

# Bacterial Growth

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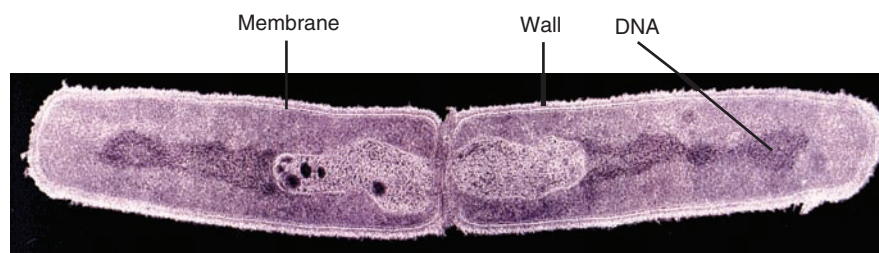
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Bacterial growth is a complex process involving numerous **anabolic** (synthesis of cell constituents and metabolites) and **catabolic** (breakdown of cell constituents and metabolites) reactions. Ultimately, these biosynthetic reactions result in 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, cannot share nutrients or protection mechanisms, and therefore never achieve a metabolic state that is efficient enough to allow exponential growth.

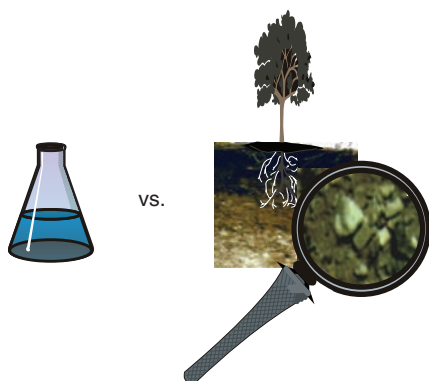
Most information available concerning the growth of microorganisms is the result of controlled laboratory studies

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 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**.



**FIGURE 3.1** Electron micrograph of *Bacillus subtilis*, a gram-positive bacterium, dividing. Magnification 31,200 $\times$ . Reprinted with permission from Madigan *et al.*, 1997.

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 (Fig. 3.2). This complexity arises from a number 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 14), microbial response to anthropogenic perturbation of the environment (Chapter 17), microbial interaction with organic and metal contaminants (Chapters 20 and 21), and survival and growth of pathogens in the environment (Chapters 22 and 27). In this chapter, we begin with a review of growth under pure culture conditions and then discuss how this is related to growth in the environment.



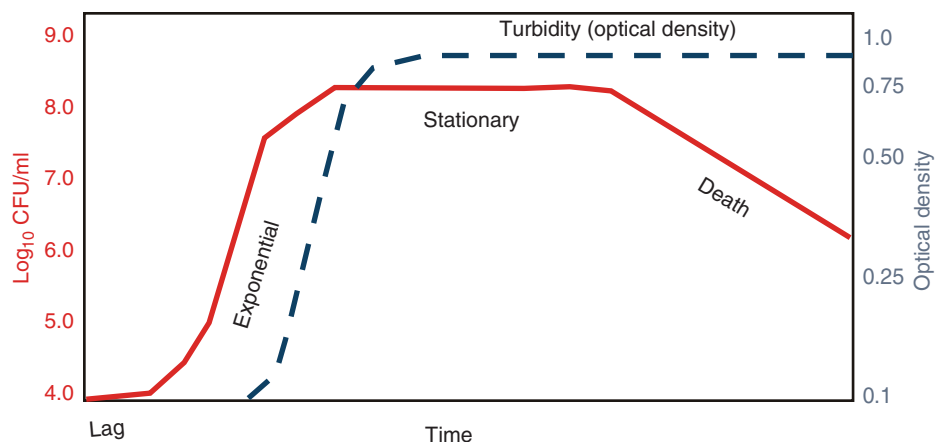
**FIGURE 3.2** Compare the complexity of growth in a flask and growth in a soil environment. Although we understand growth in a flask quite well, we still cannot always predict growth in the environment.

### 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 (Fig. 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. The lag phase is defined to transition to the exponential phase after the initial population has 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 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 not as easily taken up. As a result, initiation of cell growth and division and the transition to exponential phase may be slowed.

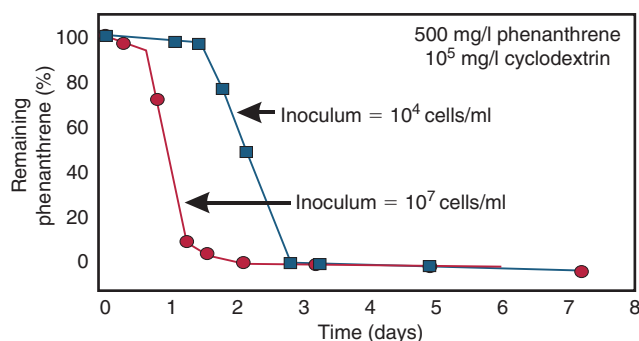


**FIGURE 3.3** A typical growth curve for a bacterial population. Compare the difference in the shape of the curves in the death phase (colony-forming units versus optical density).

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 is 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. If the inoculum is taken from a stationary phase culture, however, 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 will be observed while the cells reorganize and shift physiologically to synthesize the appropriate enzymes for glucose catabolism.

