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Microbial energetics and stoichiometry for biodegradation of aromatic compounds involving oxygenation reactions

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Abstract

Oxygenation reactions significantly alter the energy and electron flows and, consequently, the overall stoichiometry for the microbial utilization of aromatic compounds. Oxygenation reactions do not yield a net release of electrons, but require an input of electrons to reduce oxygen molecules. The biodegradation pathway of phenanthrene as a model compound was analyzed to determine the impact of oxygenation reactions on overall stoichiometry using the half-reaction method. For individual oxygenation reactions, the half-reaction method for analyzing the electron and energy flows must be modified, because the reactions do not release electrons for synthesis or energy generation. Coupling the oxygenation reaction to subsequent reaction steps provides a net electron release for the coupled reactions. Modeling results indicate that oxygenation reactions increase the oxygen requirement and reduce the cell yield, compared to the conventional mineralization represented by hydroxylation reactions in place of oxygenations. The computed yields considering oxygenation reactions conform better to empirical yields reported in the literature than do yields computed by the hydroxylation single-step methods. The coupled-reaction model also is consistent with information about the ways in which micro-organisms that degrade aromatics accumulate intermediates, regulate degradation genes, and organize enzyme clusters.

Introduction

Aromatic hydrocarbons are ubiquitous contaminants in the environment, and some are considered to be hazardous due to their toxic and carcinogenic properties (Keith and Telliard 1979). These compounds, which may consist of one or more benzene rings, can be degraded by numerous microorganisms existing in the natural environment (Gibson and Subramanian 1984; Smith 1990). The complete mineralization of the aromatic hydrocarbons produces cell mass and inorganic materials, such as CO_2 and H_2O. However, the biodegradation of the compounds involves multiple degradation steps, and metabolic intermediates often accumulate in the media (Guerin and Jones 1988; Kiyohara and Nagao 1978). Therefore, it can become necessary to analyze individual degradation steps in order to completely model the biodegradation of the compound of interest (VanBriesen and Rittmann 2000).

Mathematical modeling of the biodegradation of an electron-donor substrate typically includes cell growth, substrate consumption, and cell decay. Each reaction rate can be linked to the others by yield values, which can be obtained from experimental results or from theoretical stoichiometric relationships. The stoichiometric relationships are usually created by the method of regularities (Erickson 1979; Roels 1980; Stouthamer and van Verseveld 1985) or the method of half reactions (Heijnen et al. 1992; McCarty 1969, 1972; VanBriesen and Rittmann 2000). Heijnen and Van Dijken (1992) compared several regularity constants for the estimation of cell yield and suggested that the Gibbs energy-dissipation value provides the best tool for estimating of cell yield among black-box models. However, the regularity or

Gibbs energy-dissipation method cannot predict cell yield without experimental yield data, peculiarly for hydrocarbon substrates with more than six carbons. Furthermore, when intermediate formation is critical during multi-step substrate degradation, a modified half-reaction methodology is the best means for estimation of stoichiometric relationships (VanBriesen and Rittmann 2000). The half-reaction method estimates the cell yield based on a balancing of energy and electrons flows for the catabolic and anabolic reactions (McCarty 1969, 1972).

Microbial degradation of aromatic compounds includes dioxygenation or monooxygenation reactions, which insert hydroxyl groups into the compound in order to "activate" the molecule before ring cleavage. In general, an oxygenation reaction involves direct incorporation of molecular oxygen from O_2 rather than from water (Gibson and Subramanian 1984; Hayaishi et al. 1970). The aromatic substrate is oxidized during an oxygenation, but reduction of two molecular oxygens in O_2 to the -2 oxidation state requires four electrons. As a result, oxygenation reactions do not yield a net release of electrons, but require an input of electrons from an intracellular electron carrier such as $NADH_2^+$. This characteristic of oxygenation reactions, unlike more "normal" oxidation reactions, fundamentally alters the energy and electron flows when cells are oxidizing aromatic hydrocarbons. In particular, polycyclic aromatic hydrocarbons (PAHs) have two or more condensed rings, and, therefore, many oxygenation reactions are included for a full ring-cleavage of the compounds. For example, the complete biodegradation pathway for naphthalene, which has 2 rings, entails 4 oxygenation reactions (Gibson and Subramanian 1984), while the complete biodegradation of the 3-ringed phenanthrene has 6 oxygenations (Gibson and Subramanian 1984). The multiple oxygenation reactions can have an important impact on the yield and stoichiometry.

In this paper, we analyze the biodegradation pathway of phenanthrene, a 3-ring PAH compound, as a model compound to determine the impact of oxygenation reactions on overall stoichiometry. For this analysis, the half-reaction method following McCarty (1969) and VanBriesen and Rittmann (2000) forms the basis for analyzing multi-step degradations that involve oxygenations. Because oxygenation steps do not provide net electron gains, we must consider how the cells link other electron-releasing reactions to drive the oxygenation reactions. The feasibility of our hypothesis and relationships among several enzymatic steps are evaluated based on experimental results reported in the literature.

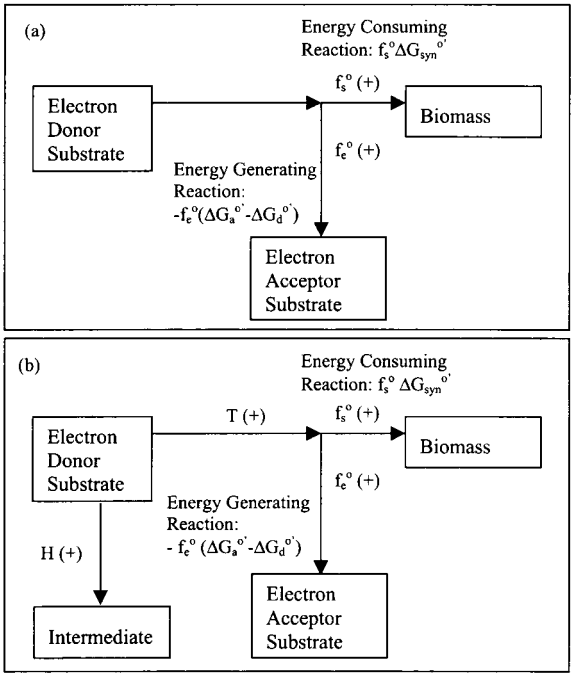

Figure 1. Electron and energy flows for biodegradation of an electron-donor substrate. (a) traditional complete mineralization and (b) intermediate formation.

Theoretical background

Energy and electron balances without oxygenation reactions

The stoichiometry of biological reactions relies upon relationships describing energy and electron balances. Generally, when an electron-donor substrate is oxidized to release available electrons, some electrons are transferred to an electron-acceptor substrate for energy generation, while the remainder of the electrons are incorporated into newly generated cell mass. The proportioning of electrons between the synthesis and energy-generating reactions defines the yield, or the ratio of bacteria produced to substrate consumed. These flows of electron and energy are shown schematically in the top panel of Figure 1.

Considering only synthesis, the energy generated by shuttling electrons from the donor to the acceptor must equal the energy invested in cell synthesis. Following McCarty (1969, 1972), the general relationship for the energy balance is

$$-f_e^0 K \Delta G_r^{0'} = f_s^0 \Delta G_{syn}^{0'} \quad (1)$$

in which $\Delta G_r^{0'}$ is the standard free energy of the energy-generating redox couple, $\Delta G_{syn}^{0'}$ is the standard free energy of the cell synthesis reactions, K is the efficiency of energy capture in the energy generation reaction, f_e^0 is the fraction of electron-donor electron equivalents sent to the acceptor to drive the energy generating redox reaction, and f_s^0 is the fraction of electron-donor electron equivalents invested in biomass via the synthesis reaction. Equation (1) says that the energy captured from transfer of f_e^0 electron equivalents from the donor to the acceptor is invested to synthesize f_s^0 electron equivalents of biomass. The efficiency of energy capture is termed K and has been computed empirically for a variety of systems (McCarty 1969). The values ranged from about 0.40 to 0.80 for aerobic and anaerobic cultures and for heterotrophic and autotrophic microorganisms. An average value, 0.60, has been used with a good success (McCarty 1969, 1972; VanBriesen and Rittmann 2000).

