Chapter 3

Bacterial Growth

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Microorganisms conduct a series of highly organized chemical reactions that collectively are known as metabolism. There are several thousand potential reactions in a microbial cell, many of which are utilized to make new cells. These reactions are known as growth metabolism. Other reactions are termed nongrowth reactions, and are needed for cellular activity such as maintenance of intracellular metabolite pools, repair of cellular structures, motility and response to environmental stress (Schaechter *et al*., 2006). In the laboratory, we can manipulate conditions so that cells are undergoing growth metabolism most of the time. In the environment it is a different story—most microorganisms are in a nongrowth state, simply surviving and awaiting new nutrient sources.

Overall, metabolism is a complex process involving numerous anabolic (synthesis of cell constituents and metabolites) and catabolic (breakdown of cell constituents and metabolites) reactions. Ultimately, these biosynthetic reactions result in cell division as shown in Figure 3.1. In a homogeneous rich culture medium, under ideal conditions, a cell can divide in as little as 10 minutes. In contrast, it has been suggested that cell division may occur as slowly as once every 100 years in some subsurface terrestrial environments. Such slow growth is the result of a combination of factors including the fact that most subsurface environments are both nutrient poor and heterogeneous. As a result, cells are likely to be isolated, and cannot share nutrients or protection mechanisms, and therefore have slower growth rates.

Most information available concerning the growth of microorganisms is the result of controlled laboratory studies using pure cultures of microorganisms. There are two approaches to the study of growth under such controlled conditions: batch culture and continuous culture. In a batch culture, the growth of a single organism or a group of organisms, called a consortium, is evaluated using a defined medium to which a fixed amount of substrate (food) is added at the outset. In continuous culture, there is a steady influx of growth medium and substrate such that the amount of available substrate always remains the same. Growth under both batch and continuous culture conditions has been well characterized physiologically and also described mathematically. This information has been used to optimize the commercial production of a variety of microbial products including antibiotics, vitamins, amino acids, enzymes, yeast, vinegar and alcoholic beverages. These materials are often produced in large batches (up to 500,000 liters), also called large-scale fermentations.

Unfortunately, it is difficult to extend our knowledge of growth under controlled laboratory conditions to an understanding of growth in natural soil or water environments, where enhanced levels of complexity are encountered (Figure 3.2). This complexity arises from a number

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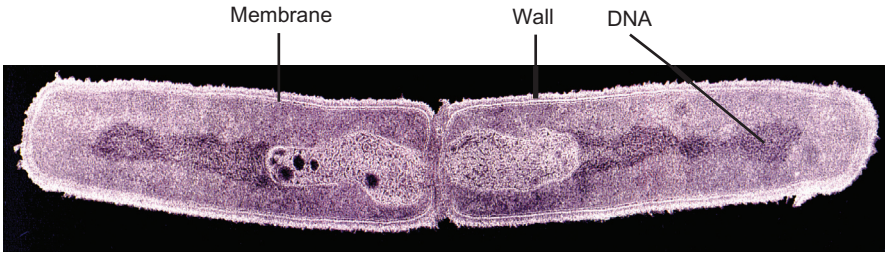


FIGURE 3.1 Gram positive *Bacillus subtilis* undergoing cell division. Reprinted with permission from Madigan and Martinko *et al*. (2006).

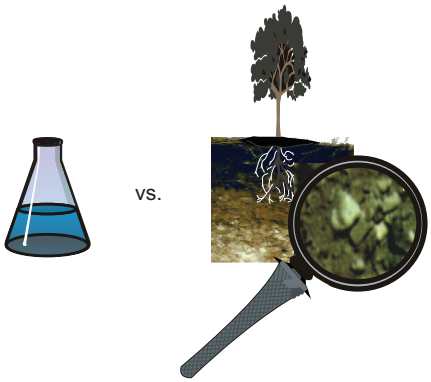


FIGURE 3.2 Compare the complexity of microbial growth in a flask and growth in a soil environment. Although we understand growth in a flask quite well, we still cannot always predict growth in the environment.

of factors, including an array of different types of solid surfaces, microenvironments that have altered physical and chemical properties, a limited nutrient status and consortia of different microorganisms all competing for the same limited nutrient supply (see Chapter 4). Thus, the current challenge facing environmental microbiologists is to understand microbial growth in natural environments. Such an understanding would facilitate our ability to predict rates of nutrient cycling (Chapter 16), microbial response to anthropogenic perturbation of the environment, microbial interaction with organic and metal contaminants (Chapters 17 and 18) and survival and growth of pathogens in the environment (Chapter 22). In this chapter, we begin with a review of growth under pure culture conditions, and then discuss how this compares to growth in the environment.

3.1 GROWTH IN PURE CULTURE IN A FLASK

Typically, to understand and define the growth of a particular microbial isolate, cells are placed in a liquid medium in which the nutrients and environmental conditions are controlled. If the medium supplies all nutrients required for growth and environmental parameters are optimal, the increase in numbers or bacterial mass can be measured as a function of time to obtain a growth curve. Several distinct growth phases can be observed within a growth curve (Figure 3.3). These include: the lag phase; the exponential or log phase; the stationary phase; and the death phase. Each of these phases represents a distinct period of growth that is associated with typical physiological changes in the cell culture. As will be seen in the following sections, the rates of growth associated with each phase are quite different.

3.1.1 The Lag Phase

The first phase observed under batch conditions is the lag phase in which the growth rate is essentially zero**.** When an inoculum is placed into fresh medium, growth begins after a period of time called the lag phase. By definition, the lag phase transitions to the exponential phase after the initial population have doubled (Yates and Smotzer, 2007). The lag phase is thought to be due to the physiological adaptation of the cell to the culture conditions. This may involve a time requirement for induction of specific messenger RNA (mRNA), and subsequent protein synthesis to meet new culture requirements. The lag phase may also be due to low initial densities of organisms that result in dilution of exoenzymes (enzymes released from the cell), and of nutrients that leak from growing cells. Normally, such materials are shared by cells in close proximity. But when cell density is low, these materials are diluted and are not taken up as easily. As a result, initiation of cell growth and division, and the transition to exponential phase growth may be delayed.

The lag phase usually lasts from minutes to several hours. The length of the lag phase can be controlled to some extent because it is dependent on the type of medium as well as on the initial inoculum size. For example, if an inoculum is taken from an exponential phase culture in trypticase soy broth (TSB), and placed into fresh TSB medium at a concentration of 10^6 cells/ml under the same growth conditions (temperature, shaking speed), there will be no noticeable lag phase. However, if the inoculum is taken from a stationary phase culture,

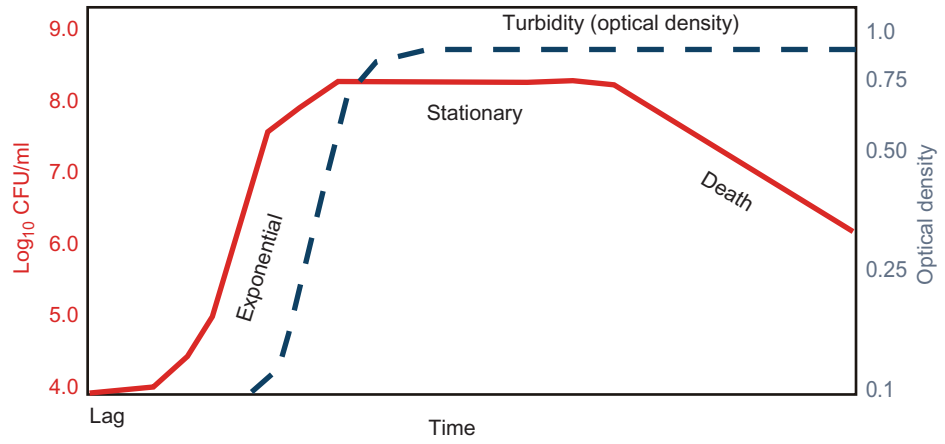


FIGURE 3.3 A typical growth curve for a bacterial population. Compare the difference in the shape of the curves in the death phase (colony-forming units versus optical density).

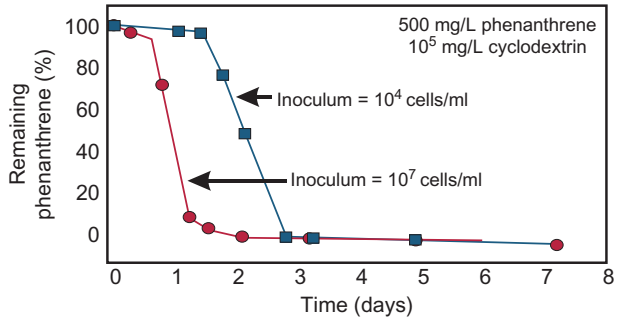


FIGURE 3.4 Effect of inoculum size on the lag phase during degradation of a polyaromatic hydrocarbon, phenanthrene. Because phenanthrene is only slightly soluble in water and is therefore not readily available for cell uptake and degradation, a solubilizing agent called cyclodextrin was added to the system. The microbes in this study were not able to utilize cyclodextrin as a source of carbon or energy. Courtesy E.M. Marlowe.

there will be a lag phase as the stationary phase cells adjust to the new conditions, and shift physiologically from stationary phase cells to exponential phase cells. Similarly, if the inoculum is placed into a medium other than TSB, for example, a mineral salts medium with glucose as the sole carbon source, a lag phase may be observed while the cells adjust physiologically to synthesize the appropriate enzymes for glucose catabolism.

Finally, if the inoculum size is small, for example 10^4 cells/ml, and one is measuring activity, such as disappearance of substrate, a lag phase will be observed until the population reaches approximately 10^6 cells/ml. This is illustrated in Figure 3.4, which compares the degradation of phenanthrene in cultures inoculated with 10^7 and with 10^4 colony-forming units (CFU) per ml. Although the degradation rate achieved is similar in both cases (compare the slope of each curve), the lag phase was 1.5 days when a low inoculum size was used (10^4 CFU/ml), in contrast to only 0.5 day when the higher inoculum was used (10^7 CFU/ml).