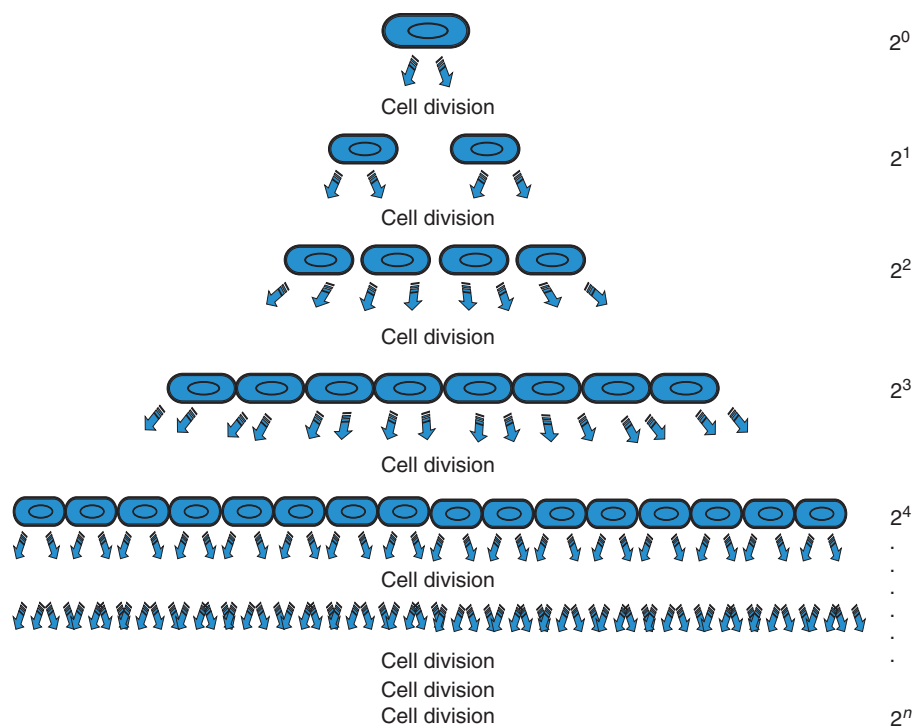
Finally, if the inoculum size is small, for example,  $10^4$  cells/ml, and one is measuring activity, such as disappearance of substrate, a lag phase will be observed until the population reaches approximately  $10^6$  cells/ml. This is illustrated in Figure 3.4, which compares the degradation of phenanthrene in cultures inoculated with  $10^7$  and with  $10^4$  colony-forming units (CFU) per milliliter. 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).



**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.

### 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 the **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  $2^0$ ,  $2^1$ ,  $2^2$ ,  $2^3$  until, after  $n$  divisions, the number of cells is  $2^n$  (Fig. 3.5).



**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 Generation Time**

*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?

Use the equation  $X = 2^n X_0$ , 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!

This can be expressed in a quantitative manner; for example, if the initial cell number is  $X_0$ , the number of cells after  $n$  doublings is  $2^n X_0$  (see Example Calculation 3.1). As can be seen from this example, if one starts with a low number of cells exponential growth does not initially produce large numbers of new cells. However, as cells accumulate after several generations, the number of new cells with each division begins to increase explosively.

In the example just given,  $X_0$  was used to represent cell number. However,  $X_0$  can also be used to represent cell mass, which is often more convenient to measure than cell number (see Chapters 10 and 11). Whether one expresses  $X_0$  in terms of cell number or in terms of cell mass, one can mathematically describe cell growth during the exponential phase using the following equation:

$$\frac{dX}{dt} = \mu X \quad (\text{Eq. 3.1})$$

where  $X$  is the number or mass of cells (mass/volume),  $t$  is time, and  $\mu$  is the specific growth rate constant (1/time). The time it takes for a cell division to occur is called the **generation time** or the **doubling time**. Equation 3.1 can be used to calculate the generation time as well as the **specific growth rate** using data generated from a growth curve such as that shown in Figure 3.3.

The generation time for a microorganism is calculated from the linear portion of a semilog plot of growth versus time. The mathematical expression for this portion of the growth curve is given by Eq. 3.1, which can be rearranged

and solved as shown in Eqs. 3.2 to 3.6 to determine the generation time (see Example Calculation 3.2):

$$\frac{dX}{dt} = \mu X \quad (\text{Eq. 3.1})$$

Rearrange:

$$\frac{dX}{X} = \mu dt \quad (\text{Eq. 3.2})$$

Integrate:

$$\int_{X_0}^X \frac{dX}{X} = \mu \int_0^t dt \quad (\text{Eq. 3.3})$$

$$\ln X = \mu t + \ln X_0 \quad \text{or} \quad X = X_0 e^{\mu t} \quad (\text{Eq. 3.4})$$

For  $X$  to be doubled:

$$\frac{X}{X_0} = 2 \quad (\text{Eq. 3.5})$$

Therefore:

$$2 = e^{\mu t} \quad (\text{Eq. 3.6})$$

where  $t$  = generation time.

**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:

$$\frac{dX}{dt} = 0 \quad (\text{Eq. 3.7})$$

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 common reason is that the carbon and energy source or an essential nutrient becomes completely used up. 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 source of nutrients. Growth on dead cells is called **endogenous metabolism**. Endogenous metabolism occurs throughout the growth cycle, but it can be best observed during 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 actually shows a small amount of growth. Again, this growth

**Example Calculation 3.2 Specific Growth Rate**

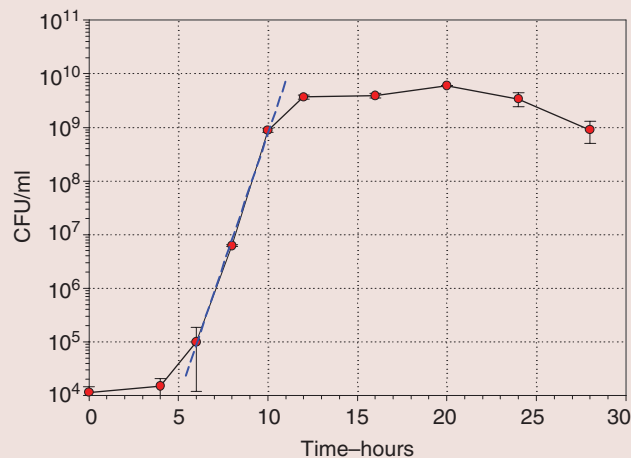
**Problem:** The following data were collected using a culture of *Pseudomonas* during growth in a minimal medium containing salicylate as a sole source of carbon and energy. Using these data, calculate the specific growth rate for the exponential phase.

