fully generalized method described in this paper is clarified with a number of examples in the text that have been elaborated in an MS Excel file that can be downloaded from the publisher's online edition of *Critical Reviews in Environmental Science and Technology* as well as the authors' Web site (http://www.bt.tudelft.nl/ebt). The type of analysis described in this paper is an integral part of our educational program, and we hope it may stimulate others to use this approach in their work as well.

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APPENDIX A: TIPS AND TRICKS TO DEFINE REACTION STOICHIOMETRIES

Closing the O, H, and Charge Balance

The numerical values for the stoichiometric coefficients in *D* or *A* can readily be derived. First, the oxidized form of the electron donor (*Ed*) should be defined in the electron donor reaction (*D*) and the reduced form of the electron acceptor (*Ea*) in reaction electron acceptor reaction (*A*). This means that the elemental balances of the central atom in the electron donor and acceptor reaction need to be closed. Typically, these can be the *C*, *N*, *S*, *Fe*, and/or *Mn*-balance.

Next, the *O* and *H* and electroneutrality balance should be solved using H_2O, H^{+1} and e^{-1}. Generalized (symbolic) solutions for calculation of the stoichiometric coefficients for H_2O, H^{+1}, and e^{-1} upon full oxidation of inorganic nitrogen and sulfur compounds and organic compounds are presented in Table A1.

Alternatively, the elemental balances for *O*, *H*, and *Charge* can be used respectively to derive numerically the reaction stoichiometry in three subsequent steps. Assuming that H_2O, H^{+1}, and e^{-1} are the three last elements of a vector containing all species participating in the reaction, the stoichiometric coefficients can be calculated using the following equations:

$$Y_{H2O} = -\sum_{i=1}^{m-3} Y_i \cdot NoO_i \tag{40}$$

$$Y_H = -\sum_{i=1}^{m-2} Y_i \cdot NoH_i \tag{41}$$

$$Y_e = \sum_{i=1}^{m-1} Y_i \cdot z_i \tag{42}$$

TABLE A1. Generalized stoichiometry of the electron donor reactions for N, S, and organic carbon containing compounds upon oxidation to the most oxidized form

Substrate	$N_xH_yO_z^v$	$S_xH_yO_z^v$	$C_xH_yO_zN_uS_w^v$
Products	NO_3^{-1}	SO_4^{-2}	HCO_3^{-1}, HS^{-1}, NH_4^{+1}
$Y_{H2O/Ed}$	$z-3\cdot x$	$z-4\cdot x$	$z-3\cdot x$
$Y_{H/Ed}$	$y-2\cdot z+6\cdot x$	$y-2\cdot z+8\cdot x$	$y-2\cdot z+5\cdot x-4\cdot u-w$
$Y_{e/Ed}$	$-v+y-2\cdot z+5\cdot x$	$-v+y-2\cdot z+6\cdot x$	$-v+y-2\cdot z+4\cdot x-3\cdot u-2\cdot w$

The stoichiometric coefficients for the products follow directly from the elemental composition of the substrates.

where m represents the number of compounds participating in the reaction and defined in the stoichiometry vector, and z_i stands for the charge of compound i.

Checking Elemental Balances

In a spreadsheet program, the elemental balance of all chemical reactions defined in a vector can easily be checked by defining the elemental composition and charge of all reactants and products in separate vectors. In case of definition of a correct stoichiometry, the sum of the product of the reaction vector with all of the elemental and charge vectors should equal zero. For a reaction equation involving n compounds, the carbon balance can be calculated according to the following equation:

$$Bal_C = \sum_{i=1}^{n} Y_i \cdot NoC_i \tag{43}$$

In Microsoft Excel, the function required for this procedure is called *Sumproduct* and requires the number of carbon atoms per mol (*NoC*) and the reaction stoichiometry vector as input. Other elemental balances and a charge balance can be setup analogously.

Derivation of a Generalized COD Equation

The chemical oxygen demand (COD) concept is a widely applied concept in the waste treatment field for characterization of the concentration of organic material in liquid streams. It is defined as the concentration of oxygen required for full oxidation of all organic compounds to carbon dioxide. For an arbitrary organic compound $C_cH_bO_oN_n^z$, the electron donor reaction can be written in generalized terms as:

$$\begin{aligned} -1 \cdot C_cH_bO_oN_n^z + Y_{NH4}^D \cdot NH_4^+ + Y_{CO2}^D \cdot CO_2 \\ + Y_{H2O}^D \cdot H_2O + Y_H^D \cdot H^+ + Y_e^D \cdot e^- \end{aligned} \tag{44}$$

Using this equation, it is implicitly assumed that the N-moiety in the organic molecule is present in the oxidation state *–III*. This equation represents five variables (x, y, z, v, u) and four elemental balances, and the electroneutrality balance can be derived:

$$\text{C - balance: } -c + Y_{CO2}^D = 0 \tag{45}$$

$$\text{H - balance: } -h + Y_H^D + 4 \cdot Y_{NH4}^D + 2 \cdot Y_{H2O}^D = 0 \tag{46}$$

$$\text{O - balance: } -o + 2 \cdot Y_{CO2}^D + Y_{H2O}^D = 0 \tag{47}$$

$$\text{N - balance: } -n + Y^D_{NH4} = 0 \tag{48}$$

$$\text{EN - balance: } -z + Y^D_H - Y^D_e + Y^D_{NH4} = 0 \tag{49}$$

This set of equations can be solved for Y^D_e:

$$Y^D_e\,[mol - e/mol] = 4 \cdot c + h - 2 \cdot o - 3 \cdot n - z$$

The electron acceptor reaction is:

$$-1 \cdot O_2 - 4 \cdot H^+ - 4 \cdot e^- + 2 \cdot H_2O = 0$$

and the electron yield on oxygen amounts is

$$Y^A_e = 4\ mol - e/mol - O_2.$$

This results in the following equation for calculation of the *COD*-equivalent concentration:

$$Y^{Cat}_{O2/S}\,[mol - O_2/mol - S] = \frac{Y^D_e}{Y^A_e} = \frac{4 \cdot c + h - 2 \cdot o - 3 \cdot n - z}{4}$$