The ratio of f_e^0/f_s^0 can be defined as A, and rearranging Equation (1) gives:

$$A = \frac{f_e^0}{f_s^0} = -\frac{\Delta G_{syn}^{0'}}{K \Delta G_r^{0'}}. \quad (2)$$

A defines the relative electron flow between cell synthesis and energy generation for the available electrons transferred from the electron-donor substrate for the reaction of interest.

The values f_s^0 and f_e^0 are coupled through the electron balance. For a direct mineralization reaction, all the electrons present in the electron donor substrate are sent either to the acceptor (f_e^0) or to biomass synthesis (f_s^0), and the sum of f_s^0 and f_e^0 must equal 1 (McCarty, 1969, 1972):

$$f_e^0 + f_s^0 = 1. \quad (3)$$

Equations (2) and (3) can be solved simultaneously to compute f_s^0 and f_e^0 as

$$f_s^0 = \frac{1}{1+A} \quad \text{and} \quad f_e^0 = \frac{A}{1+A}. \quad (4)$$

For reactions involving intermediate formation, all the electrons in the electron-donor substrate are not released, because some of the electrons are retained in the intermediates. Previously, VanBriesen and Rittmann (2000) defined T as the fraction of electrons from the donor transferred to either the energy-generation or the biomass-synthesis pathways, while H is the fraction of electrons held in the intermediates. This is shown in the bottom panel of Figure 1. Then,

$$T + H = 1 \quad (5)$$

and

$$f_s^0 + f_e^0 = T. \quad (6)$$

VanBriesen and Rittmann (2000) showed that Equations (2) and (6) could be solved simultaneously to obtain

$$f_s^0 = \frac{T}{1+A} \quad \text{and} \quad f_e^0 = \frac{TA}{1+A}. \quad (7)$$

When intermediate formation does not occur, Equation (6) reduces to Equation (3), since $T = 1.0$.

Energy and electron balances for oxygenation reactions

For oxygenation reactions, the electron balances must be modified, because not all the electrons released from the electron-donor substrate are either transferred to cell synthesis processing or retained in the intermediates. Instead, some of electrons are transferred to molecular oxygen. Thus, electrons originally in the primary electron-donor substrate follow one of four paths: transferred to the electron acceptor to generate energy, utilized to build macromolecules in biomass synthesis, utilized to reduce O_2, or sequestered in the intermediate. We define O as the fraction of electrons from the donor invested to reduce O_2 and R as the fraction of electrons released or not sequestered in the intermediate. Then, the electron balances for oxygenation reactions are given by:

$$T + H + O = 1 \quad (8)$$

$$R = T + O. \quad (9)$$

T, H, R, and O values can be computed directly by electron tracking for the substrate half reaction. This is illustrated below for phenanthrene.

The sum of f_s^0 and f_e^0 still follows Equation (6), because only transferred electrons are utilized for either energy generation or biomass synthesis. Therefore, simultaneously solving Equations (2) and (6) produces values of f_s^0 and f_e^0 that are the same as

Equation (7). Although Equation (7) is the same for oxygenation and nonoxygenation reactions, the T values differ, because $R = T$ for a nonoxygenation, while $R > T$ for an oxygenation.

Calculating and formulating the overall stoichiometry

Using the energy and electron balances described above, the electron fractions allocated to each flow can be obtained from known thermodynamic values for each half reaction. The free energy of the energy-generation redox couple in Equation (1) is computed as the difference between the free energy of the donor and acceptor half reactions:

$$\Delta G_r^{0'} = \Delta G_a^{0'} - \Delta G_d^{0'} \tag{10}$$

in which $\Delta G_d^{0'}$ is the standard free energy for the electron-donor reduction half reaction and $\Delta G_a^{0'}$ is the standard free energy for the electron-acceptor reduction half reaction, where the prime ($'$) indicates that the pH is fixed at 7.0 (McCarty 1969). Free energies for many electron-donor and electron-acceptor half reactions are tabulated (McCarty 1969, 1972; Stumm and Morgan 1996). For example, $\Delta G_a^{0'}$ for the oxygen half reaction is -78.06 kJ/electron equivalent. If the half reaction is not tabulated, the calculation of $\Delta G_d^{0'}$ is straightforward when values for the standard free energy of formation ($\Delta G_f^{0'}$) are known for all the species in the donor half reaction. If the standard free energy of formation of the electron-donor substrate or an intermediate is not known, it can be estimated using group-contribution theory (Mavrovouniotis 1990, 1991). When intermediates are involved, $\Delta G_d^{0'}$ must be computed with the actual intermediate included (VanBriesen and Rittmann 2000). When $\Delta G_d^{0'}$ is for an oxygenation reaction, O_2 must be included. In short, $\Delta G_d^{0'}$ must be for the reduction half reaction (R_d) that includes exactly the reactants and products involved. This is illustrated below for phenanthrene, and Appendix A shows how group-contribution theory is used to compute unlisted $\Delta G_f^{0'}$ values.

When NH_4^+ is available as the N source, the synthesis cost to create biomass ($\Delta G_{syn}^{0'}$) has two components: the energy required to transform the carbon in the carbon source to the oxidation state of cellular carbon and the energy required to create and assemble the cell macromolecules (McCarty 1969, 1972). The two components are summed in Equation (11):

$$\Delta G_{syn}^{0'} = \frac{\Delta G_{pyr}^{0'} - \Delta G_{cs}^{0'}}{K^m} + \Delta G_{cells}^{0'}. \tag{11}$$

$\Delta G_{cs}^{0'}$ is standard free energy for the carbon-source reduction half reaction (kJ/e-eq). $\Delta G_{pyr}^{0'}$ is standard free energy of formation of pyruvate and is 35.72 kJ/e-eq. $\Delta G_{cells}^{0'}$ is the free energy to synthesize macromolecules and was estimated empirically as 31.35 kJ/e-eq (McCarty 1969). As with the energy-generation reaction, inefficiencies in energy transfer are included via K. The exponent m accounts for the fact that the conversion from the carbon source to the oxidation state of the common organic component (pyruvate is used here) may be energy generating, giving an energy loss due to inefficiency (m is -1), or energy utilizing, giving an additional energy cost due to inefficiency (m is $+1$).