3.1.2 The Exponential Phase

The second phase of growth observed in a batch system is the exponential phase. The exponential phase is characterized by a period of exponential growth—the most rapid growth possible under the conditions present in the batch system. During exponential growth, the rate of increase of cells in the culture is proportional to the number of cells present at any particular time. There are several ways to express this concept both theoretically and mathematically. One way is to imagine that during exponential growth the number of cells increases in the geometric progression 2^0, 2^1, 2^2, 2^4 until, after n divisions, the number of cells is 2^n (Figure 3.5). This can be expressed in a quantitative manner as:

$$X = 2^n X_0 \qquad \text{(Eq. 3.1)}$$

where:

X_0 = initial concentration of cells
X = concentration after time t
n = number of generations or cell division

From Eq. 3.1 it follows that:

$$\ln X = n \ln 2 + \ln X_0 \qquad \text{(Eq. 3.2)}$$

During the exponential phase of growth, if the number of cells initially, and at any particular time thereafter, is known, the number of generations can be calculated from Eq. 3.3.

$$n = \frac{\ln X - \ln X_0}{0.693} \qquad \text{(Eq. 3.3)}$$

Example Calculation 3.1 shows that if one starts with a low number of cells, exponential growth does not initially produce large numbers of cells. However, as cells accumulate after several generations, the number of new cells with each cell division increases dramatically.

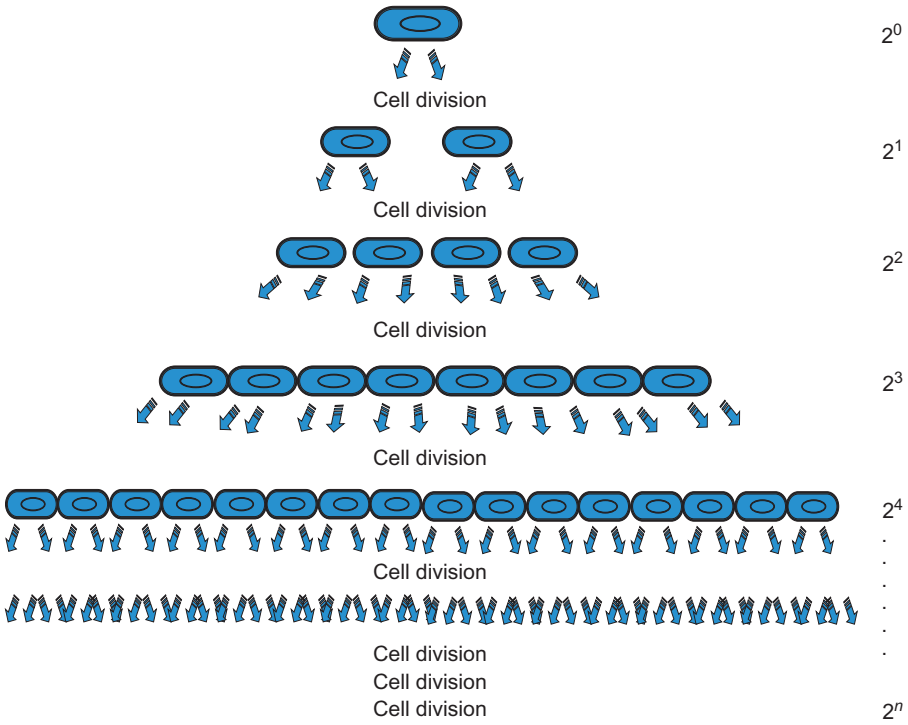


FIGURE 3.5 Exponential cell division. Each cell division results in a doubling of the cell number. At low cell numbers the increase is not very large; however, after a few generations, cell numbers increase explosively.

Example Calculation 3.1 Calculation of the Number of Cells in a Pure Culture

Problem: If one starts with 10,000 (10^4) cells in a culture that has a generation time of 2 h, how many cells will be in the culture after 4, 24, and 48 h?

From Eq. 3.1, $X = 2^n X_0$, where X_0 is the initial number of cells, n is the number of generations, and X is the number of cells after n generations.

After 4 h, $n = 4\text{h}/2\text{ h per generation} = 2$ generations:

$$X = 2^2(10^4) = 4.0 \times 10^4 \text{ cells}$$

After 24 h, $n = 12$ generations:

$$X = 2^{12}(10^4) = 4.1 \times 10^7 \text{ cells}$$

After 48 h, $n = 24$ generations:

$$X = 2^{24}(10^4) = 1.7 \times 10^{11}$$

This represents an increase of less than one order of magnitude for the 4-h culture, four orders of magnitude for the 24-h culture, and seven orders of magnitude for the 48-h culture.

Example Calculation 3.2 demonstrates how the number of generations and the mean generation time can be calculated.

As long as a culture is in the exponential or logarithmic phase, the culture is said to be undergoing balanced growth.

3.1.2.1 Mean Generation Time Versus Specific Growth Rate

Two terms that are used to describe growth in the exponential phase are generation time and specific growth rate. The generation time refers to the time needed for cell doubling while the specific growth rate is the maximum growth rate that can be achieved given the environmental conditions present (unlimited substrate, temperature, etc.). When substrate becomes limiting or toxic by-products build up, cells will leave the exponential phase and, correspondingly, the specific growth rate will decrease.

In mathematical terms in the exponential phase:

$$\frac{dX}{dt} = \mu X \qquad \text{(Eq. 3.4)}$$

where:

dX/dt = change in cell number X during time t
μ = specific growth rate expressed as reciprocal time (hours^{-1})

Example Calculation 3.2 Calculation of Mean Generation Time

Following a dilution and plating experiment, the following data were obtained:

At the beginning of exponential growth:

$$t_0 = 0$$
$$X_0 = 1000 \text{ cells/ml}$$

At time $t = 6$ hours:

$$X = 16,000 \text{ cells/ml}$$

Using Eq. 3.3:

$$n = \frac{ln\, X - ln\, X_0}{0.693}$$
$$n = \frac{ln\, 16,000 - ln\, 1000}{0.693}$$
$$\therefore n = \frac{9.7 - 6.9}{0.693}$$

And

$$n = \frac{1.204}{0.693} = 4 \text{ generations}$$

$\therefore$ Since there are 4 generations in 6 hours, the mean generation time = 6/4 = 1.5 hours.

By integration:

$$X/X_0 = e^{\mu t} \qquad \text{(Eq. 3.5)}$$

Taking the natural log (ln) of both sides:

$$\ln X/X_o = ln\, X - ln\, X_o = \mu t$$
$$\therefore \mu = \frac{ln\, X - ln\, X_0}{t} \qquad \text{(Eq. 3.6)}$$

Thus, the specific growth rate in exponential phase is the slope of the growth curve. See Example Calculation 3.3 for an illustration of how to determine specific growth rates.

To calculate the generation time (g), we consider the special case where X_0 is doubled. In the simplest case we can consider when one cell becomes two cells and $X = 2$ while $X_0 = 1$:

$$\therefore \mu = \frac{0.693 - 0}{g}$$
$$\therefore \mu = \frac{0.693}{g} \qquad \text{(Eq. 3.7)}$$

3.1.3 The Stationary Phase

The third phase of growth is the stationary phase. The stationary phase in a batch culture can be defined as a state of no net growth, which can be expressed by the following equation:

$$\frac{dX}{dt} = 0 \qquad \text{(Eq. 3.8)}$$

Although there is no net growth in stationary phase, cells still grow and divide. Growth is simply balanced by an equal number of cells dying. There are several reasons why a batch culture may reach stationary phase. One reason is that the carbon and energy source or an essential nutrient becomes limiting. When a carbon source is used up it does not necessarily mean that all growth stops. This is because dying cells can lyse and provide a recycled source of nutrients. Growth resulting from dead cells is called endogenous metabolism. Endogenous metabolism occurs throughout the growth cycle, but can be best observed during the stationary phase when growth is measured in terms of oxygen uptake or evolution of carbon dioxide. Thus, in many growth curves such as that shown in Figure 3.6, the stationary phase can actually show a small amount of growth. Again, this growth occurs after the substrate has been utilized, and reflects the use of dead cells as a source of carbon and energy. A second reason that the stationary phase may be observed is that waste products build up to a point where they begin to inhibit cell growth or are toxic to cells. This generally occurs only in cultures with high cell density. Regardless of the reason why cells enter the stationary phase, growth in the stationary phase is referred to as unbalanced growth because it is easier for the cells to synthesize some components than others. As some components become more and more limiting, cells will still keep growing and dividing as long as possible. As a result of this nutrient stress, stationary phase cells are generally smaller and rounder than cells in the exponential phase. Ultimately, since the reuse of some cell components is not 100% efficient, more cells die than new cells are produced, and the culture will enter the death phase.

3.1.4 The Death Phase

The final phase of the growth curve is the death phase, which is characterized by a net loss of culturable cells. Even in the death phase there may be individual cells that are metabolizing and dividing, but more viable cells are lost than are gained so there is a net loss of viable cells. The death phase is often exponential, although the rate of cell death is usually slower than the rate of

Example Calculation 3.3 Calculation of Specific Growth Rate

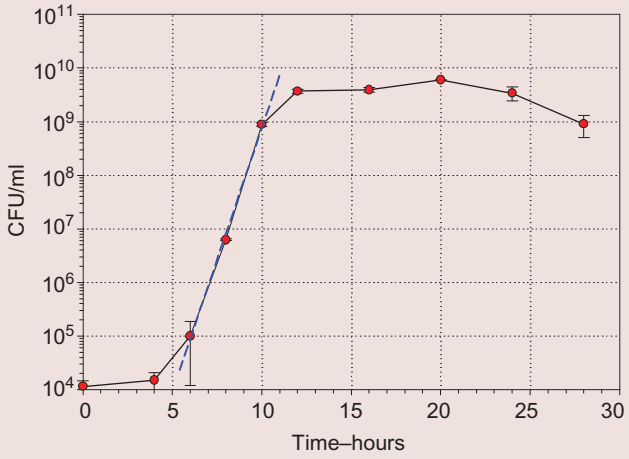
Problem: The following data were collected using a culture of *Pseudomonas* during growth in a minimal medium containing salicylate as a sole source of carbon and energy. Using these data, calculate the specific growth rate for the exponential phase.