APPENDIX B

TABLE A2. Abbreviations and constants

Symbol	Description, value, and unit
Cat	Vector describing the stoichiometry of the catabolic reaction per mol electron donor
An	Vector describing the stoichiometry of the anabolic reaction per mol biomass formed
Met	Vector describing the stoichiometry of the metabolic reaction per mol biomass formed
D	Vector describing the stoichiometry of the electron donor reaction per mol electron donor
A	Vector describing the stoichiometry of the electron acceptor reaction per mol electron acceptor
Ed, Ea	Electron donor and electron acceptor
a_S	Activity of compound S.
c_x	Molar concentration of specie x (mol dm^{-3})
Z_x	Charge of specie x
C_{IV}	Elemental mass balance concentration of all species of the central element (C) with the same oxidation state (+IV) (mol dm^{-3})
γ_C	Oxidation state of the element in the subscript (C) with the elemental state as reference state. The oxidation state is defined as the number of electrons released/taken up upon conversion to the elemental state.
E	Electrochemical potential (V)
E^0	Electrochemical reference potential (V)
E^{01}	Electrochemical reference potential corrected for a pH of 7 (V)
F	Faraday's constant (96.485309 kJ mol^{-1} V^{-1})
I	Ionic strength of a solution ($I = \sum_x c_x \cdot z_x^2$)
K	Thermodynamic equilibrium constant (—)
pK	Negative logarithm of the Equilibrium constant K (—)
pH	Negative logarithm of the proton activity (—)
R	Gas constant (8.31451 J mol^{-1} K^{-1}, or 0.082057844 Atm mol^{-1} K^{-1})
RET	Reverse electron transfer
T	Absolute temperature in Kelvin
T^0	Standard temperature (298.15 K)
V_{MG}^0	Standard molar volume of ideal gas (22.414 dm^3 mol^{-1} at 273.15 K and P=1 Atm)
$Y_{A/B}^R$	Stoichiometric coefficient for the production/consumption of compound A per compound B in reaction R.
Gf⁰, Hf⁰, Sf⁰	Standard formation values for the Gibbs energy, enthalpy and entropy, relative to the standard state.
ΔG^0, ΔH^0, ΔS^0	Standard Gibbs energy change (kJ mol^{-1}), standard enthalpy change (kJ mol^{-1}), and standard entropy change (kJ mol^{-1} K^{-1}). Standard conditions are defined as: 298.15 K, and an activity of 1 of all species in the reaction.
ΔG^{01}, ΔH^{01}, ΔS^{01}(ΔG, ΔH, ΔS)	Standard Gibbs energy change, standard enthalpy change and standard entropy change, corrected for a proton activity of 1 10^{-7} (pH 7.0)
ΔG^1, ΔH^1, ΔS^1	Actual Gibbs energy change, enthalpy change, and entropy change

APPENDIX C: THERMODYNAMIC PROPERTIES OF BIOLOGICALLY IMPORTANT COMPOUNDS

Table A3 contains basic thermodynamic and oxidation-reduction properties of a wide range of biologically important organic and inorganic compounds (Dimroth, 1983; Hanselmann, 1991; Hinz, 1986; Kelly, 1999; Poughon et al., 2001; Thauer et al., 1977). For all compounds, only the G_f^0 and H_f^0 values of the dominant form and state at pH 7 is shown. The values for other forms can be found elsewhere (Hanselmann, 1991) or can be calculated from thermodynamic equilibrium parameters (K values) (Martell & Smith, 1976; Stumm & Morgan, 1996). For G_f^0 and H_f^0 values of specific ranges of compounds like chlorinated hydrocarbons or organic solvents (Perry & Green, 1999), specific literature resources are available. An extensive list of the temperature dependencies of the Gibbs energy change of biologically important reactions has been presented by Amend & Shock (2001). In case G_f^0 and H_f^0 values of certain compounds cannot be found, they can be estimated using group contribution methods for aqueous species using the method described by Mavrovouniotis (1990, 1991).

TABLE A3. Thermodynamic and oxidation-reduction properties of organic and inorganic compounds

	Name	Structure	Phase	G_f^0 kJ mol^{-1}	H_f^0 kJ/ mol^{-1}	γ^* el mol^{-1}	COD† gO_2 g^{-1}	$Y_e^{D\ddagger}$ el mol^{-1}	ΔG_e^{01} kJ e-mol^{-1}
E	Electron	e^{-1}	aq	0.0	0.0	0.0		0.0	0.0
H	Proton	H^{+1}	aq	0.0	0.0	1.0		0.0	0.0
	Hydrogen	H_2	g	0.0	0.0	0.0		2.0	−40.0
O	Oxygen	O_2	g	0.0	0.0	0.0		0.0	0.0
	Water	H_2O	l	−237.2	−285.8	−2.0		4.0	78.6
N	Dinitrogen	N_2	g	0.0	0.0	0.0		10.0	72.1
	Nitric oxide	NO	g	86.6	90.2	2.0		3.0	38.9
	Dinotrogenoxide	N_2O	g	104.2	82.0	1.0		8.0	57.5
	Hydroxylamine	NH_2OH	g	−43.6	—	−1.0		6.0	21.2
	Hydrazine	N_2H_4	g	159.17	95.3	−2.0		14.0	28.7
	Amonium	NH_4^{+1}	aq	−79.4	−133.3	−3.0		8.0	35.0
	Nitrite	NO_2^{-1}	aq	−32.2	−107.0	3.0		2.0	39.1
	Nitrate	NO_3^{-1}	aq	−111.3	−173.0	5.0		0.0	0.0
P	Phosporous white	P	s	0.0	0.0	0.0		5.0	−84.2
	Dihydrogen phosphate	$H_2PO_4^{-1}$	aq	−1130.3	−1296.3	5.0		0.0	0.0
S	Sulfur (rhombic)	S	s	0.0	0.0	0.0		6.0	−19.2
	Bisulfide	HS^{-1}	aq	12.1	−17.6	−2.0		8.0	−20.9
	Disulfide	S_2^{-2}	aq	79.5	30.1	−1.0		14.0	−22.2
	Trisulfide	S_3^{-2}	aq	73.7	25.9	−0.7		20.0	−21.0
	Thiosulfate	$S_2O_3^{-2}$	aq	−522.5	−608.0	2.0		8.0	−22.5
	Sulfite	SO_3^{-2}	aq	−486.6	−635.5	4.0		2.0	−50.4
	Sulfate	SO_4^{-2}	aq	−744.6	−909.6	6.0		0.0	0.0
	Trithionate	$S_3O_6^{-2}$	aq	−958.0	−1167.0	3.3		8.0	−41.5
	Tetrathionate	$S_4O_6^{-2}$	aq	−1040.4	−1224.2	2.5		14.0	−26.1
Fe	Metallic iron	Fe	s	0.0	0.0	0.0		3.0	−1.5