To complete the framework for the stoichiometric relationship, half reactions for the cell synthesis, electron donor, and electron acceptor are needed. The cell synthesis half reaction can be modeled by postulating a chemical formula for the cell mass and assuming synthesis is from simple, inorganic forms of carbon and nitrogen. Hoover and Porges, (1952) suggest $C_5H_7O_2N$ as a suitable formula for cells based on elemental analysis of biomass. This formula has been widely used in biological modeling (McCarty 1972; Metcalf and Eddy 1991; VanBriesen and Rittmann 2000). VanBriesen and Rittmann (2000) previously considered the differences caused by selection of $C_5H_7O_2N$ versus $CH_2O_{0.6}N_{0.2}$, which also is used in modeling. The difference of cell yields was less than 5%. Utilizing $C_5H_7O_2N$ for cells and assuming the cells are formed from carbonate and ammonium, a half reaction (R_c) for cell synthesis can be constructed (by convention) as a reduction reaction and in terms of one equivalent of electrons (McCarty 1969; VanBriesen and Rittmann 2000):

$$\begin{aligned} \frac{5}{20}\ H_2CO_3 + \frac{1}{20}NH_4^+ + \frac{19}{20}H^+ + e^- \\ = \frac{1}{20}C_5H_7O_2N + \frac{13}{20}H_2O. \end{aligned} \tag{12}$$

The free energy required to drive this reaction is supplied from the redox reaction between an electron donor and acceptor. Bacteria prefer molecular oxygen as the electron acceptor when aromatic hydrocarbons are used for the electron donor. The half reaction (R_a)

for the utilization of molecular oxygen as the electron acceptor is:

$$\frac{1}{4}O_2(g) + H^+ + e^- = \frac{1}{2}H_2O. \qquad (13)$$

The full biodegradation stoichiometry (R_t) can be obtained by coupling the electron-donor half reaction (R_d) of interest with the half reactions for electron acceptor and cell synthesis via f_s^0, f_e^0, and T values (McCarty 1969; VanBriesen and Rittmann 2000).

$$R_t = -T R_d + f_e^0 R_a + f_s^0 R_c. \qquad (14)$$

Electron-donor half reactions for phenanthrene degradation

Each reaction step in a biodegradation pathway can have its own electron-donor reaction (R_d). To illustrate a pathway of electron-donor reactions that include oxygenations, phenanthrene, a 3-ring polycyclic aromatic hydrocarbon, is selected. Phenanthrene is degraded by some soil bacteria through one of two different routes (Cerniglia 1992; Gibson and Subramanian 1984; Smith 1990). Both routes have the same "upper pathway" for the degradation of phenanthrene to 1-hydroxy-2-naphthoate. However, the "lower pathway" is divided into two routes (Kiyohara and Nagao 1978). In one route, 1-hydroxy-2-naphthoate is oxidized to 1,2-dihydroxynaphthalene, which is further degraded via the naphthalene pathway to salicylate, which can be further metabolized. In the other pathway, the ring of 1-hydroxy-2-naphthoate is cleaved and further metabolized via the phthalate pathway (Barnsley 1983a; Kiyohara and Nagao 1978; Ribbons and Evans 1960). In this study, the naphthalene pathway was analyzed as a model system for the stoichiometry calculations. Figure 2 shows the transformation steps for phenanthrene biodegradation via the commonly illustrated naphthalene pathway (Cerniglia 1992; Eaton and Chapman 1992; Evans et al. 1965; Gibson and Subramanian 1984; Kiyohara et al. 1994; Smith 1990).

In the overall degradation of phenanthrene via the naphthalene pathway, 6 oxygenation reactions are involved; 4 are dioxygenation reactions (reactions A, C, H, and M), and 2 are monooxygenation reactions (reactions G and L). The monooxygenation reactions incorporate one oxygen atom from molecular oxygen into a substrate molecule, and the other oxygen atom goes into a water molecule produced from the reaction. The dioxygenations incorporate both oxygen atoms into a substrate molecule without producing a water molecule (Hayaishi et al. 1970). In both cases, each oxygen atom is reduced by two electrons.

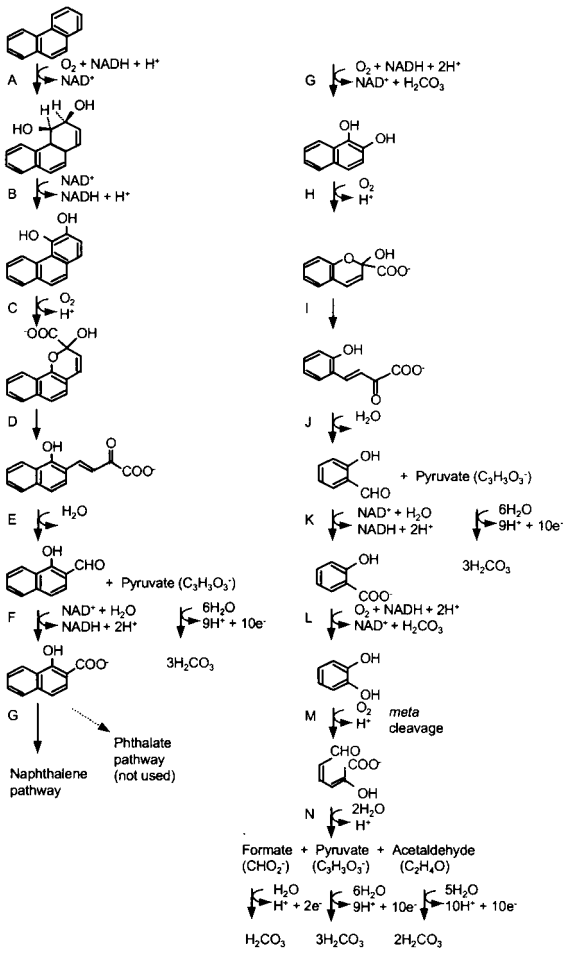

Figure 2. Biodegradation pathway of phenanthrene via the naphthalene pathway. The left column is the "upper pathway," while the right column is the naphthalene "lower pathway". Capital letters indicate enzyme reactions or enzymes. A, phenanthrene dioxygenase; B, *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene dehydrogenase; C, 3,4-dihydroxyphenanthrene dioxygenase; D, 2-hydroxy-2*H*-benzo[h]chromene-2-carboxylate isomerase; E, *cis*-4-(1′-hydroxynaphth-2′-yl)-2-.oxo-but-3-enoate hydratase-aldolase; F, 1-hydroxy-2-naphthaldehyde dehydrogenase; G, 1-hydroxy-2-naphthoate monooxygenase; H, 1,2-dihydroxynaphthalene dioxygenase; I, 2-hydroxychromene-2-carboxylate isomerase; J, *trans*-o-hydroxybenzylidenepyruvate hydratase-aldolase; K, salicylaldehyde dehydrogenase; L, salicylate monooxygenase; M, catechol 2,3-dioxygenase; N, 2-hydroxymuconate semialdehyde hydrolase with subsequent dehydrogenation and hydrogenation steps.

Table 1 summarizes the reduction half reactions (i.e., R_d) for each step. Table 1 also lists the electron-transfer parameters for one mole of substrate (H', R',

T', and O') and the standard free energy ($\Delta G_d^{0'}$) for one electron equivalent. In the phenanthrene pathway, $\Delta G_f^{0'}$ values for phenanthrene and all its intermediates were estimated using group-contribution theory (Mavrovouniotis, 1990, 1991), which is described in Appendix A. In two cases, three reactions were combined (reactions D–F and I–K), because they consist of isomerase (D, I), or hydratase-aldolase (E, J) reactions that do not involve any transfer of electrons. These non-redox reactions were combined with a subsequent dehydrogenation reaction to allow an electron transfer (i.e., $R > 0$). Appendix B illustrates how the $\Delta G_d^{0'}$ values are computed.

The first step, a dioxygenation reaction from phenanthrene to *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, illustrates the profound impact of oxygenation reactions that require a reduced electron carrier as a cosubstrate. Phenanthrene and *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene contain 66 and 64 electron equivalents per mole, respectively. Thus, reaction A releases 2 electron equivalents per mole from phenanthrene ($R' = 2$), while 4 electron equivalents ($O' = 4$) are incorporated to reduce molecular oxygen, which is incorporated into phenanthrene as 2 OH substituents. Two of the 4 electron equivalents come from phenanthrene, and the other 2 come from a reduced electron carrier, such as $NADH_2^+$ shown in Figure 2. As a result, the T' value is negative (-2), which represents that the reaction requires an input of 2 electron equivalents, rather than transferring electrons to cell synthesis or energy generation. Similar "negative" electron transfers occur for reactions G and L, which are monooxygenations.