Time(h)	Culturable Cell Count (CFU/ml)
0	1.2×10^4
4	1.5×10^4
6	1.0×10^5
8	6.2×10^6
10	8.8×10^8
12	3.7×10^9
16	3.9×10^9
20	6.1×10^9
24	3.4×10^9
28	9.2×10^8

The times to be used to determine the specific growth rate can be chosen by visual examination of a semilog plot of the data (see figure). Examination of the graph shows that the exponential phase is from approximately 6 to 10 hours. Using Eq. 3.6, which describes the exponential phase of the graph, one can determine the specific growth rate for this *Pseudomonas*. (Note that Eq. 3.4 describes a line, the slope of which is μ, the specific growth rate.) From the data given, the slope of the graph from time 6 to 10 hours is:

$$\mu = \frac{ln\ 10^9 - ln\ 10^5}{10 - 6} 2.3\ \text{hour}^{-1}$$

It should be noted that the specific growth rate and generation time calculated for growth of the *Pseudomonas* on salicylate are valid only under the experimental conditions used. For example, if the experiment were performed at a higher temperature, one would expect the specific growth rate to increase. At a lower temperature, the specific growth rate would be expected to decrease.



growth during the exponential phase. The death phase can be described by the following equation:

$$\frac{dX}{dt} = -k_d X \qquad \text{(Eq. 3.9)}$$

where k_d = the specific death rate.

It should be noted that the way in which cell growth is measured can influence the shape of the growth curve. For example, if growth is measured by optical density instead of by plate counts (compare the two curves in Figure 3.3), the onset of the death phase is not readily apparent. Similarly, if one examines the growth curve measured in terms of carbon dioxide evolution shown in Figure 3.6, again it is not possible to discern the death phase. Still, these are approaches commonly used to measure growth, because normally, the growth phases of most interest to environmental microbiologists are the lag phase, the exponential phase and the time to the onset of the stationary phase.

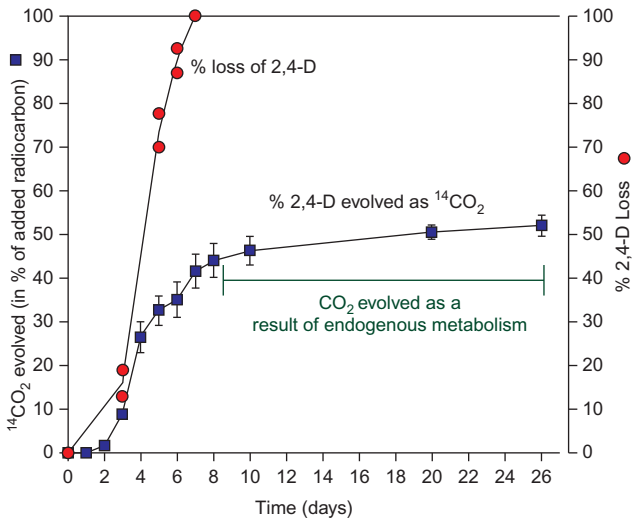


FIGURE 3.6 Mineralization of the broadleaf herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in a soil slurry under batch conditions. Note that the 2,4-D is completely utilized after 6 days but the CO_2 evolved continues to rise slowly. This is a result of endogenous metabolism. *From* Estrella *et al*. (1993).

3.1.5 Effect of Substrate Concentration on Growth

So far we have discussed each of the growth phases and have shown that each phase can be described mathematically (see Eqs. 3.1, 3.8 and 3.9). One can also write equations to allow description of the entire growth curve. Such equations become increasingly complex. For example, one of the first and simplest descriptions is the Monod equation, which was developed by Jacques Monod in the 1940s:

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad \text{(Eq. 3.10)}$$

where:

μ = the specific growth rate (1/time)
μ_{max} = the maximum specific growth rate (1/time) for the culture
S = the substrate concentration (mass/volume)
K_s = the half-saturation constant (mass/volume) also known as the affinity constant

Equation 3.10 was developed from a series of experiments performed by Monod. The results of these experiments showed that at low substrate concentrations, growth rate becomes a function of the substrate concentration (note that Eqs. 3.1 to 3.9 are independent of substrate concentration). Thus, Monod designed Eq. 3.10 to describe the relationship between the specific growth rate and the substrate concentration. There are two constants in this equation, μ_{max}, the maximum specific growth rate, and K_s, the half-saturation constant, which is defined as the substrate concentration at which growth occurs at one-half the value of μ_{max}. Both μ_{max} and K_s reflect intrinsic physiological properties of a particular type of microorganism. They also depend on the substrate being utilized and on the temperature of growth (see Information Box 3.1). Monod assumed in writing Eq. 3.10 that no nutrients other than the substrate are limiting and that no toxic by-products of metabolism build up.

As shown in Eq. 3.11, the Monod equation can be expressed in terms of cell number or cell mass (X) by equating it with Eq. 3.4:

$$\frac{dX}{dt} = \frac{\mu_{max} S X}{K_s + S} \qquad \text{(Eq. 3.11)}$$

The Monod equation has two limiting cases (see Figure 3.7). The first case is at high substrate concentration where $S >> K_s$. In this case, as shown in Eq. 3.12, the specific growth rate μ is essentially equal to μ_{max}. This simplifies the equation and the resulting relationship is zero order or independent of substrate concentration:

$$\text{for } S \gg K_s: \quad \frac{dX}{dt} = \mu_{max} X \qquad \text{(Eq. 3.12)}$$

Under these conditions, growth will occur at the maximum growth rate. There are relatively few instances in which ideal growth as described by Eq. 3.12 can occur. One such instance is under the initial conditions found in pure culture in a batch flask when substrate and nutrient levels are high. Another is under continuous culture conditions, which are discussed further in Section 3.2. It must be emphasized that this type of growth is likely to be rare under natural conditions in a soil or water environment, where either substrate or other nutrients are commonly limiting.

The second limiting case occurs at low substrate concentrations where $S << K_s$ as shown in Eq. 3.13. In this case there is a first-order dependence on substrate concentration (Figure 3.7):

$$\text{for } S \ll K_s: \quad \frac{dX}{dt} = \frac{\mu_{max} S X}{K_s} \qquad \text{(Eq. 3.13)}$$

As shown in Eq. 3.13, when the substrate concentration is low, growth (dX/dt) is dependent on the substrate concentration. Since the substrate concentration is in the numerator, as the substrate concentration decreases, the rate of growth will also decrease. This type of growth is typically found in batch flask systems at the end of the growth curve when almost all substrate has been consumed. This is also the type of growth that would be more typically expected under the conditions found in a natural environment, where substrate and nutrients are limiting.

The Monod equation can also be expressed as a function of substrate utilization given that growth is related to substrate utilization by a constant called the cell yield (Eq. 3.14):

$$\frac{dS}{dt} = -\frac{1}{Y}\frac{dX}{dt} \qquad \text{(Eq. 3.14)}$$

Information Box 3.1 The Monod Growth Constants

Both μ_{max} and K_s are constants that reflect:

- The intrinsic properties of the degrading microorganism
- The limiting substrate
- The temperature of growth

The following table provides representative values of μ_{max} and K_s for growth of different microorganisms on a variety of substrates at different temperatures and for oligotrophs and copiotrophs in soil.

Organism	Growth Temperature (°C)	Limiting Nutrient	μ_{max} (L/h)	K_s (mg/L)
Escherichia coli	37	Glucose	0.8–1.4	2–4
Escherichia coli	37	Lactose	0.8	20
Saccharomyces cerevisiae	30	Glucose	0.5–0.6	25
Pseudomonas sp.	25	Succinate	0.38	80
Pseudomonas sp.	34	Succinate	0.47	13
Oligotrophs in soil			0.01	0.01
Copiotrophs in soil			0.045	3

Source: Adapted from Blanch and Clark (1996), Miller and Bartha (1989), Zelenev *et al.* (2005).

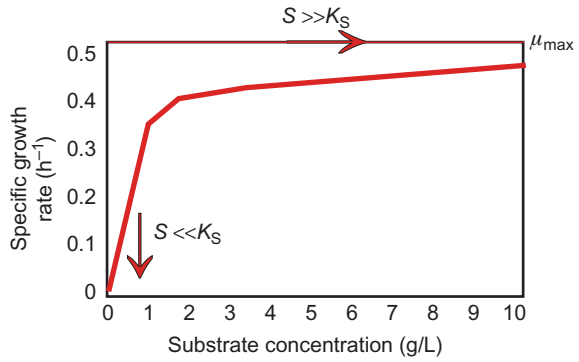


FIGURE 3.7 Dependence of the specific growth rate, μ, on the substrate concentration. The maximal growth rate, $\mu_m = 0.5\ h^{-1}$ and $K_s = 0.5$ g/L. Note that μ approaches μ_{max} when $S >> K_s$ and becomes independent of substrate concentration. When $S << K_s$, the specific growth rate is very sensitive to the substrate concentration, exhibiting a first-order dependence.

where Y = the cell yield (mass/mass). The cell yield coefficient is defined as the unit amount of cell mass produced per unit amount of substrate consumed. Thus, the more efficiently a substrate is degraded, the higher the value of the cell yield coefficient (see Section 3.3 for more details). The cell yield coefficient is dependent on both the structure of the substrate being utilized and the intrinsic physiological properties of the degrading microorganism. As shown below, Eqs. 3.11 and 3.14 can be combined to express microbial growth in terms of substrate disappearance:

$$\frac{dS}{dt} = -\frac{1}{Y}\frac{\mu_{max} S X}{K_s + S} \qquad \text{(Eq. 3.15)}$$

Figure 3.8 shows a set of growth curves constructed from a fixed set of constants. The growth data used to generate this figure were collected by determining protein as a measure of the increase in cell growth (see Chapter 11). The growth data were then used to estimate the growth constants μ_{max}, K_s and Y. Both Y and μ_{max} were estimated directly from the data. K_s was estimated using a mathematical model that performs a nonlinear regression analysis of the simultaneous solutions to the Monod equations for cell mass (Eq. 3.11) and substrate (Eq. 3.14). This set of constants was then used to model or simulate growth curves that express growth in terms of CO_2 evolution and substrate disappearance. Such models are useful because they can help to: (1) estimate growth constants such as K_s that are difficult to determine experimentally; and (2) quickly understand how changes in any of the experimental parameters affect growth without performing a long and tedious set of experiments.