Time (h)	Culturable cell count (CFU/ml)
0	$1.2 \times 10^4$
4	$1.5 \times 10^4$
6	$1.0 \times 10^5$
8	$6.2 \times 10^6$
10	$8.8 \times 10^8$
12	$3.7 \times 10^9$
16	$3.9 \times 10^9$
20	$6.1 \times 10^9$
24	$3.4 \times 10^9$
28	$9.2 \times 10^8$

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 8 hours. Using Eq. 3.4, 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  $\mu$ , the specific growth rate.) From the data given, the slope of the graph from time 6 to 10 hours is:

$$\mu = (\ln 1 \times 10^9 - \ln 1 \times 10^5) / (10 - 6) = 2.31/\text{h}$$

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.

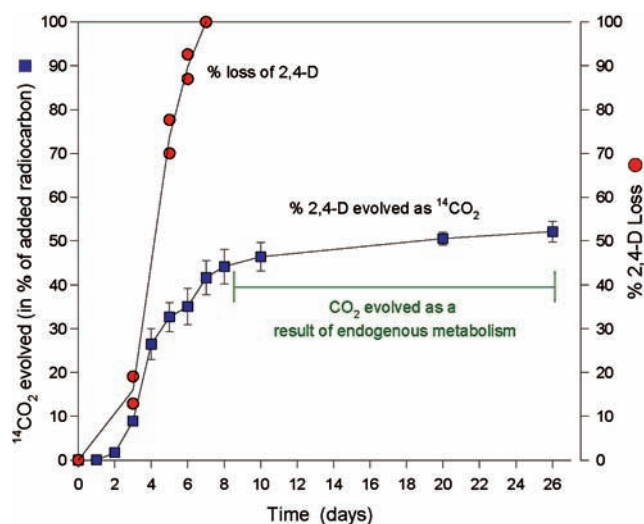


occurs after the substrate has been utilized and reflects the use of dead cells as a source of carbon and energy. A second reason that stationary phase may be observed is that waste products build up to a point where they begin to inhibit cell growth or are toxic to cells. This generally occurs only in cultures with high cell density. Regardless of the reason why cells enter stationary phase, growth in the stationary phase is unbalanced 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 (see Section 2.2.2).

### 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



**FIGURE 3.6** Mineralization of the broadleaf herbicide 2,4-dichlorophenoxy acetic 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<sub>2</sub> evolved continues to rise slowly. This is a result of endogenous metabolism. From Estrella *et al.*, 1993.

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

$$\frac{dX}{dt} = -k_d X \quad (\text{Eq. 3.8})$$

where  $k_d$  is 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 Fig. 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 commonly used approaches to measurement of growth because normally the growth phases of most interest to environmental microbiologists are the lag phase, the exponential phase, and the time to onset of the stationary phase.

### 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, 3.7, and 3.8). 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:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad (\text{Eq. 3.9})$$

where  $\mu$  is the specific growth rate (1/time),  $\mu_{\max}$  is the maximum specific growth rate (1/time) for the culture,  $S$  is the substrate concentration (mass/volume), and  $K_s$  is the half-saturation constant (mass/volume) also known as the affinity constant.

Equation 3.9 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 3.8 are independent of substrate concentration). Thus, Monod designed Eq. 3.9 to describe the relationship between the specific growth rate and the substrate concentration. There are two constants in this equation,  $\mu_{\max}$ , 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  $\mu_{\max}$ . Both  $\mu_{\max}$  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. 3.9 that no nutrients other than the substrate are limiting and that no toxic by-products of metabolism build up.

As shown in Eq. 3.10, the Monod equation can be expressed in terms of cell number or cell mass ( $X$ ) by equating it with Eq. 3.1:

$$\frac{dX}{dt} = \frac{\mu_{\max} SX}{K_s + S} \quad (\text{Eq. 3.10})$$

The Monod equation has two limiting cases (see Fig. 3.7). The first case is at high substrate concentration where  $S \gg K_s$ . In this case, as shown in Eq. 3.11, the specific growth rate  $\mu$  is essentially equal to  $\mu_{\max}$ . This simplifies the equation and the resulting relationship is zero order or independent of substrate concentration:

$$\text{For } S \gg K_s: \quad \frac{dX}{dt} = \mu_{\max} X \quad (\text{Eq. 3.11})$$

Under these conditions, growth will occur at the maximum growth rate. There are relatively few instances in which ideal growth as described by Eq. 3.11 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 unlikely to be found under natural conditions in a soil or water environment, where either substrate or other nutrients are commonly limiting.