(Continued on next page)

TABLE A3. Thermodynamic and oxidation-reduction properties of organic and inorganic compounds *(Continued)*

	Name	Structure	Phase	G_f^0 kJ mol^{-1}	H_f^0 kJ/ mol^{-1}	γ^* el mol^{-1}	COD† $gO_2\ g^{-1}$	$Y_e^{D\ddagger}$ el mol^{-1}	ΔG_e^{01} kJ e-mol^{-1}
	Ferrous iron	Fe^{+2}	aq	−78.9	−89.1	2.0		1.0	74.3
	Ferric iron	Fe^{+3}	aq	−4.6	−48.5	3.0		0.0	0.0
Mn	Manganese	Mn	s	0.0	0.0	0.0		4.0	−21.4
	Manganese II+ ion	Mn^{+2}	aq	−228.0	−220.7	2.0		2.0	71.3
	Manganese IV+ ion	Mn^{+4}	aq	−85.4	−100.4	4.0		0.0	0.0
C	Graphite	C	s	0.0	0.0	0.0	2.67	4.0	−18.8
	Carbon monoxide	CO	g	−137.2	−110.5	2.0	0.57	2.0	−47.6
	Carbon dioxide	CO_2	g	−394.4	−393.5	4.0	0.00	0.0	0.0
	Formate	CHO_2^{-1}	aq	−351.0	−410.0	2.0	0.36	2.0	−39.3
	Bicarbonate	CHO_3^{-1}	aq	−586.9	−692.0	4.0	0.00	0.0	0.0
	Formaldehyde	CH_2O	aq	−130.5	−150.2	0.0	1.07	4.0	−45.4
	Methyl amine	$CH_3NH_3^{+1}$	aq	20.7	−70.2	−2.0	1.50	6.0	−42.5
	Methane	CH_4	g	−50.7	−74.8	−4.0	4.00	8.0	−23.0
	Methanol	CH_4O	aq	−175.4	−245.9	−2.0	1.50	6.0	−36.1
	Oxalate	$C_2O_4^{-2}$	aq	−674.0	—	3.0	0.18	2.0	−52.6
	Glyoxylate	$C_2HO_3^{-1}$	aq	−486.6	−824.0	2.0	0.44	4.0	−43.8
	Acetate	$C_2H_3O_2^{-1}$	aq	−369.4	−485.8	0.0	1.08	8.0	−26.9
	Glycolate	$C_2H_3O_3^{-1}$	aq	−517.6	−648.5	0.7	0.92	10.0	−29.9
	Acetaldehyde	C_2H_4O	aq	−139.1	−212.5	−1.0	1.82	10.0	−32.8
	Glycinate	$C_2H_4O_2N^{-1}$	aq	−324.3	−324.3	1.0	0.65	6.0	−36.6
	Ethane	C_2H_6	g	−32.9	−85.0	−3.0	3.73	14.0	−25.5
	Ethanol	C_2H_6O	aq	−181.8	−288.3	−2.0	2.09	12.0	−30.4
	Ethyleneglycol	$C_2H_6O_2$	aq	−330.5	—	−1.0	1.29	10.0	−37.4
	Malonate	$C_3H_2O_4^{-2}$	aq	−700.0	—	1.3	0.63	8.0	−29.3
	Acrylate	$C_3H_3O_2^{-1}$	aq	−286.2	—	0.0	1.35	12.0	−31.1
	Pyruvate	$C_3H_3O_3^{-1}$	aq	−474.6	−596.0	0.7	0.92	10.0	−34.2
	Propionate	$C_3H_5O_2^{-1}$	aq	−361.1	−510.4	−0.7	1.53	14.0	−27.0
	Lactate	$C_3H_5O_3^{-1}$	aq	−517.1	−687.0	0.0	1.08	12.0	−31.6
	B-hydroxy propionate	$C_3H_5O_3^{-1}$	aq	−518.4	—	0.0	1.08	12.0	−31.5
	Acetone	C_3H_6O	aq	−159.8	−221.9	−1.3	2.21	16.0	−28.9
	Alaninate	$C_3H_6O_2N^{-1}$	aq	−509.9	−315.1	0.0	1.09	12.0	−15.8
	Hydrogen cysteinate	$C_3H_6O_2NS^{-1}$	aq	−291.2	−349.6	−0.7	0.80	10.0	−36.8
	Dihydroxy-acetone	$C_3H_6O_3$	aq	−445.2	—	0.0	1.07	12.0	−41.0
	Propane	C_3H_8	g	−24.0	−104.0	−2.7	3.64	20.0	−26.1
	N-propanol	C_3H_8O	aq	−175.8	−331.0	−2.0	2.40	18.0	−29.2
	2-propanol	C_3H_8O	aq	−185.3	−331.0	−2.0	2.40	18.0	−28.7
	1,3-propanediol	$C_3H_8O_2$	aq	−327.0	—	−1.3	1.68	16.0	−33.3
	Glycerol	$C_3H_8O_3$	aq	−488.5	−676.0	−0.7	1.22	14.0	−37.7
	Fumarate	$C_4H_2O_4^{-2}$	aq	−604.2	−777.0	1.0	0.84	12.0	−33.8
	Oxaloacetate	$C_4H_2O_5^{-2}$	aq	−793.8	−985.2	1.5	0.62	10	−37.3
	Sucinate	$C_4H_4O_4^{-2}$	aq	−690.2	−909.0	0.5	0.97	14.0	−28.5
	Malate	$C_4H_4O_5^{-2}$	aq	−845.0	−843.0	1.0	0.73	12.0	−33.5
	Tartrate	$C_4H_4O_6^{-2}$	aq	−1010.0	—	1.5	0.54	10.0	−39.4
	Crotonate	$C_4H_5O_2^{-1}$	aq	−277.4	—	−0.5	1.69	18.0	−29.8
	Acetoacetate	$C_4H_5O_3^{-1}$	aq	−493.7	—	0.0	1.27	16.0	−29.9
	Aspartate	$C_4H_5O_4N^{-2}$	aq	−639.1	−906.4	1.0	0.73	12.0	−34.1
	Butyrate	$C_4H_7O_2^{-1}$	aq	−352.6	−535.0	−1.0	1.84	20.0	−27.1
	B-hydroxy butyrate	$C_4H_7O_3^{-1}$	aq	−506.3	—	−0.5	1.40	18.0	−30.3
	Glycylglycinate	$C_4H_7O_3N^{-1}$	aq	−473.4	−690.3	0.3	1.03	15.0	−33.2
	Creatinine	$C_4H_8ON_3$	aq	−23.2	—	1.0	0.84	12.0	−42.7
	Ethyl actetate	$C_4H_8O_2$	aq	−355.0	−482.3	−1.0	1.82	20.0	−29.0
	Acetoin	$C_4H_8O_2$	aq	−280.0	—	−1.0	1.82	20.0	−32.7