3.2 CONTINUOUS CULTURE

Thus far, we have focused on theoretical and mathematical descriptions of batch culture growth, which is currently of great economic importance in terms of the production of a wide variety of microbial products. In contrast to batch culture, continuous culture is a system that is designed for long-term operation. Continuous culture can be operated over the long term because it is an open system (Figure 3.9), with a continuous feed of influent solution that contains nutrients and substrate. It also contains a continuous drain of effluent solution that has

cells, metabolites, waste products and any unused nutrients and substrate. The vessel that is used as a growth container in continuous culture is called a bioreactor or a chemostat. In a chemostat, one can control the flow rate and maintain a constant substrate concentration, as well as provide continuous control of pH, temperature and oxygen levels. This allows control of the rate of growth, which can be used to optimize the production of specific microbial products. For example, primary metabolites or growth-associated products, such as ethanol, are produced at high flow or dilution rates which stimulate cell growth. In contrast, a secondary metabolite or nongrowth-associated product such as an antibiotic is produced at low flow or dilution rates which maintain high cell numbers. Chemostat cultures are also being used to aid in the study of the functional genomics of growth, nutrient limitation and stress responses at the whole-organism level. The advantage of the chemostat in such studies is the constant removal of secondary growth effects that may mask or alter subtle physiological changes under batch culture conditions (Hoskisson and Hobbs, 2005).

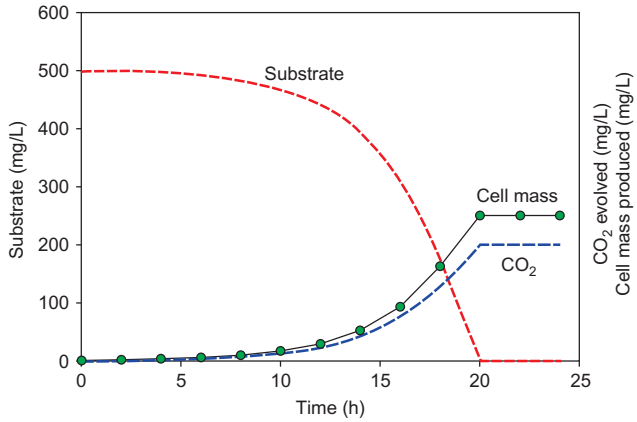


FIGURE 3.8 This figure shows the same growth curve expressed three different ways; in terms of substrate loss, in terms of CO_2 evolution and in terms of increasing cell mass. The parameters used to generate these three curves were: $\mu_{max} = 0.29\ h^{-1}$, $K_s = 10$ mg/L, $Y = 0.5$, initial substrate concentration = 500 mg/L and initial cell mass = 1 mg/L. In this experiment, cell mass was measured and so the data points are shown. The data for CO_2 evaluation and substrate loss were simulated using a model and so the data are shown using dashed lines.

Dilution rate and influent substrate concentration are the two parameters controlled in a chemostat to study microbial growth or to optimize metabolite production. The dynamics of these two parameters are shown in Figure 3.10. By controlling the dilution rate, one can control the growth rate (μ) in the chemostat, represented in this graph as doubling time (recall that during the exponential phase the growth rate is proportional to the number of cells present). By controlling the influent substrate concentration, one can control the number of cells produced or the cell yield in the chemostat since the number of cells produced will be directly proportional to the amount of substrate provided. Because the growth rate and the cell number can be controlled independently, chemostats have been an important tool for the study of the physiology of microbial growth. They are also useful in the long-term development of cultures and consortia that are acclimated to organic contaminants that are toxic and difficult to degrade. Chemostats can also produce microbial products more efficiently than batch fermentations. This is because a chemostat can essentially hold a culture in the exponential phase of growth for extended periods of time. Despite these advantages, chemostats are not yet widely used to

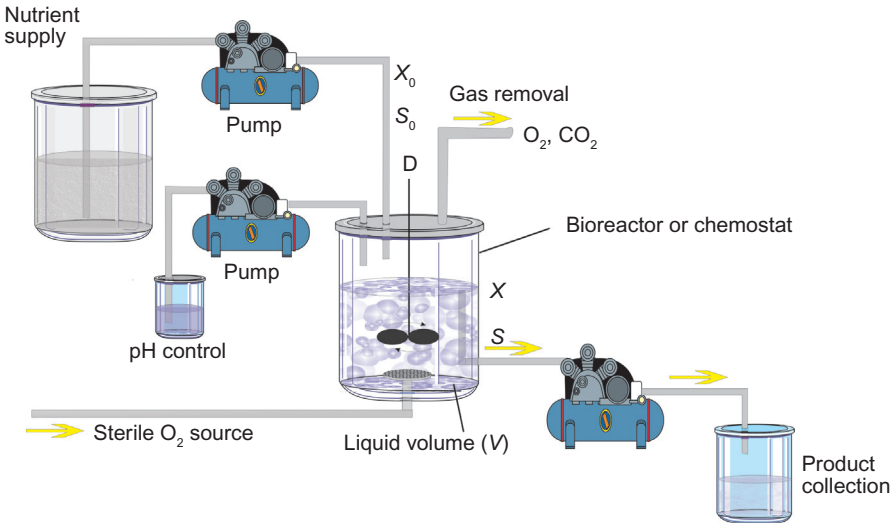


FIGURE 3.9 Schematic representation of a continuously stirred bioreactor. Indicated are some of the variables used in modeling bioreactor systems. X_0 is the dry cell weight, S_0 is the substrate concentration and D is the flow rate of nutrients into the vessel.

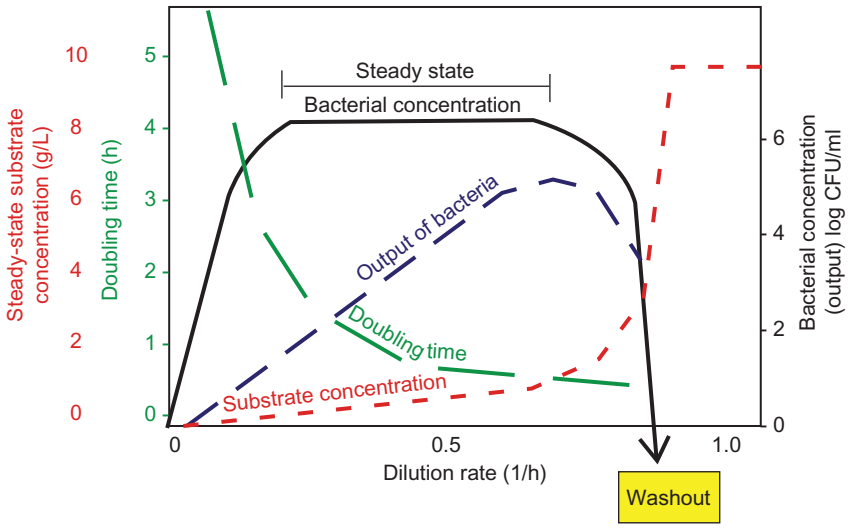


FIGURE 3.10 Steady-state relationships in the chemostat. The dilution rate is determined from the flow rate and the volume of the culture vessel. Thus, with a vessel of 1000 ml and a flow rate through the vessel of 500 ml/h, the dilution rate would be 0.5 L/h^{-1}. Note that at high dilution rates, growth cannot balance dilution and the population washes out. Thus, the substrate concentration rises to that in the medium reservoir (because there are no bacteria to use the inflowing substrate). However, throughout most of the range of dilution rates shown, the population density remains constant and the substrate concentration remains at a very low value (that is, steady state). Note that although the population density remains constant, the growth rate (doubling time) varies over a wide range. Thus, the experimenter can obtain populations with widely varying growth rates without affecting population density. Adapted with permission from Madigan and Martinko (2006).

produce commercial products because it is often difficult to maintain sterile conditions over a long period of time.

In a chemostat, the growth medium undergoes constant dilution with respect to cells due to the influx of nutrient solution (Figure 3.9). The combination of growth and dilution within the chemostat will ultimately determine growth. Thus, in a chemostat, the change in biomass with time is:

$$\frac{dX}{dt} = \mu X - DX \qquad \text{(Eq. 3.16)}$$

where:

X = the cell mass (mass/volume)
μ = the specific growth rate (1/time)
D = the dilution rate (1/time)

Examination of Eq. 3.16 shows that a steady state (no increase or decrease in biomass) will be reached when $\mu = D$. If $\mu > D$, the utilization of substrate will exceed the supply of substrate, causing the growth rate to slow until it is equal to the dilution rate. If $\mu < D$, the amount of substrate added will exceed the amount utilized. Therefore, the growth rate will increase until it is equal to the dilution rate. In either case, given time, a steady state will be established where:

$$\mu = D \qquad \text{(Eq. 3.17)}$$

Such a steady state can be achieved and maintained as long as the dilution rate does not exceed a critical rate, D_c. The critical dilution rate can be determined by combining Eqs. 3.10 and 3.17:

$$D_c = \mu_{max}\left(\frac{S}{K_s + S}\right) \qquad \text{(Eq. 3.18)}$$

Looking at Eq. 3.18, it can be seen that the operation efficiency of a chemostat can be optimized under conditions in which $S >> K_s$, and therefore $D_c \approx \mu_{max}$. But it must be remembered that when a chemostat is operating at D_c, if the dilution rate is increased further, the growth rate will not be able to increase (since it is already at μ_{max}) to offset the increase in dilution rate. The result will be washing out of cells and a decline in the operating efficiency of the chemostat. Thus, D_c is an important parameter because if the chemostat is run at dilution rates less than D_c, operation efficiency is not optimized; whereas if dilution rates exceed D_c, washout of cells will occur as shown in Figure 3.10.

3.3 GROWTH IN THE ENVIRONMENT

How is growth in the natural environment related to growth in a flask or in continuous culture? There have been several attempts to classify bacteria in soil systems on the basis of their growth characteristics and affinity for carbon substrates. The first was by Sergei Winogradsky (1856–1953), the "father of soil microbiology," who introduced the ecological classification system

of autochthonous versus zymogenous organisms. The former metabolize slowly in soil, utilizing slowly released nutrients from soil organic matter as a substrate. The latter are adapted to intervals of dormancy when substrate availability is low, or to rapid growth following the addition of fresh substrate or amendment to the soil. In addition to these two categories, there are the allochthonous organisms, which are organisms that are freshly introduced into soil and usually survive for only short periods of time (Figure 3.11).