### Information Box 3.1 The Monod Growth Constants

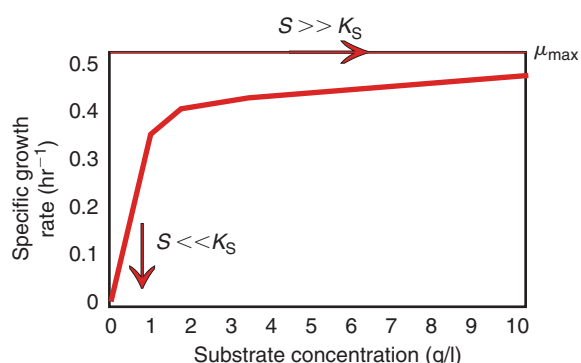
Both  $\mu_{\max}$  and  $K_s$  are constants that reflect:

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

The following table provides representative values of  $\mu_{\max}$  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	$\mu_{\max}$ (1/h)	$K_s$ (mg/l)
<i>Escherichia coli</i>	37	Glucose	0.8–1.4	2–4
<i>Escherichia coli</i>	37	Lactose	0.8	20
<i>Saccharomyces cerevisiae</i>	30	Glucose	0.5–0.6	25
<i>Pseudomonas</i> sp.	25	Succinate	0.38	80
<i>Pseudomonas</i> 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,  $\mu$ , on the substrate concentration. The maximal growth rate  $\mu_{\max} = 0.51/\text{h}$  and  $K_s = 0.5\text{ g/l}$ . Note that  $\mu$  approaches  $\mu_{\max}$  when  $S \gg K_s$  and becomes independent of substrate concentration. When  $S \ll K_s$ , the specific growth rate is very sensitive to the substrate concentration, exhibiting a first-order dependence.

The second limiting case occurs at low substrate concentrations where  $S \ll K_s$  as shown in Eq. 3.12. In this case there is a first order dependence on substrate concentration (Fig. 3.7):

$$\text{For } S \ll K_s: \quad \frac{dX}{dt} = \frac{\mu_{\max} SX}{K_s} \quad (\text{Eq. 3.12})$$

As shown in Eq. 3.12, 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 as the substrate is nearly all consumed. This is also the type of growth that would be more typically expected under conditions 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. 3.13):

$$\frac{dS}{dt} = \frac{1}{Y} \frac{dX}{dt} \quad (\text{Eq. 3.13})$$

where  $Y$  is 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 detail). 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 in Eq. 3.14, Eqs. 3.10 and 3.13 can be combined to express microbial growth in terms of substrate disappearance:

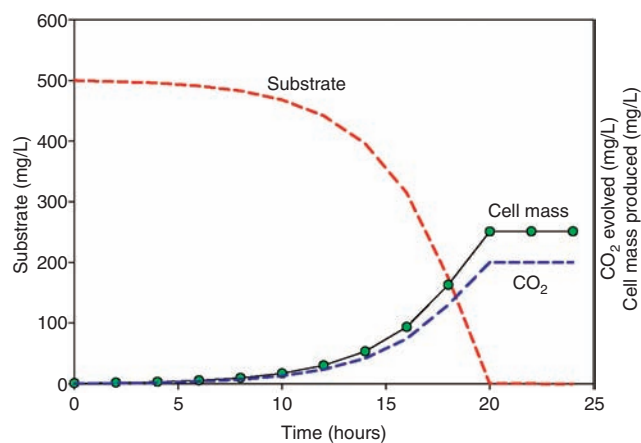
$$\frac{dS}{dt} = -\frac{1}{Y} \frac{\mu_{\max} SX}{K_s + S} \quad (\text{Eq. 3.14})$$

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  $\mu_{\max}$ ,  $K_s$ , and  $Y$ . Both  $Y$  and  $\mu_{\max}$  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. 3.10) and substrate (Eq. 3.13). This set of constants was then used to model or simulate growth curves that express growth in terms of  $\text{CO}_2$  evolution and substrate disappearance. Such models are useful because they can help one 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

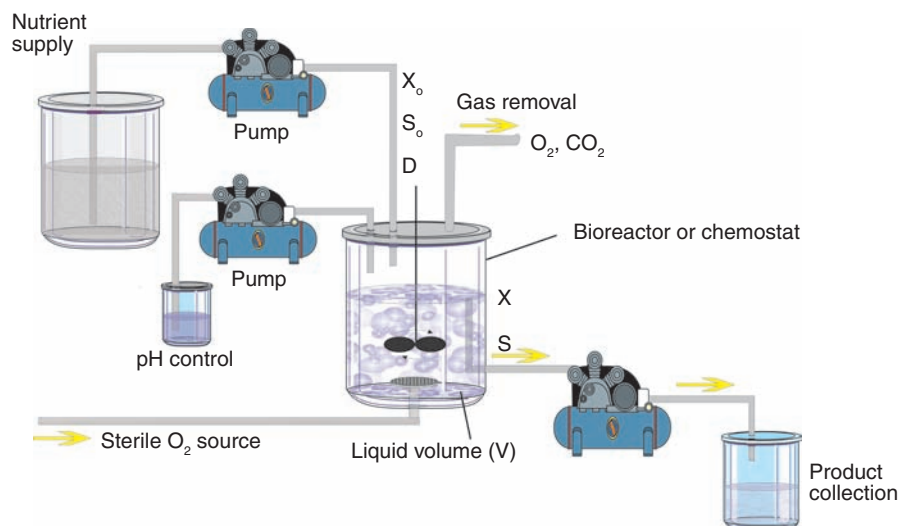
Thus far, we have focused on theoretical and mathematical descriptions of batch culture growth, which is currently of great economic importance in terms of the production of a wide variety of microbial products. In contrast to batch culture, continuous culture is a system that is designed for



**FIGURE 3.8** This figure shows the same growth curve expressed three different ways: in terms of substrate loss, in terms of  $\text{CO}_2$  evolution, and in terms of increasing cell mass. The parameters used to generate the curves were as follows:  $\mu_{\max} = 0.29 \text{ 1/h}$ ,  $K_s = 10 \text{ mg/l}$ ,  $Y = 0.5$ , initial substrate concentration =  $500 \text{ mg/l}$ , and initial cell mass =  $1 \text{ mg/l}$ . In this experiment, cell mass was measured and so the data points are shown. The data for  $\text{CO}_2$  evolution and substrate loss were simulated using a model and so those data are shown using dashed lines.

long-term operation. Continuous culture can be operated over the long term because it is an open system (Fig. 3.9) with a continuous feed of influent solution that contains nutrients and substrate, as well as a continuous drain of effluent solution that contains 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, maintain a constant substrate concentration, as well as provide continuous control of pH, temperature, and oxygen levels. As will be discussed further, 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 non-growth-associated product such as an antibiotic is produced at low flow or dilution rates, which maintains high cell numbers. Chemostat cultures are also being used to aid in 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 lies in the constant removal of metabolites, including signal molecules (see Chapter 16) or secondary metabolites that may mask or subtly alter physiological conditions under batch culture conditions (Hoskisson and Hobbs, 2005).