For one-step dioxygenation reactions (C, H, and M), the T' values are zero, because the number of electrons released from the reaction ($R' = 4$) is exactly the same with the number of electrons required for oxygen reduction ($O' = 4$). This indicates that net electron transfer does not occur ($T' = 0$). Thus, electrons removed from the donor substrate are not available for either energy generation or biomass synthesis.

For reactions that are not oxygenation reactions, the O' values are zero, because oxygen is not a cosubstrate. As a result, the T' value is identical to the R' value, which means that all electrons released from the donor half reaction are transferred to either energy generation or biomass synthesis. Quantitatively, all T' values are positive, because R' values are necessarily positive.

In total, complete oxidation of one mole of phenanthrene liberates 66 electron equivalents. However, only 42 electron equivalents are available for energy generation or biomass synthesis, because 24 electron equivalents are utilized to reduce 6 oxygen molecules in oxygenation reactions. Thus, oxygenation reactions consume a substantial fraction (36.4%) of the total electrons present in phenanthrene. These activation reactions divert electrons from synthesis and energy generation and ought to affect the biomass yield for complete mineralization. Furthermore, individual reactions can have T values that are positive, zero, or negative. Only a positive T value supplies electrons for energy generation and synthesis. Therefore, accumulation of intermediates that are further oxidized by oxygenation reactions can profoundly affect whether or not electrons become available to support biomass synthesis.

Results and discussion

Single-step mineralization

The effect of oxygenation reactions on the stoichiometry of complete mineralization is explored by comparing two scenarios: the pathway in Table 1, which involves six oxygenation steps, and an alternate pathway in which each of the six oxygenations is replaced by one or two hydroxylation reactions so that one hydroxylation is used for each oxygen atom inserted. In a hydroxylation reaction, the organic substrate is oxidized by two electrons, an OH substituent is added, and H_2O is the source of oxygen in OH. A comparison of the two methods is valuable, since the hydroxylation pathway corresponds to conventional mineralization reactions that have been used for determination of overall stoichiometry in the past.

For the case of the hydroxylation method, the standard half reaction for the electron donor, phenanthrene ($C_{14}H_{10}$), is written as a reduction for one electron equivalent:

$$\frac{14}{66}H_2CO_3 + H^+ + e^- = \frac{1}{66}C_{14}H_{10} + \frac{42}{66}H_2O$$

$$(\Delta G_d^{0'} = 25.87 \text{ kJ/e-eq}). \qquad (15)$$

This reaction indicates that full mineralization of phenanthrene yields 66 electron equivalents per mole of phenanthrene. The mineralization of phenanthrene produces 25.87 kJ per electron equivalent ($\Delta G_d^{0'}$) at pH = 7.

Table 1. Electron donor reactions and electron flows for each step of phenanthrene degradation via the naphthalene pathway

Rxn.		H'	R'	T'	O'	$\Delta G_d^{0'}$
A	$C_{14}H_{10} + O_2 + 2H^+ + 2e^- = C_{14}H_{12}O_2$	64	2	−2	4	–
	$\frac{1}{2}C_{14}H_{10} + \frac{1}{2}O_2 + H^+ + e^- = \frac{1}{2}C_{14}H_{12}O_2$ (r)					−126.82
B	$C_{14}H_{12}O_2 = C_{14}H_{10}O_2 + 2H^+ + 2e^-$	62	2	2	0	–
	$\frac{1}{2}C_{14}H_{10}O_2 + H^+ + e^- = \frac{1}{2}C_{14}H_{12}O_2$ (r)					43.30
C	$C_{14}H_{10}O_2 + O_2 = C_{14}H_9O_4^- + H^+$	58	4	0	4	–
	$C_{14}H_9O_4^- + H^+ = C_{14}H_{10}O_2 + O_2$ (r)					–
D–F	$C_{14}H_9O_4^- + 8H_2O = C_{11}H_7O_3^- + 3H_2CO_3 + 12H^+ + 12e^-$	46	12	12	0	–
	$\frac{1}{12}C_{11}H_7O_3^- + \frac{3}{12}H_2CO_3 + H^+ + e^- = \frac{1}{12}C_{14}H_9O_4^- + \frac{8}{12}H_2O$ (r)					34.40
G	$C_{11}H_7O_3^- + O_2 + 3H^+ + 2e^- = C_{10}H_8O_2 + H_2CO_3$	44	2	−2	4	–
	$\frac{1}{12}C_{11}H_7O_3^- + \frac{1}{2}O_2 + \frac{3}{2}H^+ + e^- = \frac{1}{2}C_{10}H_8O_2 + \frac{1}{2}H_2CO_3$ (r)					−167.24
H	$C_{10}H_8O_2 + O_2 = C_{10}H_7O_4^- + H^+$	40	4	0	4	–
	$C_{10}H_7O_4^- + H^+ = C_{10}H_8O_2 + O_2$ (r)					- -
I–K	$C_{10}H_7O_4^- + 8H_2O = C_7H_5O_3^- + 3H_2CO_3 + 12H^+ + 12e^-$	28	12	12	0	–
	$\frac{1}{12}C_7H_5O_3^- + \frac{3}{12}H_2CO_3 + H^+ + e^- = \frac{1}{12}C_{10}H_7O_4^- + \frac{8}{12}H_2O$ (r)					35.32
L	$C_7H_5O_3^- + O_2 + 3H^+ + 2e^- = C_6H_6O_2 + H_2CO_3$	26	2	−2	4	–
	$\frac{1}{2}C_7H_5O_3^- + \frac{1}{2}O_2 + \frac{3}{2}H^+ + e^- = \frac{1}{2}C_6H_6O_2 + \frac{1}{2}H_2CO_3$ (r)					−167.24
M	$C_6H_6O_2 + O_2 = C_6H_5O_4^- + H^+$	22	4	0	4	–
	$C_6H_5O_4^- + H^+ = C_6H_6O_2 + O_2$ (r)					–
N	$C_6H_5O_4^- + 14H_2O = 6H_2CO_3 + 21H^+ + 22e^-$	0	22	22	0	–
	$\frac{6}{22}H_2CO_3 + \frac{21}{22}H^+ + e^- = \frac{1}{22}C_6H_5O_4^- + \frac{14}{22}H_2O$ (r)					36.70
1 step	$C_{14}H_{10} + 30H_2O + 6O_2 = 14H_2CO_3 + 42H^+ + 42e^-$	0	66	42	24	–
	$\frac{14}{42}H_2CO_3 + H^+ + e^- = \frac{1}{42}C_{14}H_{10} + \frac{30}{42}H_2O + \frac{6}{42}O_2$ (r)					85.52

1. All free energies are in kJ/electron equivalent.
2. (r) represents a standard half reaction as a reduction form with one electron equivalent.
3. H', R', T', and O' represent the total number of electrons held in intermediates, not held in intermediates, transferred to energy or synthesis reactions, and used to reduce O_2, respectively. H, R, T, and O are computed by dividing H', R', T', and O' by the number of electrons originally in the donor substrate.
4. Electrons are associated with an intracellular electron carrier, such as $NADH_2^+$, as shown in Figure 2.