Current terminology distinguishes soil microbes as either oligotrophs, those that prefer low substrate concentrations, or copiotrophs, those that prefer high substrate concentrations. This is similar to the concept of r and K selection. Organisms that respond to added nutrients with rapid growth rates are designated as r-strategists, while K-strategists have low but consistent growth rates and numbers in low nutrient environments. In reality, a soil community normally has a continuum of microorganisms with various levels of nutrient requirements ranging from obligate r-strategists, or copiotrophs, to obligate K-strategists, or oligotrophs. Typical maximum growth rates (μ_{max}) and affinity constants (K_s) for these two groups of microbes are given in Information Box 3.1.

When considering oligotrophic microbes in the environment, it is unlikely that they exhibit the stages of growth observed in batch flask and continuous culture.

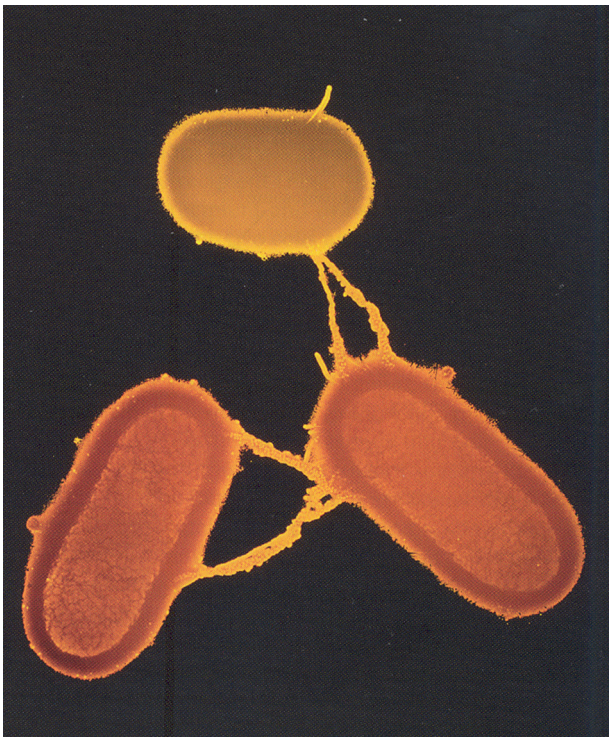


FIGURE 3.11 Conjugating cells of *Escherichia coli*. False-color transmission electron micrograph (TEM) of a male *E. coli* bacterium (bottom right) conjugating with two females. This male has attached two F-pili to each of the females.

These microbes metabolize slowly, and as a result have long generation times. Frequently, they use energy obtained from metabolism simply for cell maintenance. On the other hand, copiotrophic organisms may exhibit high rates of metabolism and perhaps exponential growth for short periods, or may be found in a dormant state. Note also that exponential growth does not normally persist for long periods of time in the environment. Rather, bacteria frequently alternate between periods of growth and nongrowth, i.e., constantly entering and leaving the stationary phase (Schaechter *et al*., 2006). Dormant cells are often rounded and small (approximately 0.3 μm) in comparison with healthy laboratory specimens, which range from 1 to 2 μm in size. Dormant cells may become viable but nonculturable (VBNC) with time because of extended starvation conditions or because cells become reversibly damaged (Roszak and Collwell, 1987). VBNC microbes are thus difficult to culture because of cell stress and damage. Specific microbes may also be difficult to culture for several reasons, which are discussed in Section 10.3.1. New approaches for enhanced cultivation of soil bacteria are defined in Section 10.3.1.1.

Both of these cases contribute to the fact that direct counts from environmental samples, which include all viable cells, are often one to two orders of magnitude higher than culturable counts, which include only cells capable of growth on the culture medium used. When a soil culture is plated on a solid medium, a subset of the community which is dominated by copiotrophs quickly takes advantage and begins to metabolize actively. In a sense, this is similar to the reaction by microbes in a batch flask when nutrients are added. Studies utilizing both culture-dependent and culture-independent methodologies on the same samples have shown that culturing bacteria from soil actually selects for less abundant species, or members of the "soil rare biosphere" (Shade *et al*., 2012) (see also Case Study 4.2 and Section 10.3.1.2). Thus, these microbes can exhibit the growth stages described in Section 3.2 for batch and continuous culture, but the pattern of the stages is quite different as described in the following sections.

3.3.1 The Lag Phase

The lag phase observed in a natural environment can be much longer than the lag phase normally observed in a batch culture. In some cases, this longer lag phase may be caused by very small numbers of initial populations that are capable of metabolizing the added substrate. Note also that chemicals which humans may consider to be organic contaminants can be a useful source of substrate for growth for those microbes with the necessary set of enzymes to degrade the contaminant. In this case, neither a significant disappearance of the contaminant nor a

significant increase in cell numbers will be observed for several generations. Note that in a pure culture, the transition between the lag and exponential phase is defined to have occurred after the initial population has doubled. However, this is a troublesome definition to impose in the environment, where it is difficult to accurately measure the doubling of a small subset of the microbial community that is responding to the addition of nutrients including contaminants. Alternatively, populations capable of metabolizing the substrate may be dormant or injured, and require time to recover physiologically and resume metabolic activities. Further complicating growth in the environment is the fact that generation times are usually much longer than those measured under ideal laboratory conditions. This is due to a combination of limited nutrient availability and environmental conditions such as temperature or moisture that are not optimal for specific microbes. Thus, it is not unusual to observe lag periods of months or even years after an initial application of a newly synthesized anthropogenic pesticide, for significant degradation to be observed. However, once an environment has been exposed to a new pesticide and developed a community for its degradation, the degradation of succeeding pesticide applications will occur with shorter and shorter lag periods. This phenomenon is called acclimation or adaptation, and has been observed with successive applications of many pesticides including the broad-leaf herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Newby *et al*., 2000).

A second explanation for long lag periods in the environment is that a community with microbes genetically capable of utilizing a specific carbon source may not initially be present within the existing population. This situation may require a mutation or a gene transfer event to introduce appropriate degradative genes into indigenous microbes. For example, one of the first documented cases of gene transfer in soil was the transfer of the plasmid pJP4 from an introduced organism into an indigenous soil population. The plasmid transfer resulted in rapid and complete degradation of the herbicide 2,4-D within a microcosm (Case Study 3.1). In this study, gene transfer to indigenous soil recipients was followed by growth and survival of the transconjugants at levels significant enough to affect degradation. There are still few such studies, and the likelihood and frequency of gene transfer in the environment are topics that are currently under debate.

3.3.2 The Exponential Phase

In the environment, the second phase of growth, exponential growth, occurs for only very brief periods of time following addition of a substrate. Such substrate might be crop residues, vegetative litter, root residues or contaminants added to or spilled into the environment. Following substrate addition, it is the zymogenous cells, many of which are initially dormant, that can most quickly respond to added nutrients. Upon substrate addition, these dormant cells become physiologically active and briefly enter the exponential phase until the substrate is utilized, or until some limiting factor causes a decline in substrate degradation. Thus, in many environmental samples, bacteria alternate between short periods of exponential or balanced growth and subsequent nongrowth or unbalanced growth (Schaechter *et al*., 2006). Thus, such bacteria may constantly leave and re-enter the stationary phase. As shown in Table 3.1, culturable cell counts rapidly increase one to two orders of magnitude in response to the addition of 1% glucose. In this experiment, four different soils were left untreated or were amended with 1% glucose and incubated at room temperature for 1 week.

Because nutrient levels and other factors, e.g., temperature or moisture, are seldom ideal, it is rare for cells in the environment to achieve a growth rate equal to μ_{max}. Thus, rates of growth in the environment are slower than growth rates measured under laboratory conditions. This is illustrated in Table 3.2, which compares the metabolism or degradation rates for wheat and rye straw in a laboratory environment with degradation rates in natural environments. These include: a Nigerian tropical soil that undergoes some dry periods; an English soil that is exposed to a moderate, wet climate; and a soil from Saskatoon, Canada, that is subjected to cold winters and dry summers. As shown in Table 3.2, the relative rate of straw degradation under laboratory conditions is twice as fast as in the Nigerian soil, eight times faster than in the English soil and 18 times faster than in the Canadian soil. This example illustrates the importance of understanding that there can be large differences between degradation rates in the laboratory and in natural environments. This understanding is crucial when attempting to predict degradation rates for contaminants in an environment.

3.3.3 The Stationary and Death Phases

Stationary phase in the laboratory is a period of time where there is active cell growth that is matched by cell death. In batch culture, cell numbers increase rapidly to levels as high as 10^{10} to 10^{11} CFU/ml. At this point, either the substrate is completely utilized or waste metabolites inhibit further growth. Recall that most cells are only likely to achieve an exponential phase for brief periods of time because of nutrient limitations and environmental stress. Rather, they are likely to be either dormant or in a maintenance state. Cells that do undergo growth in response to a nutrient amendment will quickly utilize the added food source. However, even with an added food source, cultural counts rarely exceed 10^8 to 10^9 CFU/g soil except perhaps on some root surfaces. At this point, cells will either die or, in order to prolong survival, they

Case Study 3.1 Gene Transfer Experiment

Biodegradation of contaminants in soil requires the presence of appropriate degradative genes within the soil population. If degradative genes are not present within the soil population, the duration of the lag phase for degradation of the contaminant may range from months to years. One strategy for stimulating biodegradation is to "introduce" degrading microbes into the soil. Unfortunately, unless selective pressure exists to allow the introduced organism to survive and grow, it will die within a few weeks as a result of abiotic stress and competition from indigenous microbes. DiGiovanni *et al.* (1996) demonstrated that an alternative to "introduced microbes" is "introduced genes." In this study the introduced microbe was *Ralstonia eutrophus* JMP134. JMP134 carries an 80-kb plasmid, pJP4, that encodes the initial enzymes necessary for the degradation of the herbicide 2,4-D. A series of soil microcosms were set up and contaminated with 2,4-D. In control microcosms, there was slow, incomplete degradation of the 2,4-D over a 9-week period (see figure). In a second set of microcosms, JMP134 was added to give a final inoculum of 10^5 CFU/g dry soil. In these microcosms, rapid degradation of 2,4-D occurred after a 1-week lag phase and the 2,4-D was completely degraded after 4 weeks. The scientists examined the microcosm 2,4-D-degrading population very carefully during this study. What they found was surprising. They could not recover viable JMP134 microbes after the first week. However, during weeks 2 and 3 they isolated two new organisms that could degrade 2,4-D. Upon closer examination, both organisms, *Pseudomonas glathei and Burkholderia caryophylli*, and found to be carrying the pJP4 plasmid! Finally, during week 5 a third 2,4-D degrader was isolated, *Burkholderia cepacia*. This isolate also carried the pJP4 plas-mid. Subsequent addition of 2,4-D to the microcosms resulted in rapid degradation of the herbicide, primarily by the third isolate, *B. cepacia*. Although it is clear from this research that the pJP4 plasmid was transferred from the introduced microbe to several indigenous populations, it is not clear how the transfer occurred. There are two possibilities: cell-to-cell contact and transfer of the plasmid via conjugation or death, and lysis of the JMP134 cells to release the pJP4 plasmid which the indigenous populations then took up, a process called transformation.