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 ( $\mu$ ) in the chemostat, represented in this graph as doubling time (recall that during exponential phase the growth rate is proportional to the number of cells present). By controlling the influent substrate concentration, one can



**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.



control the number of cells produced or the cell yield in the chemostat (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 in studying the physiology of microbial growth and also 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. Despite these advantages, chemostats are not yet widely used to produce commercial products because it is often difficult to maintain sterile conditions over time.

In a chemostat, the growth medium undergoes constant dilution with respect to cells due to the influx of nutrient solution (Fig. 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

$$\frac{dX}{dt} = \mu X - DX \quad (3.15)$$

where  $X$  is the cell mass (mass/volume),  $\mu$  is the specific growth rate (1/time), and  $D$  is the dilution rate (1/time).

Examination of Eq. 3.15 shows that a steady state (no increase or decrease in biomass) will be reached when

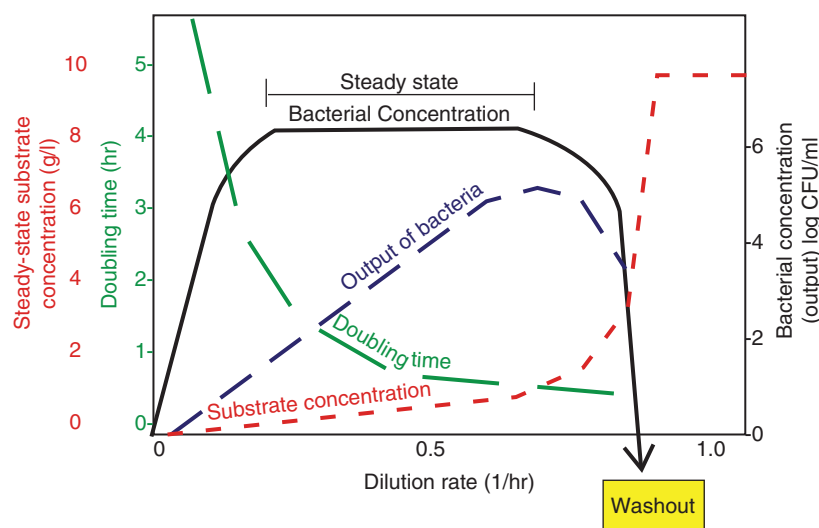
$\mu = D$ . If  $\mu > D$ , the utilization of substrate will exceed the supply of substrate, causing the growth rate to slow until it is equal to the dilution rate. If  $\mu < D$ , the amount of substrate added will exceed the amount utilized. Therefore the growth rate will increase until it is equal to the dilution rate. In either case, given time, a steady state will be established where

$$\mu = D \quad (3.16)$$

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. 3.9 and 3.16:

$$D_c = \mu_{\max} \left( \frac{S}{K_s + S} \right) \quad (3.17)$$

Looking at Eq. 3.17, it can be seen that the operation efficiency of a chemostat can be optimized under conditions in which  $S \gg K_s$ , 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  $\mu_{\max}$ ) 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.



**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 1/h. 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 (i.e., 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 *et al.*, 1997.

### 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 and rapid growth, depending on substrate availability, 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 introduced into soil and usually survive for only short periods of time.

Current terminology distinguishes soil microbes as either **oligotrophs**, those that prefer low substrate concentrations, or **copiotrophs**, those that prefer high substrate concentrations. There is also the similar concept of ***r* selection** 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 ( $\mu_{\max}$ ) 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. These microbes metabolize slowly and as a result have long generation times, and they often 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. Dormant cells are often rounded and small (approximately 0.3  $\mu\text{m}$ ) in comparison with healthy laboratory specimens, which range from 1 to 2  $\mu\text{m}$  in size. Dormant cells may become **viable but nonculturable (VBNC)** with time because of extended starvation conditions or because cells become reversibly damaged. VBNC are thus difficult to culture because of cell stress and damage. In addition, many environmental microbes are **viable but difficult to culture (VBDC)**. VBDC cells are difficult to culture for several reasons. First we know little about their physiology and associated nutrient and growth requirements. Often VBDC microbes are highly oligotrophic and require special low-nutrient media. For example, some VBDC will not grow on plates that use agar as the gelling agent, and require an alternate agent such as gellan gum. Also many VBDC microbes are very slow growing, taking several weeks to months to produce visible growth on an agar plate or in liquid culture (see Information Box 3.2).

The existence of VBNC and VBDC is reflected in the fact that **direct counts** from environmental samples, which include all cells, are 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

#### Information Box 3.2 Typical Soil Bacterial Community Composition

Since the late 1990s advances in analysis of microbial communities based on DNA extraction and analysis of the 16S rRNA gene rather than culture has revealed that there are entire bacteria phyla that are viable but difficult to culture. The table here was adapted from Janssen (2006) and indicates the most common bacterial phyla in 21 soil samples that were examined using community DNA analysis (see Chapter 13) rather than culture-based techniques.