For the case of the oxygenation method, complete mineralization of one mole phenanthrene incorporates six moles of O_2, and the standard reduction half reaction is

$$\frac{14}{42}H_2CO_3 + H^+ + e^- = \frac{1}{42}C_{14}H_{10} + \frac{30}{42}H_2O + \frac{6}{42}O_2(g)$$

$$(\Delta G_d^{0'} = 85.52 \text{ kJ/e-eq}). \quad (16)$$

This reaction indicates that only 42 electron equivalents per mole of phenanthrene are available for energy generation or biomass synthesis for the actual degradation involving oxygenation, and the other 24 electron equivalents are invested to reduce oxygen molecules. The free energy for reaction 16 is $\Delta G_d^{0'}$ = 85.52 kJ/e-eq. Thus, mineralization with oxygenations is more energy yielding (per electron transferred from the donor to the acceptor) than oxidation without oxygenations. This extra energy yield partially offsets the loss of 24 electrons shunted to O_2 reductions.

Table 2 summarizes all the key parameters for the estimation of the overall stoichiometry for one-step mineralization of phenanthrene using Equations (2), (7), (10), and (11). In these calculations, pyruvate is assumed to be a carbon source for both methods, because pyruvate is always released as an intermediate in the biodegradation pathway. The f_s^0 value (0.482) in the oxygenation method is 26.4% lower than f_s^0 (0.655) in the hydroxylation method. However, this decrease is not as much as the electron loss to oxygenation reactions (i.e., 24/66 or 36.4%). This result quantities the off-setting effect of the increased free energy of the electron-donor reaction when oxygenation reactions are considered.

Table 2. Key parameters and calculated values for single-step mineralization of phenanthrene by hydroxylation and oxygenation methods

	Hydroxylation method	Oxygenation method
$\Delta G_d^{0'}$	25.87	85.52
$\Delta G_r^{0'}$	−103.96	−163.61
T	66/66	42/66
H	0/66	0/66
O	0/66	24/66
A	0.503	0.319
f_s^0	0.665	0.482
f_e^0	0.335	0.154

$\Delta G_a^{0'} = -78.06$, $\Delta G_{cs}^{0'} = 35.72$, $\Delta G_{syn}^{0'} = 31.35$, $\Delta G_{pyr}^{0'}$ =35.72, $\Delta G_{cells}^{0'} = 31.35$, m =1, $K = 0.6$.
All free energies are in kJ/electron equivalent.

Substituting T, f_s^0, and f_e^0 values into Equation (14) predicts the following overall stoichiometric equations for each method:

Hydroxylation method:

$$C_{14}H_{10} + 2.196NH_4^+ + 5.520O_2(g) + 2.412H_2O = 3.020H_2CO_3 + 2.196H^+ + 2.196C_5H_7O_2N \quad (17)$$

Oxygenation method:

$$C_{14}H_{10} + 1.592NH_4^+ + 8.541O_2(g) + 4.225H_2O = 6.041H_2CO_3 + 1.592H^+ + 1.592C_5H_7O_2N. \quad (18)$$

The oxygenation method gives a higher oxygen requirement and a lower cell yield. Thus, conventional mineralization, which is represented by the hydroxylation method, should significantly overestimate cell yield or underestimate oxygen requirement for polycyclic aromatic hydrocarbons, in which many oxygenation reactions are involved. The validity of this prediction is evaluated against experimental results in a later section.

Multi-step degradation involving oxygenations

Complete phenanthrene degradation actually consists of multiple enzyme reactions in series, and accumulation of intermediates sequesters electrons that otherwise could be used for energy and synthesis. Therefore, the energetics and stoichiometry for each step should be analyzed. Phenanthrene degradation includes several oxygenation reactions that have zero or negative electron flows ($T \leq 0$). Thus, an oxygenation reaction fundamentally alters electron and energy balances (e.g., Equations (7) and (8)), which require that the donor reaction releases electrons, or $T > 0$. To perform the electron and energy balances for reactions with $T \leq 0$, we first had to consider how the microorganisms supply electrons for situations in which $T \leq 0$.

When $T = 0$ (i.e., reactions C, H, and M), the reaction releases no electrons and cannot supply any electrons for energy generation and synthesis. In other words, $f_s^0 = f_e^0 = 0$. However, these reactions can release energy. Thus, reactions with $T = 0$ have a mismatch between energy generation and electron transfer. We reconcile this mismatch by coupling any oxygenation reaction having $T = 0$ with subsequent reactions in the pathway so that a net electron release results. For example, reaction C ($T = 0$) is combined with reaction D, E, and F to yield a 12-electron release.

When $T < 0$, the oxygenation requires an input of electrons from another source. Reaction A, G, and L have $T < 0$. In parallel to the situation for $T = 0$, we couple subsequent reactions to the oxygenation to give a positive T. The 3 coupled reactions having $T > 0$ are A–F, G–K, and L–N. Electron-donor reactions and electron flows for the set of coupled reaction are summarized in Table 3. The biodegradation pathway of phenanthrene via the coupled-reaction model is shown in Figure 3.

Coupling reactions make it possible to apply Equations (2) and (7) to compute f_s^0 and f_e^0. The parameters and f_s^0 values for each step are shown in Table 4. Since pyruvate is released as an intermediate in all the coupled reactions, this compound is assumed to be the carbon source. Each coupled reaction produces a positive electron transfer (T) and, consequently, positive f_s^0 and f_e^0 values. Substituting the electron fraction values into Equation (14) provides overall stoichiometric equation for each step degradation reaction. Table 5 summarizes the mole-based stoichiometric coefficients for each step. The sum of the molar cell yield for the coupled reactions (1.589 mole cells/mole substrate) is similar to that in the single-step reaction (1.592 mole cells/mole substrate).

Comparison of alternatives

Table 6 compares normalized electron fractions used for synthesis for each reaction. For each reaction step, $f_s^0(m)$ values are f_s^0 for that step normalized to the number of electrons in phenanthrene.

Table 3. Electron donor reactions and electron flows for the coupled reaction for phenanthrene degradation

Rxn.		H'	R'	T'	O'	$\Delta G_d^{0'}$
A–F	$C_{14}H_{10} + 2O_2 + 8H_2O = C_{11}H_7O_3^- + 3H_2CO_3 + 13H^+ + 12e^-$	46	20	12	8	–
	$\frac{1}{12}C_{11}H_7O_3^- + \frac{3}{12}H_2CO_3 + \frac{13}{12}H^+ + e^- = \frac{1}{12}C_{14}H_{10} + \frac{2}{12}O_2 + \frac{8}{12}H_2O$ (r)					90.83
G–K	$C_{11}H_7O_3^- + 2O_2 + 8H_2O = C_7H_5O_3^- + 4H_2CO_3 + 10H^+ + 10e^-$	28	18	10	8	–
	$\frac{1}{10}C_7H_5O_3^- + \frac{4}{10}H_2CO_3 + H^+ + e^- = \frac{1}{10}C_{11}H_7O_3^- + \frac{2}{10}O_2 + \frac{8}{10}H_2O$ (r)					108.43
L–N	$C_7H_5O_3^- + 2O_2 + 14H_2O = 7H_2CO_3 + 19H^+ + 20e^-$	0	28	20	8	–
	$\frac{7}{20}H_2CO_3 + \frac{19}{20}H^+ + e^- = \frac{1}{20}C_7H_5O_3^- + \frac{2}{20}O_2 + \frac{14}{20}H_2O$ (r)					70.93
1 step	$C_{14}H_{10} + 30H_2O + 6O_2 = 14H_2CO_3 + 42H^+ + 42e^-$	0	66	42	24	–
	$\frac{14}{42}H_2CO_3 + H^+ + e^- = \frac{1}{42}C_{14}H_{10} + \frac{30}{42}H_2O + \frac{6}{42}O_2$ (r)					85.52

All free energies are in kJ/electron equivalent.
(r) represents a standard half reaction as a reduction form with one electron equivalent.
O_2 refers to gas-phase oxygen, or O_2(g).