(Continued on next page)

TABLE A3. Thermodynamic and oxidation-reduction properties of organic and inorganic compounds *(Continued)*

Name	Structure	Phase	G_f^0 kJ mol^{-1}	H_f^0 kJ/ mol^{-1}	γ^* el mol^{-1}	COD† gO_2 g^{-1}	$Y_e^{D\ddagger}$ el mol^{-1}	ΔG_e^{01} kJ e-mol^{-1}
Creatine	$C_4H_8O_2N_3^{-1}$	aq	−177.9	−128.2	1.0	0.74	12.0	−42.9
Asparagine (dipolar)	$C_4H_8O_3N$	aq	−526.3	−766.6	0.3	1.02	15.0	−32.3
N-butanol	$C_4H_{10}O$	aq	−171.8	—	−2.0	2.59	24.0	−28.6
2,3-butanediol	$C_4H_{10}O_2$	aq	−322.0	—	−1.5	1.96	22.0	−31.5
Oxalosuccinate	$C_5H_4O_5^{-2}$	aq	−1139.6	—	0.8	0.89	16.0	−11.4
Glutamate	$C_5H_7O_4N^{-2}$	aq	−643.9	−940.3	0.4	0.99	18.0	−31.1
Valerate	$C_5H_9O_2^{-1}$	aq	−344.3	−560.0	−1.2	2.06	26.0	−27.1
Valinate	$C_5H_{10}O_2N^{-1}$	aq	−307.6	−567.8	−0.8	1.66	24.0	−29.2
Glutamine	$C_5H_{10}O_3N_2$	aq	−528.4	−805.5	0.4	0.99	18.0	−31.0
Citrate	$C_6H_5O_7^{-3}$	aq	−1168.3	−1515.0	1.0	0.76	18.0	−32.4
Cystinate	$C_6H_{10}O_4N_2S^{-2}$	aq	−562.7	—	0.0	0.85	20.0	−35.8
Caproate	$C_6H_{11}O_2^{-1}$	aq	−336.0	—	−1.3	2.23	32.0	−27.1
Gluconate	$C_6H_{11}O_7^{-1}$	aq	−1154.0	—	0.3	0.90	22.0	−38.0
Leucinate	$C_6H_{12}O_2N^{-1}$	aq	−296.8	−601.0	−1.0	1.85	30.0	−28.9
Glucose	$C_6H_{12}O_6$	aq	−919.8	−1264.2	0.0	1.07	24.0	−39.7
Mannnitol	$C_6H_{14}O_6$	aq	−942.6	—	−0.3	1.14	26.0	−38.9
Benzoate	$C_7H_5O_2^{-1}$	aq	−218.6	—	−0.3	1.98	30.0	−27.4
Sucrose	$C_{12}H_{22}O_{11}$	aq	−1552.4	−2217.3	0.0	1.12	48.0	−40.8
A-lactose	$C_{12}H_{22}O_{11}$	aq	−1565.9	−2233.8	0.0	1.12	48.0	−40.5
A-maltose	$C_{12}H_{22}O_{11}$	aq	−1574.6	−2239.7	0.0	1.12	48.0	−40.3
Palmitate	$C_{16}H_{31}O_2^{-1}$	aq	−259.6	−891.4	−1.8	2.89	92.0	−27.1
Biomass	$CH_{1.8}O_{0.5}N_{0.2}$	s	−67.0	−91.0	−0.2	1.37	4.2	−33.9

*The γ values for N and S compounds ($N_xH_yO_z^v$ and $S_xH_yO_z^v$) are calculated using $\gamma = v - y + 2 \cdot z$. For organic compounds ($C_xH_yO_zN^{-III}{}_u^{-II}{}_w^v$ with $\gamma_N = -3$ and $\gamma_S = -2$), the degree of reduction is calculated using $\gamma = v - y + 2 \cdot z + 3 \cdot u - 2 \cdot w$.

†The COD-content is calculated using the equation derived in Eq. (A.3).

‡The last two columns show the electron yield for oxidation to the most oxidized form in an electron donor reaction (Y_e^D) and the Gibbs energy change per electron of the reaction (ΔG_e^{01}).