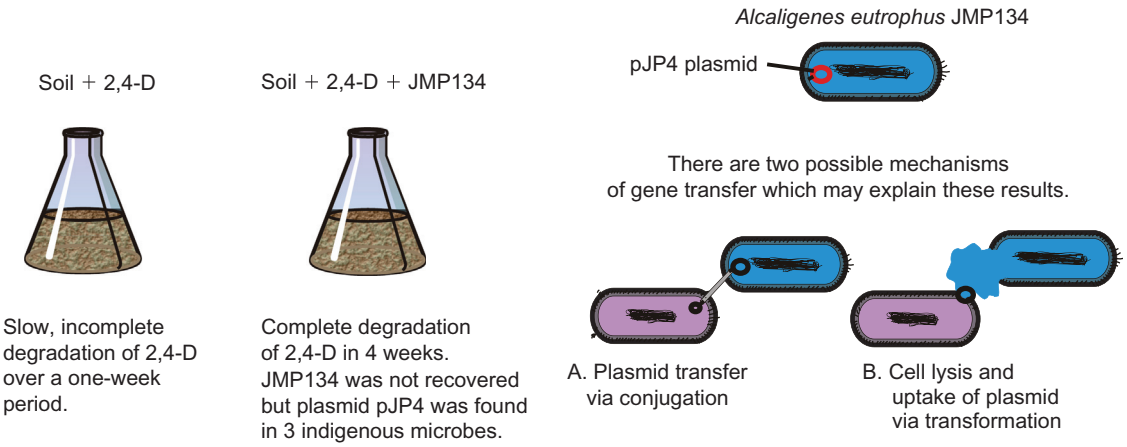


TABLE 3.1 Culturable Counts in Unamended and Glucose-Amended Soils[a]

Soil	Unamended (CFU/g soil)	1% Glucose (CFU/g soil)	Log Increase
Pima	5.6×10^5	4.6×10^7	1.9
Brazito	1.1×10^6	1.1×10^8	2.0
Clover Springs	1.4×10^7	1.9×10^8	1.1
Mt. Lemmon	1.4×10^6	8.3×10^7	1.7

Courtesy E.M. Jutras.
[a]*Each soil was incubated for approximately 1 week and then culturable counts were determined using R2A agar.*

can re-enter the dormant phase until a new source of nutrients becomes available. Thus, stationary phase periods of growth are likely to be very short, similar to that for exponential growth. In contrast, the death phase in environmental samples can certainly be observed, at least in terms of culturable counts. Once added nutrients are consumed, both living and dead cells become prey for protozoa that act as microbial predators. Bacteriophage can also infect and lyse significant portions of the living bacterial community. Dead cells are also quickly scavenged by other microbes in the vicinity, which reuse available carbon and nitrogen substrate. Thus, culturable cell numbers increase in response to nutrient addition (see Table 3.1), but will decrease again just as quickly to former background levels after all nutrients have been utilized.

3.4 MASS BALANCE OF GROWTH

During growth there is normally an increase in cell mass which is reflected in an increase in the number of cells. In this case, one can say that the cells are metabolizing substrate under growth conditions. However, in some cases, when the concentration of substrate or some other nutrient is limiting, utilization of the substrate occurs without production of new cells. In this case, the energy from substrate utilization is used to meet the maintenance requirements of the cell under nongrowth conditions. The level of energy required to maintain a cell is called the maintenance energy (Neidhardt *et al*., 1990).

Under either growth or nongrowth conditions, the amount of energy obtained by a microorganism through the oxidation of a substrate is reflected in the amount of cell mass produced, or the cell yield (*Y*). As discussed in Section 3.1.5, the cell yield coefficient is defined as the amount of cell mass produced per amount of substrate consumed. Although the cell yield is a constant, the value of the cell yield is dependent on the substrate being utilized. In general, the more reduced the substrate, the larger the amount of energy that can be obtained through its oxidation. For example, it is generally assumed that approximately half of the carbon in a molecule of sugar or organic acid will be used to build new cell mass, and half will be evolved as CO_2, corresponding to a cell yield of approximately 0.4. Note that the glucose ($C_6H_{12}O_6$) molecule is partially oxidized because the molecule contains six atoms of oxygen. One can compare this to a very low cell yield of 0.05 for pentachlorophenol, which is highly oxidized due to the presence of five chlorine atoms, and a very high cell yield of 1.49 for octadecane, which is completely reduced (Figure 3.12). As these examples show, some substrates support higher levels of growth and the production of more cell mass than others.

We can explore further why there are such differences in cell yield for these three substrates. As microbes have evolved, standard catabolic pathways have developed for common carbohydrate- and protein-containing substrates. For these types of substrates, approximately half of the carbon is used to build new cell mass. This translates into a cell yield of approximately 0.4 for a sugar such as glucose (see Example Calculation 3.4). However, since industrialization began in the late 1800s, many new molecules have been manufactured for which there are no standard catabolic pathways. Pentachlorophenol is an example of such a molecule. This material has been commercially produced since 1936, and is one of the major chemicals used to treat wood and utility poles. To utilize a molecule such as pentachlorophenol, which appeared in the environment relatively recently on an evolutionary scale, a microbe must alter the chemical structure to allow use of standard catabolic pathways. For pentachlorophenol, which has five carbon–chlorine bonds, this means

TABLE 3.2 Effect of Environment on Decomposition Rate of Plant Residues Added to Soil

Residue	Half-life (days)[a]	μ[b]	
		(1/days)	Relative Rate[c]
Wheat straw, laboratory	9	0.08	1
Rye straw, Nigeria	17	0.04	0.5
Rye straw, England	75	0.01	0.125
Wheat straw, Saskatoon	160	0.003	0.05

From *Paul and Clark (1989)*.
[a]*The half-life is the amount of time required for degradation of half of the straw initially added.*
[b]*μ is the specific growth rate constant.*
[c]*The relative rate of degradation of wheat straw under laboratory conditions is assumed to be 1. The degradation rates for straw in each of the soils were then compared with this value.*

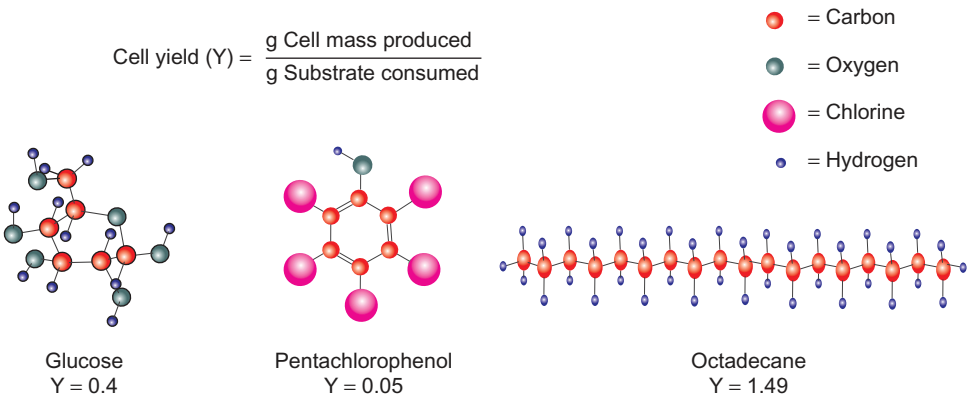


FIGURE 3.12 Cell yield values for various substrates. Note that the cell yield depends on the structure of the substrate.

Example Calculation 3.4 Conversion of Substrate Carbon into Cell Mass and Carbon Dioxide during Growth

Problem: A bacterial culture is grown using glucose as the sole source of carbon and energy. The cell yield value is determined by dry weight analysis to be 0.4 (i.e., 0.4 g cell mass was produced per 1 g glucose utilized). What percentage of the substrate (glucose) carbon will be found as cell mass and as CO_2?

Assume that you start with 1 mole of carbohydrate ($C_6H_{12}O_6$, molecular weight = 180 g/mol):

$$(\text{substrate mass})(\text{cell yield}) = \text{cell mass produced}$$
$$(180\text{ g})(0.4) = 72\text{ g}$$

Cell mass can be estimated as $C_5H_7NO_2$ (molecular weight = 113 g/mol):

$$\text{mol cell mass} = \frac{72\text{ g cell mass}}{113\text{ g/mol cell mass}} = 0.64\text{ mol cell mass}$$

In terms of carbon,

For cell mass: (0.64 mol cell mass)(5 mol C/mol cell mass)(12 g/mol C) = 38.4 g carbon

For substrate: (1 mol substrate)(6 mol C/mol substrate)(12 g/mol C) = 72 g carbon

The percentage of substrate carbon found in cell mass is

$$\frac{38.4\text{ g carbon}}{72\text{ g carbon}}(100) = 53\%$$

and by difference, 47% of the carbon is released as CO_2.

Question

Calculate the carbon found as cell mass and CO_2 for a microorganism that grows on octadecane ($C_{18}H_{36}$), Y = 1.49, and on pentachlorophenol (C_6HOCl_5), Y = 0.05.

Answer

For octadecane, 93% of the substrate carbon is found in cell mass and 7% is evolved as CO_2. For pentachlorophenol, 10% of the substrate carbon is found in cell mass and 90% is evolved as CO_2.

that a microbe must expend a great deal of energy to break the strong carbon–halogen bonds before the substrate can be metabolized to produce energy. Because so much energy is required to remove the chlorines from pentachlorophenol, relatively little energy is left to build new cell mass. This results in a very low cell yield value.