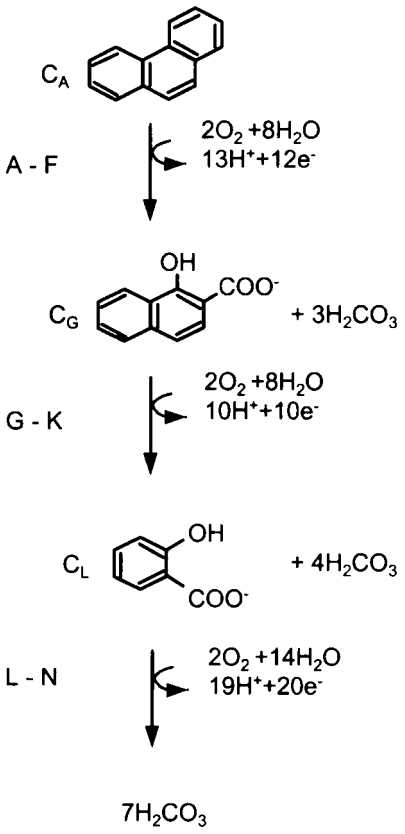

Figure 3. Release of electrons during degradation of phenanthrene via the coupled-reaction model. C_A, phenanthrene; C_G, 1-hydroxy-2-naphthoate; and C_L, salicylate. Note that coupled reactions G–K and L–N divide the naphthalene "lower pathway" into two segments. Also note that e^- are held by intracellular carriers, such as $NADH_2^+$.

Table 4. Key parameters and calculated values for the coupled reaction for phenanthrene degradation

	A–F	G–K	L–N
$\Delta G_d^{0'}$	90.83	108.43	70.93
$\Delta G_r^{0'}$	−168.91	−186.51	−148.98
T	12/66	10/46	20/28
H	46/66	28/46	0/28
O	8/66	8/46	8/28
A	0.309	0.280	0.351
f_s^0	0.139	0.170	0.529
f_e^0	0.043	0.048	0.185

$\Delta G_a^{0'} = -78.06$, $\Delta G_{pyr}^{0'} = 35.72$, $\Delta G_{cs}^{0'} = 35.72$, $\Delta G_{syn}^{0'} = 31.35$, $\Delta G_{cells}^{0'} = 31.35$, $m = 1$, $K = 0.6$.
All free energies are in kJ/electron equivalent.

Table 5. Mole-based stoichiometric coefficient for steps in coupled reaction for phenanthrene degradation

	A–F	G–K	L–N	sum	1 step (eq. 18)
C_A	−1	0	0	−1	−1
C_G	1	−1	0	0	0
C_L	0	1	−1	0	0
O_2	−2.709	−2.547	−3.298	−8.554	−8.541
H^+	1.458	0.391	−0.260	1.589	1.592
H_2O	−0.625	−1.828	−1.779	−4.232	−4.225
H_2CO_3	0.709	2.047	3.298	6.054	6.041
NH_4^+	−0.458	−0.391	−0.740	−1.589	−1.592
$C_5H_7O_2N$	0.458	0.391	0.740	1.589	1.592

Ci represents the reactant in the reaction i.
Negative and positive values represent a reactant and product, respectively.

Table 6. Comparison of normalized electron fractions for cell synthesis in each of the approaches

	Reaction	T	f_s^0	$f_s^0(m)$[a]	$f_s^0(s)$[b]	True yield[c]
Coupled reaction	A–F	12/66	0.139	0.139	0.138	–
	G–K	10/46	0.170	0.118	0.115	–
	L–N	20/28	0.529	0.224	0.229	–
	Sum	–	–	0.481	0.482	1.01
Single-step Oxygenation		42/66	0.482	0.482	0.482	1.01
Single-step Hydroxylation		66/66	0.665	0.665	0.665	1.39
Single-step Ignoring all oxygenations		42/66	0.423	0.423	0.423	0.89

[a] f_s^0 in each step of multi-step degradation, normalized to the number of electrons in an initial donor substrate, $f_s^0(m) = f_s^0 \times T'/(T \times EQS)$.
[b] Fraction of original electrons going to synthesis if the f_s^0 of the total reaction is apportioned according to the number of electrons released in each step, $f_s^0(s) = f_s^0(\text{single-step}) \times T'/[T(\text{single-step}) \times EQS]$.
[c] g cells/g phenanthrene.

$$f_s^0(m) = \frac{f_s^0 \cdot T'}{T \cdot EQS} \tag{19}$$

in which EQS is the number of electron equivalents in the original substrate (66 e-eq/mole for phenanthrene). These are compared to $f_s^0(s)$, which is the fraction of original electrons going to synthesis if the f_s^0 of the total reaction is apportioned according to the number of electrons released in each step.

$$f_s^0(s) = \frac{f_s^0(\text{single-step}) \cdot T'}{T(\text{single-step}) \cdot EQS}. \tag{20}$$

The comparison of $f_s^0(m)$ to $f_s^0(s)$ in Table 6 shows that a step-by-step computation of f_s^0 does not yield values that are weighted averages of the single-step oxygenation method. The difference between $f_s^0(m)$ and $f_s^0(s)$ depends on the free energy for the energy-generating redox couple ($\Delta G_r^{0'}$) alone, because the synthesis cost in all the coupled reactions is the same as the single-step oxygenation reaction. For example, the second coupled reaction (G–K) gives a higher $f_s^0(m)$ value than an $f_s^0(s)$ value, because $\Delta G_r^{0'}$ for this reaction (−186.51 kJ/e-eq) is lower than that for the single-step reaction (−163.61 kJ/e-eq), while the sum of each computational approach is very similar.

Using a molecular weight for cells of 113 g/mole (for $C_5H_7O_2N$), the cell yields (Table 6) are 1.39, 1.01, and 1.01 g cells/g phenanthrene for hydroxylation single-step, oxygenation single-step, and the sum of coupled reactions, respectively. The yield is 0.89 g cells/g phenanthrene if the 6 oxygenation reactions and the 24 electrons involved in them are eliminated from the computation of $\Delta G_r^{0'}$. Table 7 summarizes experimentally estimated yield values for phenanthrene. In some cases, lower yield values have been reported (Aichinger et al. 1992; Bouchez et al. 1996; Keuth and Rehm 1991; Stucki and Alexander 1987; Weissenfels et al. 1990; Wodzinski and Johnson 1968). However, the experimental conditions in these studies significantly affected the apparent yield values, making them incomparable to our model predictions. In many cases, maintenance or decay reduced the apparent cell yield, in particular with low specific growth rates and long-term cultivation (e.g., 10 days in Bouchez et al. (1996) and Stucki and Alexander (1987)). In other cases, accumulations of intermediates or dead-end products (e.g., Aichinger et al. 1992; Bouchez et al. 1996; Keuth and Rehm 1991; Wodzinski and Johnson 1968) sequestered electrons and carbon ($H > 0$), thereby reducing the observed yields per phenanthrene consumed. Finally, non-optimal nutrient sources could bring about lower cell yield values; e.g., nitrate, used in Bouchez et al. (1996) and Weissenfels et al. (1990), or unknown nitrogen sources, used in Guha and Jaffe (1996), incurs additional synthesis costs (McCarty 1969) that reduce yields. Only the study of Zhang et al. (1994) avoided these complications and provided yield values directly comparable to our predictions. The yields from the two oxygenation methods conform best to the comparable experimental yield reported in the literature, 1.0 g cells/g phenanthrene (Zhang et al. 1997), than does the hydroxylation method (1.39 g cells/g phenanthrene) or ignoring all energy associated with oxygenation reactions (0.89 g cells/g phenanthrene).