APPENDIX D: GENERALIZED GROWTH STOICHIOMETRY CALCULATIONS

Introduction

In this appendix, a generalized method is described that allows for estimation of the overall growth stoichiometry for organoheterotrophic growth on the organic carbon compounds shown in Table A3. We have used the dissipation and the TEEM method to estimate the biomass yield on all organic carbon sources/electron donors with oxygen, nitrate, sulphate, and carbon dioxide as electron acceptor. Ammonium is assumed as *N*-source in all calculations. The results obtained are presented in Table A5.

In order to derive the overall stoichiometry of the metabolism, first the stoichiometry of the catabolism (*Cat*) and the anabolism (*An*) need to be established. What need to be defined are the carbon source annex electron donor, the electron acceptor, and the *N* source. Coupling of the catabolism and the anabolism can be established with the biomass yield. Biomass yield values have been estimated from the Gibbs energy change of the catabolism and the anabolism using the Gibbs energy dissipation method and the TEEM method.

Definition of the Catabolic Reaction (*Cat*)

The stoichiometric coefficients in the electron donor reaction (HCO_3^{-1}, H_2O, H^{+1}, e^{-1}) are calculated from the elemental composition of the carbon source annex electron donor ($C_{cc}H_{cb}O_{co}N_{cn}S_{cs}^{cz}$) using the equations for organic carbon oxidation in Table A4. The stoichiometries of the electron acceptor reactions are obtained from Table A4. Closing the electron balance according to Eq. (7) yields the stoichiometry of the catabolic reaction. The standard Gibbs energy change of the catabolic reaction corrected for a pH of 7 can subsequently be calculated for the different electron acceptors from the stoichiometry and tabulated G_f^0 values using Eqs. (21) and (22).

Definition of the Anabolic Reaction (*An*)

In the anabolic reaction equation, biomass (X, $CH_{1.8}O_{0.5}N_{0.2}$) is assumed to be produced from a carbon source (*Cs*) with a generalized elemental

TABLE A4. Electron acceptor reactions considered in the growth analysis

E-acceptor	O_2	NO_3^{-1}	SO_4^{-2}	CO_2
Product	H_2O	N_2	HS^{-1}	CH_4
Y_e^A[mol mol Ea^{-1}]	−4	−5	−8	−8
ΔG^{01}[kJ mol Ea^{-1}]	−314.6	−360.6	167.5	188.9

composition of $C_{cc}H_{cb}O_{co}N_{cn}S_{cs}^{cz}$. For N and S containing carbon sources, ammonium (NH_4^{+1}) and bisulphide (HS^{-1}) are assumed as the inorganic end product of the anabolism. If insufficient N is available in the carbon source, ammonium is assumed as N source. The generalized stoichiometric equation is:

$$An* = c \cdot C_{cc}H_{cb}O_{co}N_{cn}^{cz} + n \cdot {NH_4}^{+1} + s \cdot HS^{-1} + 1 \cdot CH_{1.8}O_{0.5}N_{0.2} + a \cdot H_2O + b \cdot H^{+1} + d \cdot e^{-1} = 0$$

The stoichiometric coefficients for An* can be found by solving the set of six equations—five elemental balances (C, H, O, N, S) and the charge balance—with the six unknown stoichiometric coefficients (Y_{Cs}^{An*}, Y_{NH4}^{An*}, Y_{HS}^{An*}, Y_{H2O}^{An*}, Y_H^{An*}, Y_e^{An*}) and the assumption that we derive the stoichiometry per mol biomass formed ($Y_X^{An*} = 1$):

$$Y_{Cs}^{An*} = \frac{-1}{cc}$$

$$Y_{NH4}^{An*} = \frac{cn}{cc} - 0.2$$

$$Y_{HS}^{An*} = \frac{cs}{cc}$$

$$Y_{H2O}^{An*} = \frac{co}{cc} - 0.5$$

$$Y_H^{An*} = \frac{ch - 2 \cdot co - 4 \cdot cn - cs}{cc}$$

$$Y_e^{An*} = -\frac{0.2 \cdot cc - ch + 2 \cdot co + 3 \cdot cn + 2 \cdot cs + cz}{cc}$$

Using the stoichiometry, the standard Gibbs energy change of *An** corrected for a pH of 7 (ΔG_{An*}^{01}) can be calculated using Eqs. (21) and (22).

The overall stoichiometry of the anabolism depends on the oxidation state of the carbon source/electron donor, as described previously. If the carbon source is more reduced than biomass ($Y_e^{An*} > 0$), the stoichiometry of the anabolic reaction depends on the electron acceptor considered. In this case, the stoichiometric coefficient for the carbon source in *An* equals the coefficient in *An**:

$$Y_{Cs}^{An} = Y_{Cs}^{An*} = \frac{-1}{cc},$$

and the standard Gibbs energy change of the anabolism corrected for a pH of 7 equals:

$$\Delta G_{An}^{01} = \Delta G_{An*}^{01} - \frac{Y_e^{An*}}{Y_e^A} \cdot \Delta G_A^{01}.$$

If the carbon source is more oxidized than biomass ($Y_e^{An*} < 0$), the electrons required for reduction of the carbon source to biomass are obtained by the electron donor reaction (D). Because the carbon source and the electron donor are the same in this example, the stoichiometric coefficient for carbon source in the anabolic reaction becomes:

$$Y_{Cs}^{An} = Y_{Cs}^{An*} - \frac{Y_e^{An*}}{Y_e^D} \cdot Y_{Cs}^D,$$

and the corresponding Gibbs energy change equals:

$$\Delta G_{An}^{01} = \Delta G_{An*}^{01} - \frac{Y_e^{An*}}{Y_e^D} \cdot \Delta G_D^{01}.$$

Now, the stoichiometry and the Gibbs energy change of the catabolic and the anabolic reaction have been derived. To establish an overall description of the growth system, a link needs to be established between the two reaction equations.