In contrast, why is the cell yield so high for a hydrocarbon such as octadecane? Octadecane is a hydrocarbon typical of those found in petroleum products (see Chapter 17). Because petroleum is an ancient mixture of molecules formed on early Earth, standard catabolic pathways exist for most petroleum components, including octadecane. The cell yield value for growth on octadecane is high because octadecane is a saturated molecule (the molecule contains no oxygen, only carbon–hydrogen bonds). Such a highly reduced hydrocarbon stores more energy than a molecule that is partially oxidized such as glucose (glucose contains six oxygen molecules). This energy is released during metabolism, allowing the microbe to obtain more energy from the degradation of octadecane than from the degradation of glucose. This in turn is reflected in a higher cell yield value.

3.4.1 Aerobic Conditions

Under aerobic conditions, microorganisms metabolize substrates by a process known as aerobic respiration. The complete oxidation of a substrate under aerobic conditions is represented by the mass balance equation:

$$\underset{\text{substrate}}{(C_6H_{12}O_6)} + 6\underset{\text{oxygen}}{(O_2)} \rightarrow 6\underset{\text{carbon dioxide}}{(CO_2)} + 6\underset{\text{water}}{(H_2O)} \quad \text{(Eq. 3.19)}$$

In Eq. 3.19, the substrate is a carbohydrate such as glucose, which can be represented by the formula $C_6H_{12}O_6$. Oxidation of glucose by microorganisms is more complex than shown in this equation because some of the substrate carbon is utilized to build new cell mass, and is therefore not completely oxidized. Thus, aerobic microbial oxidation of glucose can be more completely described by the following, slightly more complex, mass balance equation:

$$a\underset{\text{substrate}}{(C_6H_{12}O_6)} + b\underset{\text{nitrogen source}}{(NH_3)} + c\underset{\text{oxygen}}{(O_2)} \rightarrow d\underset{\text{cell mass}}{(C_5H_7NO_2)} + e\underset{\text{carbon dioxide}}{(CO_2)} + f\underset{\text{water}}{(H_2O)} \quad \text{(Eq. 3.20)}$$

where *a*, *b*, *c*, *d*, *e* and *f* represent mole numbers.

It should be emphasized that the degradation process is the same whether the substrate is readily utilized (glucose) or only slowly utilized as in the case of a contaminant such as benzene. Equation 3.20 differs from Eq. 3.19 in two ways: it represents the production of new cell mass, estimated by the formula $C_5H_7NO_2$, and in order to balance the equation, it has a nitrogen source on the reactant side, shown here as ammonia (NH_3).

The mass balance equation has a number of practical applications. It can be used to estimate the amount of oxygen or nitrogen required for growth and utilization of a particular substrate. This is useful for wastewater treatment (Chapter 25), for production of high value microbial products (e.g., antibiotics or vitamins) and for remediation of contaminated sites (see Chapter 17 and Example Calculation 3.5).

3.4.2 Anaerobic Conditions

The amount of oxygen in the atmosphere (21%) ensures aerobic degradation for the overwhelming proportion of the organic matter produced annually. In the absence of oxygen, organic substrates can be mineralized to carbon dioxide by fermentation or by anaerobic respiration, although these are less efficient processes than aerobic respiration (see Information Box 3.2). In general, anaerobic metabolism is restricted to water-saturated niches such as sediments, isolated water bodies within lakes and oceans, and microenvironments in soils. Anaerobic degradation requires alternative electron acceptors, either an organic compound for fermentation, or one of a series of inorganic electron acceptors for anaerobic respiration (Table 3.3). During anaerobic respiration, the terminal electron acceptor used depends on availability, and follows a sequence that corresponds to the electron affinity of the electron acceptors. Examples of alternative electron acceptors in order of decreasing electron affinity are: nitrate (nitrate-reducing conditions); manganese (manganese-reducing conditions); iron (iron-reducing conditions); sulfate (sulfate-reducing conditions); and carbonate (methanogenic conditions). More recently, additional terminal electron acceptors have been identified, among them arsenate, arsenite, selenate and uranium IV (Stolz *et al.*, 2006). These may be important in environments where they can be found in abundance.

Often, under anaerobic conditions, organic compounds are degraded by an interactive group or consortium of microorganisms. Individuals within the consortium each carry out different, specialized reactions that together lead to complete mineralization of the compound (Stams *et al.*, 2006). The final step of anaerobic degradation is methanogenesis, which occurs when other inorganic electron acceptors such as nitrate and sulfate are exhausted. Methanogenesis results in the production of methane and is the most important type of metabolism in anoxic freshwater lake sediments. Methanogenesis is also important in anaerobic treatment of sewage sludge, in which the supply of nitrate or sulfate is very small compared with the input of organic substrate. In this case, even though the concentrations of nitrate and sulfate are low, they are of basic importance for the establishment and maintenance of a sufficiently low electron potential that allows proliferation of the complex methanogenic microbial community.

Mass balance equations very similar to that for aerobic respiration can be written for anaerobic respiration. For example, the following equation can be used to describe the transformation of organic matter into methane (CH_4) and CO_2:

$$C_nH_aO_b + \left(n - \frac{a}{2} - \frac{b}{4}\right)H_2O \rightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right)CO_2 + \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right)CH_4 \quad \text{(Eq. 3.21)}$$

where n, a and b represent mole numbers.

Note that after biodegradation occurs, the substrate carbon is found either in its most oxidized form, CO_2, or in its most reduced form, CH_4. This is called disproportionation of organic carbon. The ratio of methane to carbon dioxide found in the gas mixture which results from anaerobic degradation depends on the oxidation state of the substrate used. Carbohydrates are converted to approximately equal amounts of CH_4 and CO_2. Substrates that are more reduced such as methanol or lipids produce relatively higher amounts of methane, whereas substrates that are more oxidized such as formic acid or oxalic acid produce relatively less methane.

QUESTIONS AND PROBLEMS

1. Draw a growth curve of substrate disappearance as a function of time. Label and define each stage of growth.
2. Calculate the time it will take to increase the cell number from 10^4 CFU/ml to 10^8 CFU/ml assuming a generation time of 1.5 h.
3. From the following data calculate the mean generation time:
   a. At the beginning of exponential growth when time $t = 0$, initial cell concentration = 2500 per ml
   b. At time t = 8 hours cell concentration = 10,000 per ml
4. Differentiate between metabolism during growth and nongrowth conditions.
5. You are given a microorganism that has a maximum growth rate (μ_{max}) of 0.39 h^{-1}. Under ideal conditions (maximum growth rate is achieved), how long will it take to obtain 1×10^{10} CFU/ml if you begin with an inoculum of 2×10^7 CFU/ml?
6. Is there any way to increase the growth rate observed in Question 3?
7. Write the Monod equation and define each of the constants.

Information Box 3.2 Biological Generation of Energy

Growth and metabolism requires energy, which is usually stored and transferred in the form adenosine triphosphate (ATP). As with any chemical reaction, metabolic reactions are subject to the second law of thermodynamics which says:

> *In a chemical reaction, only part of the energy is used to do work. The rest of the energy is lost as entropy. For any chemical reaction the free energy ΔG is the amount of energy available for work.*

For the reaction $A + B \rightleftharpoons C + D$, the thermodynamic equilibrium constant, K_{eq}, is defined as

$$K_{eq} = \frac{[C][D]}{[A][B]}$$

Case 1: If the formation of products (C and D) is favored, then K_{eq} will be positive. That is, if $[C][D] > [A][B]$ then $K_{eq} > 1$. In this case, the logarithm of K_{eq} is also positive; for example, if $K_{eq} = 2$, then $\log K_{eq} = 0.301$.

Case 2: If product formation is not favored, then K_{eq} will be less than 1. That is, if $[A][B] > [C][D]$ then $K_{eq} < 1$. In this case, $\log K_{eq}$ will be negative; for example, if $K_{eq} = 0.2$, $\log Keq = -0.699$.

Thus, for a reaction to have a positive K_{eq} and proceed as written (from left to right), the energy of the products must be lower than the energy of the reactants, meaning that overall, energy is released during the reaction.

The relationship between K_{eq} and the Gibbs free energy change (ΔG^0) is given by

$$\Delta G^0 = -RT \log K_{eq}$$

where R is the universal gas constant and T the absolute temperature (K).

ΔG^0 *is negative* when $\log K_{eq}$ is positive and the reaction will proceed spontaneously.

ΔG^0 *is positive* when $\log K_{eq}$ is negative and the reaction will not proceed as written.

Thus we can use ΔG values for any biochemical reaction mediated by microbes to determine whether energy is liberated for work and how much energy is liberated. Soil organisms can generate energy via several mechanisms, which can be divided into three main categories: photosynthesis, respiration, and fermentation.

1. **Photosynthesis**

$$2H_2O + CO_2 \xleftrightarrow{\text{light}} \underset{\text{biomass}}{CH_2O} + O_2 + H_2O$$
$$\Delta G^0 \simeq +115 \text{ kcal/mol}$$

Note that ΔG^0 is positive and so this reaction is not favorable. It requires reaction energy supplied by sunlight. The fixed organic carbon is then metabolized to generate energy via respiration. Examples of photosynthetic soil organisms are *Rhodospirillum, Chromatium*, and *Chlorobium*.