We used the same methods to estimate yield values for another PAH compound, naphthalene, for which experimental yield values are more often reported in the literature. The coupled-reaction model has 2 steps and the intermediate is salicylate. The predicted yield values are 1.411, 1.059, 1.058, and 0.94 g cells/g naphthalene for hydroxylation single-step, oxygenation single-step, the sum of coupled reactions, and ignoring all oxygenation reactions, respectively. Similar to the case of phenanthrene, the yields for the two oxygenation methods conform best to the two com-

Table 7. Cell yield values and cultivation conditions reported in the literature. Boldface type indicates that the experimental conditions did not conform to our model conditions

Reference	Reported yield value	Estimated yield value	Cultivation time	Intermediate production	N source
Wodzinski & Johnson (1968)	0.4[a]	–	**NI**[g]	**Yes**	NH_4^+
Stucki & Alexander (1987)	3.5[b]	0.55[a]	**10 d**	**NI**[g]	NH_4^+
Weissenfels et al. (1990)	0.24[c]	0.45[a]	2 d	ND[h]	**NH_4NO_3**
Keuth & Rehm (1991)	74.4[d]	0.84[a]	30 h	**Yes**	NH_4^+
Aichinger et al. (1992)	0.33[e]	0.69[a]	**NI**[g]	**0.08**[e]	**NI**[g]
Stringfellow & Aitken (1994)	1.3[a]	–	**NI**[g]	ND[h]	NH_4^+
Guha & Jaffe (1996)	0.392[a]	–	24 h	**NI**[g]	**NI**[g]
Bouchez et al. (1996)	35[f]	0.63[a]	**10 d**	**14% C**	**NH_4NO_3**
Zhang et al. (1997)	1.0[a]	–	120 h	**NI**[g]	NH_4^+
Jahan et al. (1999)	1.02[a]	–	120 h	**NI**[g]	**NO_3^-**

[a]g cells/g phenanthrene. [b]g protein/mol C. [c]mg protein/mg C. [d]mg protein/mmol phenanthrene. [e]mg COD/mg COD. [f]% carbon. [g]NI, not indicated. [h]ND, not detected.

parable yields reported in the literature: 0.93 ± 0.12 g cells/g naphthalene (Buitron and Capdeville 1993) and 1.2 g cells/g naphthalene (Volkering et al. 1993).

A physiological basis for the coupled-reaction model is supported by other information reported in the literature. The first supporting evidence is the typical pattern of intermediate accumulation. The intermediate 1-hydroxy-2-naphthoate, which is the compound produced from the first-step in the coupled-reaction model, is the most frequently accumulated intermediate during phenanthrene degradation (Evans et al. 1965; Guerin and Jones 1988; Kiyohara and Nagao 1978). Although not frequently occurring, salicylate, the compound produced from the second coupled step, also is accumulated in the media during phenanthrene degradation via the naphthalene pathway or during naphthalene degradation (Evans et al. 1965; Wodzinski and Johnson 1968). On the other hand, other intermediate compounds are not found frequently in the media during phenanthrene degradation. For example, 1-hydroxy-2-naphthoate, instead of 3,4-dihydroxyphenanthrene (the product of reaction B), was detected during the enzyme reactions of crude cell extracts (Barnsley 1983b; Ensley et al. 1982; Evans et al. 1965).

The second type of supporting evidence is the organization of gene regulation. The upper pathway of phenanthrene degradation to 1-hydroxy-2-naphthoate is very similar to the pathway for naphthalene degradation to salicylate (Kiyohara et al. 1994). Sanseverino et al. (1993) demonstrated that NAH7, a plasmid encoding naphthalene degradation, and other NAH7-like plasmids could also mediate metabolism of phenanthrene and anthracene. Yang et al. (1994) also demonstrated that a single gene cluster can encode multiple PAH degradations. The biochemistry and genetics of the naphthalene degradation pathway contained on the NAH7 plasmid has been well characterized (Yen and Serdar 1988). Naphthalene-oxidation genes are organized in two operons. The first operon includes genes *nahABCDEF*, coding for the upper pathway, and the second operon includes genes *nahGHIJK*, coding for the lower pathway (salicylate oxidation via the catechol meta-cleavage) (Yen and Gunsalus 1985). Thus, the genes needed for the coupled reactions seem to be regulated as gene clusters in each operon. This suggests that cells may regulate several sequential reactions in order to link electron-requiring and -producing reactions. Thus, regulating the expressions of genes to correspond to the coupled reactions seems to be an efficient and economic strategy for ensuring an adequate supply of electron and energy for all the individual reactions.

The third type of support is the organization of enzymes for the coupled reactions into *multi-enzyme complexes* having coupled catalytic functions. In some cases, enzymes are bound to the surface of a membrane in adsorptive arrays or are more strongly incorporated into the lipid bilayer of the membrane as integral arrays (Friedrich 1984). Through structural organization, the proximity of active sites may then render it possible for the product of the first enzyme to get rapid access to the second enzyme. This physical juxtaposition of enzymes in a sequence can markedly accelerate the overall reaction rate without the spilling of intermediates (Gaertner 1978).

The location of enzymes involved in phenanthrene or naphthalene degradation has not been intensively studied. Several enzymes involved in degradation of naphthalene, phenanthrene, or benzene were purified from the supernatant of cell extracts, indicating that these enzymes probably are not integrated into the lipid bilayer (Axcell and Geary 1975; Ensley et al. 1982; Evans et al. 1965). Nevertheless, the enzymes may be associated with the cell membrane in adsorptive arrays, which may readily be separated from the membrane (Kurganov 1985). This hypothesis seems reasonable, since phenanthrene or naphthalene should be strongly associated with cell membrane surfaces due to their hydrophobic properties (Stringfellow and Alvarez-Cohen 1999); then, having the enzyme reactions occur at the membrane is an economic strategy, as addressed by Robertson and Button (1987) for toluene degradation. Barnsley (1983b) observed that the maximum rate of oxidation of naphthalene measured with whole cells was 348 nmol/min-mg protein, whereas with uncentrifuged disrupted cells it was 30 nmol/min-mg protein. For centrifuged extracts of the same organism, Ensley et al. (1982) reported a maximum rate of 4.6 nmol/min-mg protein. This indicates that separation of enzymes from cell structure may significantly reduce the enzyme activity. Thus, the enzymes in the coupled reactions may be associated with the membrane to some extent to take advantage of physiological proximity.

Conclusions

We evaluated the impact of oxygenation reactions on thermodynamic relationships and overall stoichiometry for biodegradation of phenanthrene as a model polynuclear aromatic compound. In particular, we used the half-reaction method for analyzing multi-step degradations that involved oxygenations. Since oxygenation reactions do not yield a net release of electrons, but require an input of electrons to reduce oxygen molecules, we extended the conventional energetic relationships to consider how electrons are supplied to these reactions.

Oxygenation reactions significantly altered the energy and electron flows, and this changed the stoichiometry for the complete mineralization of phenanthrene. Oxygenation reactions increased the oxygen requirement and reduced the cell yield, compared to the conventional mineralization represented by the hydroxylation method. The decrease in yield was not as large as the diversion of electrons to reduce O_2, due to the off-setting effect of the increased free energy release when O_2 is a direct cosubstrate.