Bacterial phylum	Percent of total soil community <sup>a</sup>	Relative ease or difficulty of culture <sup>b</sup>
<i>Proteobacteria</i>	40 ± 20	Easy
<i>Acidobacteria</i>	20 ± 12	Difficult
<i>Actinobacteria</i>	13 ± 12	Easy
<i>Verrucomicrobia</i>	7 ± 6	Difficult
<i>Bacteroidetes</i>	5 ± 5	Easy
<i>Chloroflexi</i>	3 ± 3	Difficult
<i>Planctomycetes</i>	2 ± 2	Difficult
<i>Gemmatimonadetes</i>	2 ± 1	Difficult
<i>Firmicutes</i>	2 ± 2	Easy
Other	5 ± 2	
Unknown	2 ± 2	

<sup>a</sup>Based on analysis of 2920 clones from 21 different soil samples.

<sup>b</sup>Those phyla labeled easy have members that can be isolated from environmental samples using R2A agar within a relatively short time-frame (a few days to 2 weeks). Those phyla labeled difficult to culture require special media usually with very low amounts of organic carbon and often take weeks or months to grow. In many cases only a very few members of the difficult phyla have been isolated in culture.

plated on a soil 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. Thus, these microbes can exhibit the growth stages described in Section 3.1 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 initial populations that are capable of utilizing the added 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 occur after the initial population has doubled. However, this is a difficult definition to impose on environmental samples where it is hard to accurately measure the doubling of a small subset of the microbial community that is responding to the addition of nutrients.) Alternatively, degrading populations 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 suboptimal environmental conditions such as temperature or moisture that cause stress. Thus, it is not unusual to observe lag periods of months or even years after an initial application of a new pesticide. However, once an environment has been exposed to a particular pesticide and developed a community for its degradation, the disappearance 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).

A second explanation for long lag periods in environmental samples is that the capacity for degradation of an added carbon source may not initially be present within existing populations. This situation may require a mutation or a gene transfer event to introduce appropriate degradative genes into a suitable population. For example, one of the first documented cases of gene transfer in soil was the transfer of the plasmid pJP4 from an introduced organism to the indigenous soil population. The plasmid transfer resulted in rapid and complete degradation of the herbicide 2,4-D within the microcosm (see 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 among environmental microbiologists.

### 3.3.2 The Exponential Phase

In the environment the second phase of growth, exponential growth, occurs for only very brief periods following addition of a substrate. Such substrate might be crop residues, vegetative litter, root residues, or contaminants added to or spilled into the environment. As stated earlier, it is the copiotrophic cells, many of which are initially dormant, that respond most quickly 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. As shown in Table 3.1, culturable cell counts 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  $\mu_{\max}$ . Thus, rates of degradation in the environment are slower than degradation rates measured under laboratory conditions. This is illustrated in Table 3.2, which compares the 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, 8 times faster than in the English soil, and 20 times faster than in the Canadian soil. This example illustrates the importance of understanding that there can be a huge difference 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.

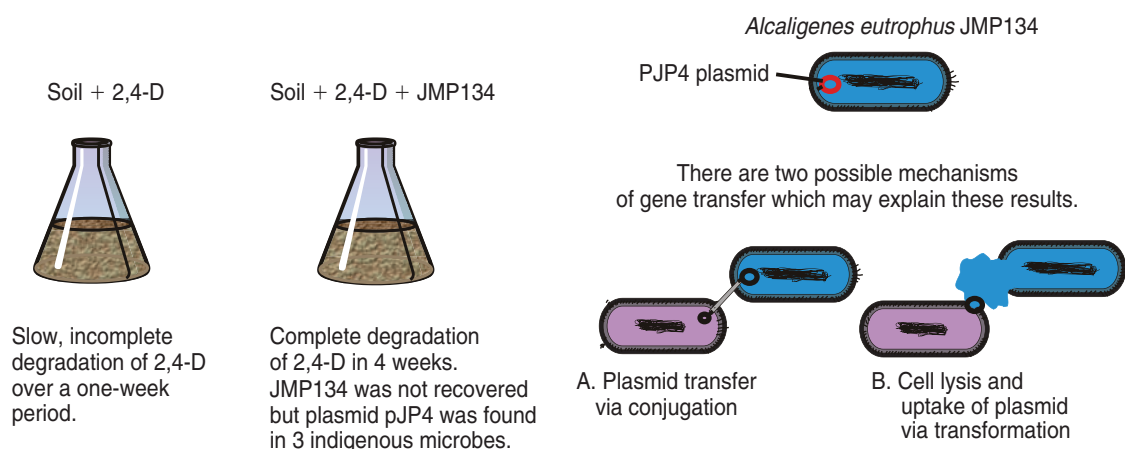
### 3.3.3 The Stationary and Death Phases

Stationary phase in the laboratory is a period 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 cells have become so dense that further growth is inhibited. In the environment, the stationary phase is most likely of short duration if it exists at all. Recall that most cells never achieve an exponential phase because of nutrient limitations and environmental stress. Rather they are in dormancy or in a maintenance

### 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*, were 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 plasmid. 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<sup>a</sup>

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

Courtesy E. M. Jutras.

<sup>a</sup> Each soil was incubated for approximately 1 week and then culturable counts were determined using R2A agar.