Estimation of the Biomass Yield and Gibbs Energy Dissipation

The Gibbs Energy Dissipation Method

For estimation of the biomass yield according to dissipation method, first ΔG_{Dis} needs to be estimated from the oxidation state and carbon chain length of the carbon source according to Eq. (34). The biomass yield is subsequently estimated from the Gibbs energy change of the catabolism (ΔG_{Cat}), anabolism (ΔG_{An}), and dissipation term (ΔG_{Dis}) using Eqs. (32) and (37).

TEEM Method

To enable coupling of the catabolic and anabolic reaction equations, the multiplication factor for the catabolic reaction (λ_{Cat}) is estimated using Eq. (39). Because ammonium (or organic nitrogen) is assumed as N-source in the catabolic anabolic reaction, the $\Delta G_N \cdot \eta^n$ value equals 0. The value for m equals 1 if the anabolic reaction is bioenergetically favorable ($\Delta G_{An} < 0$), and $m = -1$ if $\Delta G_{An} > 0$. The biomass yield is calculated from λ_{Cat} using Eq. (37).

TABLE A5. Bioenergetic properties (ΔG_{An} and ΔG_{Cat}) and estimated biomass yield values ($mol\ X\ Cmol\ Ed^{-1}$) for heterotrophic growth with different electron acceptors according to the dissipation method ($Y^{Dis}_{X/Ed}$) and the TEEM method ($Y^{Teem}_{X/Ed}$)

			O_2				NO_3^{-1}				SO_4^{-2}				CO_2			
C-source/e-donor	Structural formula	ΔG_{Dis}	ΔG_{An}	ΔG_{Cat}	$Y^{dis}_{X/Ed}$	$Y^{Teem}_{X/Ed}$	ΔG_{An}	ΔG_{Cat}	$Y^{dis}_{X/Ed}$	$Y^{Teem}_{X/Ed}$	ΔG_{An}	ΔG_{Cat}	$Y^{dis}_{X/Ed}$	$Y^{Teem}_{X/Ed}$	ΔG_{An}	ΔG_{Cat}	$Y^{dis}_{X/Ed}$	$Y^{Teem}_{X/Ed}$
Graphite	C	562.1	63.7	−389.7	−0.38	−0.29	63.7	−363.5	−0.36	−0.27	63.7	8.7	—	—	63.7	17.0	—	—
Carbon monoxide	CO	676.1	−57.3	−252.5	−0.22	−0.25	−57.3	−239.4	−0.21	−0.24	−57.3	−53.3	−0.07	−0.09	−57.3	−49.1	−0.07	−0.08
Formate	CHO_2^{-1}	676.1	−22.5	−235.9	−0.21	−0.23	−22.5	−222.8	−0.20	−0.22	−22.5	−36.7	−0.05	−0.06	−22.5	−32.6	−0.05	−0.05
Formaldehyde	CH_2O	562.1	−48.3	−496.4	−0.48	−0.48	−48.3	−470.2	−0.47	−0.47	−48.3	−98.0	−0.16	−0.16	−48.3	−89.7	−0.15	−0.15
Methyl amine	$CH_3NH_3^{+1}$	723.2	−254.1	−727.0	−0.61	−0.71	−242.3	−687.8	−0.59	−0.69	−74.8	−129.5	−0.17	−0.21	−71.1	−117.0	−0.15	−0.19
Methane	CH_4	1113.1	−340.4	−813.3	−0.51	−0.79	−315.6	−761.1	−0.49	−0.76	38.0	−16.7	−0.01	−0.02	45.9	0.0	—	—
Methanol	CH_4O	723.2	−215.8	−688.7	−0.58	−0.67	−204.0	−649.5	−0.56	−0.65	−36.5	−91.2	−0.12	−0.15	−32.8	−78.7	−0.10	−0.13
Oxalate	$C_2O_4^{-2}$	896.8	−78.5	−262.6	−0.10	−0.13	−78.5	−249.5	−0.09	−0.12	−78.5	−63.4	−0.03	−0.05	−78.5	−59.2	−0.03	−0.05
Glyoxylate	$C_2HO_3^{-1}$	645.7	−41.6	−490.0	−0.22	−0.24	−41.6	−463.8	−0.21	−0.23	−41.6	−91.6	−0.07	−0.07	−41.6	−83.3	−0.06	−0.07
Acetate	$C_2H_3O_2^{-1}$	457.1	29.6	−844.4	−0.45	−0.36	29.6	−792.1	−0.44	−0.34	29.6	−47.7	−0.05	−0.03	29.6	−31.1	−0.03	−0.02
Glycolate	$C_2H_3O_3^{-1}$	398.0	16.8	−1085.8	−0.42	−0.32	16.8	−1020.5	−0.40	−0.31	16.8	−90.0	−0.07	−0.04	16.8	−69.2	−0.05	−0.03