2. **Respiration**

a. *Aerobic heterotrophic respiration:* Many bacteria function as either obligate or facultative aerobic chemoheterotrophs including *Pseudomonas, Bacillus*, and *E. coli*.

$$\underset{\text{succinic acid}}{C_4H_6O_4} + 3.5O_2 \leftrightarrow 4CO_2 + 3H_2O$$
$$\Delta G^0(25°)C - 1569.25 \text{ kJ/mol}$$

b. *Anaerobic heterotrophic respiration*: Anaerobic chemoheterotrophs utilize alternate terminal electron acceptors and organic compounds from the electron donor. The examples below use the same electron donor (succinic acid, $C_4H_6O_4$) with three different terminal electron acceptors (nitrate, iron, sulfate).

$$C_4H_6O_4 + 2.8NO_3^- + 2.8H^+ \leftrightarrow 1.4N_2 + 4CO_2 + 4.4H_2O$$
$$\Delta G^0(25°C) = -1507.59 \text{ kJ/mol}$$

$$C_4H_6O_4 + 14Fe^{3+} + 4H_2O \leftrightarrow 4CO_2 + 14Fe^{2+} + 14H^+$$
$$\Delta G^0(25°C) = -891.00 \text{ kJ/mol}$$

$$C_4H_6O_4 + 1.75SO_4^2 + 3.5H^+ \leftrightarrow 4CO_2 + 1.75H_2S + 12H_2O$$
$$\Delta G^0(25°C) = -257.60 \text{ kJ/mol}$$

c. *Aerobic autotrophic respiration*: The reactions carried out by the obligately aerobic chemoautotrophs *Nitrosomonas* and *Nitrobacter* are known as nitrification:

$$NH_3 + 1.5O_2 \rightarrow H^+ + NO_2^- + H_2O \text{ (}Nitrosomonas\text{)}$$
$$\Delta G^0(25°C) = -267.50 \text{ kJ/mol}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \text{ (}Nitrobacter\text{)}$$
$$\Delta G^0(25°C) = -86.96 \text{ kJ/mol}$$

The following two reactions are examples of chemoautotrophic sulfur oxidation:

$$H_2S + 0.5O_2 \rightarrow S^0 + H_2O \text{ (}Beggiatoa\text{)}$$
$$\Delta G^0(25°C) = -217.53 \text{ kJ/mol}$$

$$S^0 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \text{ (}Thiobacillus\ thiooxidans\text{)}$$
$$\Delta G^0(25°C) = -532.09 \text{ kJ/mol}$$

The next reaction involves the chemoautotrophic degradation of carbon monoxide:

$$CO + 0.5O_2 \rightarrow CO_2 \quad \Delta G^0(25°C) = -274.24 \text{ kJ/mol}$$

d. *Anaerobic autotrophic respiration*: *Thiobacillus denitrificans* can utilize nitrate as a terminal electron acceptor.

$$S^0 + 1.2NO_3^- + 0.4H_2O \rightarrow SO_4^{2-} + 0.6N_2$$
$$\Delta G^0(25°C) = -505.59 \text{ kJ/mol}$$

3. **Fermentation**

$$\underset{\text{ethanol}}{C_2H_5OH} + CO_2 \rightarrow 1.5\underset{\text{acetic acid}}{CH_3COOH} \quad \Delta G^0(25°C) = -27.45 \text{ kJ/mol}$$

All of the preceding reactions illustrate how organisms mediate reactions involved in biogeochemical cycling of carbon, nitrogen and sulfur (Chapter 14). The $\Delta G^0(25°C)$ values provided were compiled from Amend and Shock (2001).

Example Calculation 3.5 Leaking Underground Storage Tanks

The Environmental Protection Agency (EPA) estimates that more than 1 million underground storage tanks (USTs) have been in service in the United States alone. Over 436,000 of these have had confirmed releases into the environment. Although regulations now require USTs to be upgraded, leaking USTs continue to be reported at a rate of 20,000/year and a cleanup backlog of > 139,000 USTs still exists. In this exercise we will calculate the amount of oxygen and nitrogen necessary for the remediation of a leaking UST site that has released 10,000 gallons of gasoline. To simplify the problem, we will assume that octane (C_8H_{18}) is a good representative of all petroleum constituents found in gasoline. We will use the mass balance equation to calculate the biological oxygen demand (BOD) and the nitrogen demand:

$$\underset{\text{octane}}{a(C_8H_{18})} + \underset{\text{ammonia}}{b(NH_3)} + \underset{\text{oxygen}}{c(O_2)} \rightarrow \underset{\text{cell mass}}{d(C_5H_7NO_2)} + \underset{\text{carbon dioxide}}{e(CO_2)} + \underset{\text{water}}{f(H_2O)}$$

In this equation, the coefficients *a* through *f* indicate the number of moles for each component. To solve the mass balance equation we must be able to relate the amount of cell mass produced to the amount of substrate (octane) consumed. This is done using the cell yield Y where

$$Y = \frac{\text{mass of cell mass produced}}{\text{mass of substrate consumed}}$$

Literature indicates that a reasonable cell yield value for octane is 1.2. Using the cell yield we can calculate the coefficient *d*. We will start with 1 mole of substrate ($a = 1$) and use the following equation:

$$d(\text{MW cell mass}) = a(\text{MW octane})(Y)$$
$$d(113\ \text{g/mol}) = 1(114\ \text{g/mol})(1.2)$$
$$\therefore d = 1.2$$

We can then solve for the other coefficients by balancing the equation. We start with nitrogen. We know that there is one N on the right side of the equation in the biomass term. Examining the left side of the equation, we see that there is similarly one N as ammonia. We can set up a simple relationship for nitrogen and use this to solve for coefficient *b*:

$$b(1\ \text{mol nitrogen}) = d(1\ \text{mol nitrogen})$$
$$b(1) = 1.2(1)$$
$$b = 1.2$$

Next we balance carbon and solve for coefficient *e*:

$$e(1\ \text{mol carbon}) = a(8\ \text{mol carbon}) - d(5\ \text{mol carbon})$$
$$e(1) = 1(8) - 1.2(5)$$
$$e = 2.0$$

Next we balance hydrogen and solve for coefficient *f*:

$$f(2\ \text{mol hydrogen}) = a(18\ \text{mol hydrogen}) + b(3\ \text{mol hydrogen}) - d(7\ \text{mol hydrogen})$$
$$f(2) = 1(18) + 1.2(3) - 1.2(7)$$
$$f = 6.6$$

Finally we balance oxygen and solve for coefficient *c*:

$$c(2\ \text{mol oxygen}) = d(2\ \text{mol oxygen}) + e(2\ \text{mol oxygen}) + f(1\ \text{mol oxygen})$$
$$c(2) = 1.2(2) + 2(2.0) + 6.6(1)$$
$$c = 6.5$$

Thus, the solved mass balance equation is $1(C_8H_{18}) + 1.2(NH_3) + 6.5(O_2) \rightarrow 1.2(C_5H_7NO_2) + 2.0(CO_2) + 6.6(H_2O)$.
Now we use this mass balance equation to determine how much nitrogen and oxygen will be needed to remediate the site.
First we convert gallons of gasoline into moles of octane using the assumption that octane is a good representative of gasoline.
Recall that we started with 10,000 gallons of gasoline:

$$\text{Convert to liters (L)}: 10,000\ \text{gallons}\ (3.78\ L/\text{gallon}) = 3.78 \times 10^4\ \text{L gasoline}$$
$$\text{Convert to grams (g)}: 3.78 \times 10^4\ \text{L gasoline}\ (690\ \text{g gasoline}/L) = 2.6 \times 10^7\ \text{g gasoline in the site}$$
$$\text{Convert to moles}: = \frac{2.6 \times 10^7\ \text{g gasoline}}{114\ \text{g octane/mol}} = 2.3 \times 10^5\ \text{mol octane in the site}$$

Now we ask, how much nitrogen is needed to remediate this spill? From the mass balance equation we know that we need 1.2 mol NH_3/mol octane (see coefficient *b*).

$$(1.2 \text{ mol } NH_3/\text{mol octane})(2.3 \times 10^5 \text{ mol octane in the site})(17 \text{ g } NH_3/\text{mol}) = 4.7 \times 10^6 \text{ g } NH_3$$
$$\Rightarrow 4.7 \times 10^6 \text{ g}(1 \text{ kg}/1000 \text{ g})(2.2046 \text{ lb/kg}) = 10000 \text{ lb or 5 tons of } NH_3!$$

Finally we ask, how much oxygen is needed to remediate this spill? From the mass balance equation we know that we need 6.5 mol O_2/mol octane (see coefficient *c*).

$$(6.5 \text{ mol } O_2/\text{mol octane})(2.3 \times 10^5 \text{ mol octane in the site}) = 1.5 \times 10^6 \text{ mol } O_2$$

A gas takes up 22.4 liters/mol, but remember that air is only 21% oxygen.

$$(1.5 \times 10^6 \text{ mol } O_2)(22.4 \text{ L/mol air})(\text{L mol air}/0.21 \text{ mol } O_2) = 1.6 \times 10^8 \text{ L air}$$
$$1 \text{ cubic foot of air} = 28.33 \text{ L}$$
$$\Rightarrow 1.6 \times 10^8 \text{ L air (1 cubic foot}/28.33 \text{ L gas}) = 5.5 \times 10^6 \text{ cubic feet of air or enough air to fill a football field to a height of 100 ft!}$$

From Pepper *et al.*, 2006.

TABLE 3.3 Relationship Between Respiration, Redox Potential and Typical Electron Acceptors and Products[a]

Type of Respiration	Reduction reaction Electron Acceptor → Product	Reduction Potential (V)	Oxidation Reaction Electron Donor → Product	Oxidation Potential (V)	Difference (V)
Aerobic	$O_2 - H_2O$	+0.81	$CH_2O - CO_2$	−0.47	−1.28
Denitrification	$NO_3 - N_2$	+0.75	$CH_2O - CO_2$	−0.47	−1.22
Manganese reduction	$Mn^{4+} - Mn^{2+}$	+0.55	$CH_2O - CO_2$	−0.47	−1.02
Nitrate reduction	$NO_3 - NH_4^+$	+0.36	$CH_2O - CO_2$	−0.47	−0.83
Sulfate reduction	$SO_4^{2-} - HS^-, H_2S$	−0.22	$CH_2O - CO_2$	−0.47	−0.25
Methanogenesis	$CO_2 - CH_4$	−0.25	$CH_2O - CO_2$	−0.47	−0.22

Adapted from *Zehnder and Stumm (1988)*.
[a]*Biodegradation reactions can be considered a series of oxidation–reduction reactions. The amount of energy obtained by cells is dependent on the difference in energy between the oxidation and reduction reactions. As shown in this table, using the same electron donor in each case but varying the electron acceptor, oxygen as a terminal electron acceptor provides the most energy for cell growth and methanogenesis provides the least.*

8. There are two special cases when the Monod equation can be simplified. Describe these cases and the simplified Monod equation that results.
9. List terminal electron acceptors used in anaerobic respiration in the order of preference (from an energy standpoint).
10. Define disproportionation.
11. Define the term critical dilution rate, D_c, and explain what happens in continuous culture when D is greater than D_c.
12. Compare the characteristics of each of the growth phases (lag, log, stationary and death) for batch culture and environmental systems.
13. Compare and contrast the copiotrophic and oligotrophic style of life in the environment.

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