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, enter a dormant phase again until new nutrients become available. Thus, the stationary phase is likely to be very short if indeed it does occur. In contrast, the death phase can certainly be observed, at least in terms of culturable counts. In fact, the death phase is often a mirror reflection of the growth phase. Once added nutrients are consumed, both living and dead cells become prey for protozoa that act as microbial predators. Complicating the issue in both is the presence of bacteriophage that can infect and lyse significant portions of the living bacterial

community. Dead cells are also quickly scavenged by other microbes in the vicinity. Thus, culturable cell numbers increase in response to nutrient addition (see Table 3.1) but will decrease again just as quickly to the background level after the 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** (Niedhardt *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 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  $\text{CO}_2$  corresponding to a cell yield of approximately 0.4. Note that glucose ( $\text{C}_6\text{H}_{12}\text{O}_6$ ) 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 (Fig. 3.11). 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 3.3). However, since industrialization began in the late 1800s, many new molecules have been manufactured for which there are no standard catabolic

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

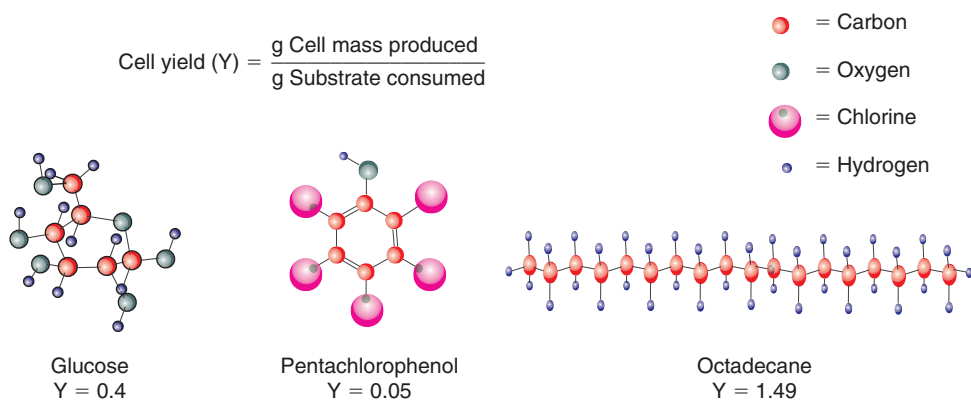
Residue	Half-life (days) <sup>a</sup>	$\mu^b$	
		(1/days)	Relative rate <sup>c</sup>
Wheat straw, laboratory	9	0.08	1
Rye straw, Nigeria	17	0.04	0.5
Rye straw, England	75	0.01	0.125
Wheat straw, Saskatoon	160	0.003	0.05

From Paul and Clark, 1989.

<sup>a</sup>The half-life is the amount of time required for degradation of half of the straw initially added.

<sup>b</sup> $\mu$  is the specific growth rate constant.

<sup>c</sup>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 3.11** Cell yield values for various substrates. Note that the cell yield depends on the structure of the substrate.

**Example Calculation 3.3 Distribution of 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<sub>2</sub>?

Assume that you start with 1 mole of carbohydrate (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, molecular weight = 180 g/mol):

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

Cell mass can be estimated as C<sub>5</sub>H<sub>7</sub>NO<sub>2</sub> (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<sub>2</sub>.

**Question**

Calculate the carbon found as cell mass and CO<sub>2</sub> for a microorganism that grows on octadecane (C<sub>18</sub>H<sub>36</sub>), Y = 1.49, and on pentachlorophenol (C<sub>6</sub>HOCl<sub>5</sub>), Y = 0.05.

**Answer**

For octadecane, 93% of the substrate carbon is found in cell mass and 7% is evolved as CO<sub>2</sub>. For pentachlorophenol, 10% of the substrate carbon is found in cell mass and 90% is evolved as CO<sub>2</sub>.

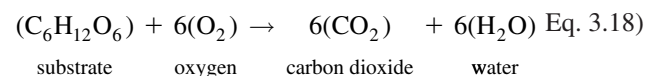
pathways. Pentachlorophenol is an example of such a molecule. This material has been commercially produced since 1936 and is one of the major chemicals used to treat wood and utility poles. To utilize a molecule like pentachlorophenol, which appeared in the environment relatively recently on an evolutionary 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 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 20). 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, yielding more energy from the degradation of octadecane than from the degradation of glucose. This in turn is reflected in a higher cell yield value.

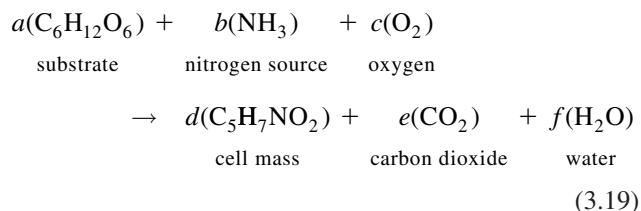
**3.4.1 Aerobic Conditions**

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:



In Eq. 3.18, the substrate is a carbohydrate such as glucose, which can be represented by the formula C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>.

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:



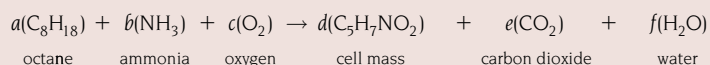
where  $a$ ,  $b$ ,  $c$ ,  $d$ ,  $e$ , and  $f$  represent mole numbers.

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 3.19 differs from Eq. 3.18 in two ways: it represents the production of new cell mass, estimated by the formula  $\text{C}_5\text{H}_7\text{NO}_2$ , and in order to balance the equation, it has a nitrogen source on the reactant side, shown here as ammonia ( $\text{NH}_3$ ).

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 24), for production of high value microbial products (e.g., antibiotics or vitamins), and for remediation of contaminated sites (see Chapter 20 and Example Calculation 3.4).

#### Example Calculation 3.4 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 ( $\text{C}_8\text{H}_{18}$ ) 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:



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:

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

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  $b$ :

$$\begin{aligned}
 b(1 \text{ mol nitrogen}) &= d(1 \text{ mol nitrogen}) \\
 b(1) &= 1.2(1) \\
 b &= 1.2
 \end{aligned}$$

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

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

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

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

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

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

Thus, the solved mass balance equation is  $1(\text{C}_8\text{H}_{18}) + 1.2(\text{NH}_3) + 6.5(\text{O}_2) \rightarrow 1.2(\text{C}_5\text{H}_7\text{NO}_2) + 2.0(\text{CO}_2) + 6.6(\text{H}_2\text{O})$ .