Acetaldehyde	C_2H_4O	549.4	−84.4	−1114.5	−0.55	−0.54	−79.2	−1049.2	−0.53	−0.52	−4.7	−118.7	−0.10	−0.10	−3.1	−97.9	−0.08	−0.08
Glycinate	$C_2H_4O_2N^{-1}$	503.4	−11.3	−691.7	−0.35	−0.34	−11.3	−652.5	−0.34	−0.33	−11.3	−94.2	−0.08	−0.08	−11.3	−81.7	−0.07	−0.07
Ethanol	C_2H_6O	731.2	−181.7	−1309.1	−0.54	−0.64	−169.9	−1230.7	−0.52	−0.61	−2.4	−114.1	−0.07	−0.09	1.3	−89.2	−0.06	−0.07
Ethyleneglycol	$C_2H_6O_2$	549.4	−107.3	−1160.4	−0.57	−0.56	−102.1	−1095.1	−0.55	−0.55	−27.7	−164.6	−0.14	−0.13	−26.0	−143.8	−0.12	−0.12
Malonate	$C_3H_2O_4^{-2}$	502.9	19.6	−863.5	−0.29	−0.25	19.6	−811.2	−0.29	−0.24	19.6	−66.8	−0.04	−0.03	19.6	−50.1	−0.03	−0.02
Acrylate	$C_3H_3O_2^{-1}$	372.6	11.8	−1317.2	−0.52	−0.40	11.8	−1238.8	−0.50	−0.39	11.8	−122.2	−0.10	−0.06	11.8	−97.3	−0.08	−0.05
Pyruvate	$C_3H_3O_3^{-1}$	398.0	−1.2	−1128.8	−0.43	−0.37	−1.2	−1063.5	−0.42	−0.35	−1.2	−133.0	−0.10	−0.07	−1.2	−112.2	−0.08	−0.06
Propionate	$C_3H_5O_2^{-1}$	453.1	−20.3	−1479.5	−0.53	−0.48	−17.3	−1388.1	−0.51	−0.46	26.2	−85.4	−0.06	−0.04	27.1	−56.2	−0.04	−0.03
Lactate	$C_3H_5O_3^{-1}$	372.6	9.6	−1323.5	−0.52	−0.41	9.6	−1245.1	−0.51	−0.39	9.6	−128.5	−0.10	−0.06	9.6	−103.6	−0.08	−0.05
B-hydroxy propionate	$C_3H_5O_3^{-1}$	372.6	10.1	−1322.2	−0.52	−0.41	10.1	−1243.8	−0.51	−0.39	10.1	−127.2	−0.10	−0.06	10.1	−102.3	−0.08	−0.05
Acetone	C_3H_6O	600.6	−100.7	−1720.8	−0.53	−0.56	−93.3	−1616.3	−0.52	−0.54	12.1	−127.4	−0.06	−0.06	14.5	−94.1	−0.05	−0.05
Alaninate	$C_3H_6O_2N^{-1}$	372.6	8.1	−1327.8	−0.52	−0.41	8.1	−1249.4	−0.51	−0.40	8.1	−132.8	−0.10	−0.07	8.1	−107.8	−0.09	−0.06
Hydrogen cysteinate	$C_3H_6O_2NS^{-1}$	453.1	−8.0	−1154.4	−0.41	−0.37	−8.0	−1089.1	−0.40	−0.36	−8.0	−158.6	−0.10	−0.09	−8.0	−137.8	−0.09	−0.08
Dihydroxy-acetone	$C_3H_6O_3$	372.6	−29.5	−1435.4	−0.57	−0.46	−29.5	−1357.0	−0.55	−0.45	−29.5	−240.4	−0.19	−0.13	−29.5	−215.4	−0.17	−0.12
N-propanol	C_3H_8O	836.9	−174.5	−1942.0	−0.49	−0.63	−162.7	−1824.4	−0.47	−0.61	4.8	−149.5	−0.06	−0.08	8.5	−112.0	−0.04	−0.06
2-propanol	C_3H_8O	836.9	−171.3	−1932.4	−0.49	−0.63	−159.5	−1814.9	−0.47	−0.60	8.0	−139.9	−0.05	−0.07	11.7	−102.5	−0.04	−0.05
Glycerol	$C_3H_8O_3$	453.1	−70.2	−1629.2	−0.59	−0.53	−67.2	−1537.8	−0.57	−0.51	−23.7	−235.1	−0.15	−0.13	−22.8	−206.0	−0.14	−0.11
Fumarate	$C_4H_2O_4^{-2}$	414.4	0.7	−1348.9	−0.38	−0.33	0.7	−1270.5	−0.37	−0.32	0.7	−153.9	−0.08	−0.06	0.7	−128.9	−0.07	−0.05
Sucinate	$C_4H_4O_4^{-2}$	322.1	22.8	−1500.1	−0.47	−0.33	22.8	−1408.6	−0.46	−0.31	22.8	−105.9	−0.07	−0.04	22.8	−76.8	−0.05	−0.03
Malate	$C_4H_4O_5^{-2}$	414.4	2.0	−1345.2	−0.38	−0.32	2.0	−1266.9	−0.37	−0.31	2.0	−150.2	−0.08	−0.06	2.0	−125.3	−0.07	−0.05
Tartrate	$C_4H_4O_6^{-2}$	573.5	−22.9	−1180.3	−0.28	−0.29	−22.9	−1115.0	−0.27	−0.28	−22.9	−184.5	−0.07	−0.08	−22.9	−163.7	−0.07	−0.07
Crotonate	$C_4H_5O_2^{-1}$	391.1	−15.4	−1952.9	−0.57	−0.47	−13.4	−1835.3	−0.55	−0.46	14.5	−160.4	−0.09	−0.06	15.1	−122.9	−0.07	−0.05