For individual oxygenation reactions, the half-reaction method for analyzing the electron and energy flows is inadequate, because the reactions do not release electrons for synthesis or energy generation. We overcame this complication by coupling the oxygenation reaction to subsequent reaction steps so that the coupled reaction had a net electron release. We predicted the stoichiometry of each coupled reaction, as well as the sum of the coupled reactions. The yield for the sum of coupled reactions was similar to a single-step mineralization involving oxygenations, much smaller than for single-step mineralization in which hydroxylations replace all oxygenations, and larger than ignoring all energy released from oxygenation reactions

Oxygenation methods, single step or coupled, conformed better to empirical yields reported in the literature than did the hydroxylation single-step method or ignoring energy released from oxygenation reactions. Reported observations on what intermediates frequently accumulate, the regulation of the PAH-degrading genes, and the location of enzyme clusters also support that the coupled reactions have a solid physiological basis.

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Appendix A

Estimating standard free energy of formation using group contribution theory

In order to complete stoichiometric relationships for biological degradation reactions using half-reaction method, it is necessary to know the standard free energy of the donor, acceptor, and cell synthesis reactions. For relevant electron-acceptors and common organic electrondonors, $\Delta G_d^{0'}$ values are tabulated (McCarty 1969, 1971; Stumm and Morgan 1996). If the half reaction is not tabulated, the $\Delta G_d^{0'}$ value can be calculated from the standard free energy of formation ($\Delta G_f^{0'}$) for all species in the half reaction. For the

phenanthrene pathway, since the $\Delta G_f^{0'}$ values for the electron-donor substrate and intermediates were not found in the literature, the values were estimated using group contribution theory (Mavrovouniotis 1990, 1991).

The calculations of the standard free energies of formation for phenanthrene ($C_{14}H_{10}$), 1-hydroxy-2-naphthoate ($C_{11}H_7O_3^-$), and salicylate ($C_7H_5O_3^-$), which are relevant to coupled-reaction model, are shown in Table A1. The chemical structure for each compound is shown in Figure 3.

Table A1. Estimation of the free energy of formation for the compounds involved in the coupled-reactions in the phenanthrene pathway from contributions of groups

Compound	Group or correction	Number of occurrences	Contribution (kJ/mol)	Total contribution (kJ/mol)
$C_{14}H_{10}$	Origin[a]	1	−98.65	−98.65
	Correction for hydrocarbon	1	16.72	16.72
	—CH=[b]	10	35.11	351.1
	>C=[c]	4	10.45	41.8
	Total			310.99
$C_{11}H_7O_3^-$	Origin[a]	1	−98.65	−98.65
	—CH=[b]	6	35.11	210.67
	>C=[c]	2	10.45	20.9
	>C=[d]	2	6.27	12.54
	—OH[e]	1	−132.92	−132.92
	—COO^-	1	−300.96	−300.96
	Total			−288.42
$C_7H_5O_3^-$	Origin[a]	1	−98.65	−98.65
	—CH=[b]	4	35.11	140.45
	>C=[d]	2	6.27	12.54
	—OH[e]	1	−132.92	−132.92
	—COO^-	1	−300.96	−300.96
	Total			−379.54

[a] A contribution that must be added to every compound.
[b] Participating in a benzene ring.
[c] Participating in two fused benzene rings.
[d] The formal double bond and a formal single bond participating in a benzene ring.
[e] Attached to benzene ring.

Appendix B

Calculation of the free energy for the electron-donor half reaction

The overall free energy of the donor half reaction can be computed using the free energy of formations of all reactants and products in the half reaction. For example, for coupled reaction A–F, the electron-donor half reaction is:

$$\frac{1}{12}C_{11}H_7O_3^- + \frac{3}{12}H_2CO_3 + \frac{13}{12}H^+ + e^- = \frac{1}{12}C_{14}H_{10} + \frac{2}{12}O_2(g) + \frac{8}{12}H_2O.$$

For this reaction, the $\Delta G_f^{0'}$ values tabulated in the literature are −237.0, 0, −39.96, 0, and −622.4 kJ/mole for H_2O, $O_2(g)$, e^-, H^+ (with pH = 7.0), and H_2CO_3, respectively (Stumm and Morgan 1996), and the estimated values are 310.99 and −288.42 for $C_{14}H_{10}$ and $C_{11}H_7O_3^-$, respectively (see Appendix A). The free energy for this reaction is calculated as:

$$\begin{aligned}\Delta G_d^{0'}(\text{reaction}) &= \sum \Delta G_f^{0'}(\text{products}) - \sum \Delta G_f^{0'}(\text{reactants}) \\ &= \left(\tfrac{310.99}{12} + \tfrac{2\times 0}{12} + \tfrac{8\times(-237)}{12}\right) - \left(\tfrac{-288.42}{12} + \tfrac{3\times(-622.4)}{12} + \tfrac{13\times(-39.96)}{12} + 0\right) \\ &= 90.83 \text{ kJ/e-eq.}\end{aligned}$$

Nomenclature

A	electron equivalents of substrate converted for energy per electron equivalent of cells synthesized.
EQS	electron equivalents in one mole of substrate, e-eq/mole substrate.
f_e^0	fraction of electron-donor electron equivalents used for energy generation.
f_s^0	fraction of electron-donor electron equivalents used for cell synthesis.
$f_s^0(m)$	f_s^0 in each step of multi-step degradation, normalized to the number of electrons in an initial donor substrate.
$f_s^0(s)$	fraction of original electrons going to synthesis if the f_s^0 of the total reaction is apportioned according to the number of electrons released in each step.
$\Delta G_a^{0'}$	standard free energy for the electron acceptor reduction half reaction, kJ/e-eq.
$\Delta G_{cells}^{0'}$	standard free energy to synthesize macromolecules, kJ/e-eq.
$\Delta G_{cs}^{0'}$	standard free energy for the carbon source reduction half reaction, kJ/e-eq.

$\Delta G_d^{0'}$ standard free energy for the electron donor reduction half reaction, kJ/e-eq.

$\Delta G_r^{0'}$ standard free energy for the energy generating redox couple, kJ/e-eq.

$\Delta G_{syn}^{0'}$ standard free energy for the cell synthesis reaction, kJ/e-eq.

$\Delta G_{pyr}^{0'}$ standard free energy of formation of pyruvate, kJ/e-eq.

H fraction of electrons held in an intermediate per electron equivalents for a given step in the biodegradation pathway.

H' fraction of electrons held in an intermediate per mole substrate for a given step in the biodegradation pathway.

K efficiency of energy transfer.

m constant, equal to $+1$ when $\Delta G_{pyr}^{0'} - \Delta G_{cs}^{0'}$ is positive, and -1 when $\Delta G_{pyr}^{0'} - \Delta G_{cs}^{0'}$ is negative.

O fraction of electrons invested to oxygenation reaction per electron equivalents for a given step in the biodegradation pathway.

O' fraction of electrons invested to oxygenation reaction per mole substrate for a given step in the biodegradation pathway.

R fraction of electrons not sequestered in the intermediate per electron equivalents for a given step in the biodegradation pathway.

R' fraction of electrons not sequestered in the intermediate per mole substrate for a given step in the biodegradation pathway.

R_a reduction half reaction for electron-donor in terms of one electron equivalent.

R_c reduction half reaction for cell synthesis in terms of one electron equivalent.

R_d reduction half reaction for electron-acceptor in terms of one electron equivalent.

R_t overall stoichiometric equation for electron-donor biodegradation.

T fraction of electrons transferred from the substrate to either energy generation or biomass synthesis per electron equivalents for a given step in the biodegradation pathway.

T' fraction of electrons transferred from the substrate to either energy generation or biomass synthesis per mole substrate for a given step in the biodegradation pathway.

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