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:

$$\text{Convert to liters (l): } 10,000 \text{ gallons } (3.78 \text{ l/gallon}) = 3.78 \times 10^4 \text{ l gasoline}$$

$$\text{Convert to grams (g): } 3.78 \times 10^4 \text{ l gasoline } (690 \text{ g gasoline/l}) = 2.6 \times 10^7 \text{ g gasoline in the site}$$

$$\text{Convert to moles: } \frac{2.6 \times 10^7 \text{ g gasoline}}{114 \text{ g octane/mol}} = 2.3 \times 10^5 \text{ mol octane in the site}$$

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  $\text{NH}_3$ /mol octane (see coefficient  $b$ ).

$$\begin{aligned} (1.2 \text{ mol NH}_3/\text{mol octane})(2.3 \times 10^5 \text{ mol octane in the site})(17 \text{ g NH}_3/\text{mol}) &= 4.7 \times 10^6 \text{ g NH}_3 \\ \Rightarrow 4.7 \times 10^6 \text{ g } (1 \text{ kg}/1000 \text{ g})(2.2046 \text{ lb}/\text{kg}) &= 10,000 \text{ lb or 5 tons of NH}_3! \end{aligned}$$

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  $\text{O}_2$ /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.

$$\begin{aligned} (1.5 \times 10^6 \text{ mol O}_2)(22.4 \text{ l/mol air})(1 \text{ mol air}/0.21 \text{ mol O}_2) &= 1.6 \times 10^8 \text{ l air} \\ 1 \text{ cubic foot of air} &= 28.33 \text{ l} \\ \Rightarrow 1.6 \times 10^8 \text{ l air } (1 \text{ cubic foot}/28.33 \text{ l gas}) &= 5.5 \times 10^6 \text{ cubic feet of air or enough air to fill a} \\ &\text{football field to a height of 100 ft!} \end{aligned}$$

From Pepper, *et al.*, 2006.

### 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. In general, anaerobic degradation is restricted to 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). In 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),



**TABLE 3.3** Relationship between Respiration, Redox Potential, and Typical Electron Acceptors and Products<sup>a</sup>

Type of respiration	Reduction reaction electron acceptor <sup>→</sup> product	Reduction potential (V)	Oxidation reaction electron donor <sup>→</sup> product	Oxidation potential (V)	Difference (V)
Aerobic	O <sub>2</sub> – H <sub>2</sub> O	+0.81	CH <sub>2</sub> O – CO <sub>2</sub>	–0.47	–1.28
Denitrification	NO <sub>3</sub> <sup>–</sup> – N <sub>2</sub>	+0.75	CH <sub>2</sub> O – CO <sub>2</sub>	–0.47	–1.22
Manganese reduction	Mn <sup>4+</sup> – Mn <sup>2+</sup>	+0.55	CH <sub>2</sub> O – CO <sub>2</sub>	–0.47	–1.02
Nitrate reduction	NO <sub>3</sub> <sup>–</sup> – NH <sub>4</sub> <sup>+</sup>	+0.36	CH <sub>2</sub> O – CO <sub>2</sub>	–0.47	–0.83
Sulfate reduction	SO <sub>4</sub> <sup>2–</sup> – HS <sup>–</sup> , H <sub>2</sub> S	–0.22	CH <sub>2</sub> O – CO <sub>2</sub>	–0.47	–0.25
Methanogenesis	CO <sub>2</sub> – CH <sub>4</sub>	–0.25	CH <sub>2</sub> O – CO <sub>2</sub>	–0.47	–0.22

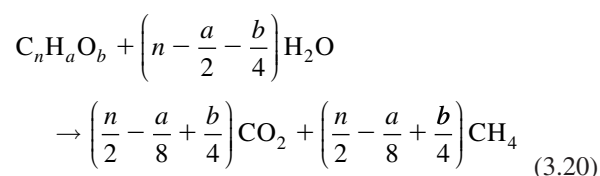
Adapted from Zehnder and Stumm, 1988, reproduced by permission of John Wiley and Sons, Inc.

<sup>a</sup>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.

iron (iron-reducing conditions), sulfate (sulfate-reducing conditions), and carbonate (methanogenic conditions). 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<sub>4</sub>) and CO<sub>2</sub>:



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<sub>2</sub>, or in its most reduced form, CH<sub>4</sub>. This is called **disproportionation** of organic carbon. The ratio of methane to carbon dioxide found in the gas mixture formed as a result of anaerobic degradation depends on the oxidation state of the substrate used. Carbohydrates are converted to approximately equal amounts of CH<sub>4</sub> and CO<sub>2</sub>. 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<sup>4</sup> to 10<sup>8</sup> CFU/ml assuming a generation time of 1.5 h.
3. You are given a microorganism that has a maximum growth rate ( $\mu_{\max}$ ) of 0.39 1/h. Under ideal conditions (maximum growth rate is achieved), how long will it take to obtain 1 × 10<sup>10</sup> CFU/ml if you begin with an inoculum of 2 × 10<sup>7</sup> CFU/ml?
4. Is there any way to increase the growth rate observed in question 3?
5. Write the Monod equation and define each of the constants.
6. There are two special cases when the Monod equation can be simplified. Describe these cases and the simplified Monod equation that results.
7. List terminal electron acceptors used in anaerobic respiration in the order of preference (from an energy standpoint).

8. Define disproportionation.
9. Define the term critical dilution rate,  $D_c$ , and explain what happens in continuous culture when  $D$  is greater than  $D_c$ .
10. Compare the characteristics of each of the growth phases (lag, log, stationary, and death) for batch culture and environmental systems.
11. Compare and contrast the copiotrophic and oligotrophic life-styles in the environment.

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