β−ketobutyrate	$C_4H_5O_3^{-1}$	310.0	17.0	−1736.6	−0.55	−0.39	17.0	−1632.1	−0.54	−0.37	17.0	−143.3	−0.10	−0.05	17.0	−110.0	−0.08	−0.04
Aspartate	$C_4H_5O_4N^{-2}$	414.4	−0.8	−1353.4	−0.38	−0.33	−0.8	−1275.1	−0.37	−0.32	−0.8	−158.5	−0.08	−0.06	−0.8	−133.5	−0.07	−0.06
Butyrate	$C_4H_7O_2^{-1}$	535.4	−55.9	−2114.9	−0.52	−0.51	−50.6	−1984.2	−0.51	−0.49	23.8	−123.2	−0.05	−0.04	25.5	−81.6	−0.04	−0.03
β−hydroxy butyrate	$C_4H_7O_3-^1$	391.1	−17.4	−1961.2	−0.57	−0.48	−15.5	−1843.6	−0.55	−0.46	12.4	−168.7	−0.09	−0.06	13.1	−131.2	−0.08	−0.05
Glycylglycinate	$C_4H_7O_3N^{-1}$	295.0	3.1	−1677.7	−0.55	−0.40	3.1	−1579.7	−0.53	−0.39	3.1	−184.0	−0.13	−0.07	3.1	−152.8	−0.11	−0.06
Creatinine	$C_4H_8ON_3$	414.4	-36.9	−1456.6	−0.41	−0.35	−36.9	−1378.2	−0.40	−0.34	−36.9	−261.6	−0.14	−0.11	−36.9	−236.6	−0.13	−0.10
Ethyl actetate	$C_4H_8O_2$	535.4	−65.2	−2152.4	−0.53	−0.52	−60.0	−2021.8	−0.52	−0.50	14.4	−160.7	−0.07	−0.06	16.1	−119.1	−0.05	−0.04
Acetoin	$C_4H_8O_2$	535.4	−84.0	−2227.4	−0.55	−0.54	−78.8	−2096.8	−0.53	−0.52	−4.3	−235.8	−0.10	−0.10	−2.7	−194.2	−0.08	−0.08
Creatine	$C_4H_8O_2N_3-^1$	414.4	−37.8	−1459.1	−0.41	−0.35	−37.8	−1380.7	−0.40	−0.34	−37.8	−264.1	−0.14	−0.11	−37.8	−239.1	−0.13	−0.10
Asparagine (dipolar)	$C_4H_8O_3N$	295.0	6.7	−1664.8	−0.54	−0.39	6.7	−1566.8	−0.53	−0.38	6.7	−171.1	−0.12	−0.07	6.7	−139.8	−0.10	−0.05
2,3-butanediol	$C_4H_{10}O_2$	762.2	−132.8	−2422.6	−0.49	−0.59	−124.3	−2278.9	−0.47	−0.57	−3.3	−231.8	−0.07	−0.09	−0.6	−186.0	−0.06	−0.08
Oxalosuccinate	$C_5H_4O_5^{-2}$	359.2	94.8	−1440.3	−0.35	−0.19	94.8	−1335.8	−0.33	−0.18	94.8	153.0	N/A	N/A	94.8	186.3	N/A	N/A
Glutamate	$C_5H_7O_4N^{-2}$	271.4	11.9	−1975.5	−0.53	−0.36	11.9	−1857.9	−0.52	−0.35	11.9	−183.0	−0.11	−0.05	11.9	−145.6	−0.09	−0.04
Valerate	$C_5H_9O_2^{-1}$	754.1	−77.1	−2750.0	−0.45	−0.53	−70.6	−2580.2	−0.43	−0.51	22.4	−160.9	−0.04	−0.04	24.5	−106.8	−0.03	−0.03
Valinate	$C_5H_{10}O_2N^{-1}$	513.4	−44.9	−2589.0	−0.52	−0.50	−41.0	−2432.2	−0.51	−0.49	14.8	−199.0	−0.07	−0.06	16.1	−149.0	−0.05	−0.04
Glutamine	$C_5H_{10}O_3N_2$	271.4	12.5	−1973.2	−0.53	−0.36	12.5	−1855.6	−0.52	−0.35	12.5	−180.7	−0.11	−0.05	12.5	−143.3	−0.09	−0.04
Citrate	$C_6H_5O_7^{-3}$	491.8	6.5	−1998.5	−0.35	−0.31	6.5	−1881.0	−0.33	−0.30	6.5	−206.1	−0.06	−0.05	6.5	−168.6	−0.05	−0.04
Cystinate	$C_6H_{10}O_4N_2S^{-2}$	261.1	−5.7	−2288.6	−0.52	−0.37	−5.7	−2157.9	−0.51	−0.36	−5.7	−296.9	−0.16	−0.08	−5.7	−255.3	−0.14	−0.07
Caproate	$C_6H_{11}O_2^{-1}$	1197.1	−91.3	−3385.2	−0.34	−0.55	−83.9	−3176.2	−0.32	−0.53	21.5	−198.6	−0.03	−0.05	23.9	−132.0	−0.02	−0.03
Gluconate	$C_6H_{11}O_7^{-1}$	248.3	−17.2	−2567.2	−0.59	−0.42	−17.2	−2423.5	−0.58	−0.40	−17.2	−376.4	−0.21	−0.10	−17.2	−330.6	−0.19	−0.09
Leucinate	$C_6H_{12}O_2N^{-1}$	803.5	−64.9	−3226.6	−0.42	−0.52	−59.7	−3030.7	−0.40	−0.50	14.8	−239.2	−0.05	−0.06	16.4	−176.7	−0.03	−0.04
Glucose	$C_6H_{12}O_6$	261.1	−24.4	−2841.3	−0.65	−0.46	−24.4	−2684.6	−0.63	−0.45	−24.4	−451.4	−0.24	−0.12	−24.4	−401.4	−0.22	−0.11
Mannnitol	$C_6H_{14}O_6$	360.2	−36.4	−3055.7	−0.61	−0.49	−35.6	−2885.9	−0.60	−0.48	−23.1	−466.6	−0.19	−0.13	−22.9	−412.5	−0.17	−0.11
Benzoate	$C_7H_5O_2^{-1}$	349.7	18.5	−3180.6	−0.55	−0.40	19.0	−2984.7	−0.54	−0.39	27.0	−193.2	−0.07	−0.04	27.2	−130.7	−0.05	−0.03
Sucrose	$C_{12}H_{22}O_{11}$	261.1	−28.7	−5732.6	−0.65	−0.46	−28.7	−5419.1	−0.64	−0.45	−28.7	−952.7	−0.25	−0.13	−28.7	−852.8	−0.23	−0.12
A-lactose	$C_{12}H_{22}O_{11}$	261.1	−27.6	−5719.1	−0.65	−0.46	−27.6	−5405.6	−0.64	−0.45	−27.6	−939.2	−0.25	−0.13	−27.6	−839.3	−0.23	−0.12
A−maltose	$C_{12}H_{22}O_{11}$	261.1	−26.8	−5710.4	−0.65	−0.46	−26.8	−5396.9	−0.64	−0.45	−26.8	−930.5	−0.25	−0.13	−26.8	−830.6	−0.23	−0.11
Palmitate	$C_{16}H_{31}O_2^{-1}$	457.1	−135.3	−9730.2	−0.65	−0.59	−125.1	−9129.3	−0.63	−0.57	19.1	−568.6	−0.07	−0.05	22.3	−377.1	−0.05	−0.03