

## Exponential growth and bacterial growth laws

Bacterial populations in balanced exponential growth play a central role for quantitative biology. Thus, it is crucial to have techniques for precise measurements of the exponential growth rate of bacterial populations. Exponential growth has implications for the distribution of cell ages in the population. Further, many aspects of the physiology and composition of the bacterial cell depend only on the growth rate: in particular, the concentration of ribosomes, the DNA content, and the size of the cell increase with growth rate in ways that can often be calculated starting from a few plausible assumptions. These phenomenological “growth laws” are conceptually analogous to laws in physics such as Ohm’s law or the ideal gas law: they enable quantitative predictions about the behavior of the system even if the microscopic origins of the laws are not fully understood. Relatively few such laws are known in biology but their identification and corroboration is an active field of research in biological physics. In this experiment, exponential growth of an *Escherichia coli* population is observed in growth media of different nutrient quality and the cell sizes are measured to validate a phenomenological law that relates the total protein content of the cell to its growth rate.

### Background and introduction

#### Theory

##### *Exponential growth*

Consider a population of  $N$  bacteria. If the population is provided with an excess of all nutrients that are required for growth, it will reach a steady state of exponential growth after some time. In this state, each cell doubles and divides once during its generation time. The number of cells  $N$  obeys

$$\dot{N} = \mu N. \quad (1)$$

Here,  $\mu$  is the (specific) growth rate. Starting from  $N_0$  cells at time  $t = 0$ , the solution of this simple differential equation is

$$N(t) = N_0 \exp(\mu t)$$

i.e. the population grows exponentially. The **doubling time**  $\tau$  is often used instead of  $\mu$ ; it is defined as the time it takes for  $N$  to double.

The **cell age distribution** of a bacterial population in steady state exponential growth is not homogeneous. Here, the “age”  $a$  of a cell is defined as the time that has passed since the last cell division (which led to the birth of the cell). Measuring cell age in units of the generation time, the cell age probability density distribution (Sueoka & Yoshikawa 1965) is

$$\rho(a) = (\ln 2) 2^{1-a}.$$

We see from this relation that there are exactly twice as many cells with age 0 (i.e. directly after cell division) than cells with age 1 (i.e. directly before cell division). Since cell age is closely related to cell size (cells right after division have half the size as right before division), this implies that the cell size distribution in an exponentially growing population of bacteria is not symmetric but skewed towards small cell sizes.

### The bacterial growth curve

Exponential growth cannot continue indefinitely as the nutrients are bound to be depleted at some point. A more realistic model that takes this into account is described by the **logistic growth equation**

$$\dot{N} = \mu \left(1 - \frac{N}{K}\right) N$$

Here,  $K$  is the **carrying capacity**. For  $N \ll K$ , this reduces to Eq. (1). The logistic growth model describes that the growth rate decreases as  $N$  approaches the carrying capacity  $K$ . For  $t \rightarrow \infty$  growth stops completely and the population size approaches  $N_{\infty} = K$ : the population has grown to saturation and has reached stationary phase (where cells have stopped dividing but remain viable). The final number of cells is also called **growth yield**.

Another aspect of the growth curve is the **lag phase**: it is roughly defined as the time  $\tau_{\text{lag}}$  a population of bacteria in stationary phase needs to start growing after being provided with an excess of nutrients. The complete growth curve (Figure 1) also includes the death phase in which cells that remain in stationary phase for a long time start dying.

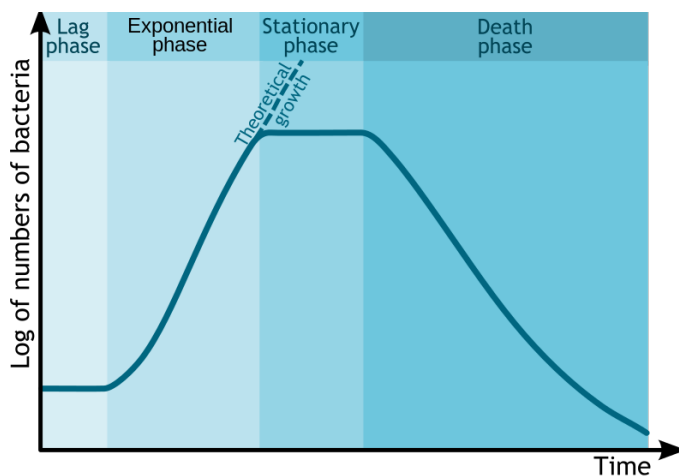


Figure 1. Schematic of the growth curve. Source: Wikipedia.

### Properties of growth parameters

The three parameters characterizing the growth curve  $\tau_{\text{lag}}$ ,  $K$ , and  $\mu$  depend on the nutrients that are provided (Bremer & Dennis 2008) and on other properties of the environment such as temperature, pH, and osmolality. The lag phase  $\tau_{\text{lag}}$  and the growth yield  $K$  depend on the history of the system. In particular,  $\tau_{\text{lag}}$  increases the longer the cells were in stationary phase before being provided with fresh nutrients. Similarly,  $K$  depends on the number of cells and the amount of nutrients that were initially present and on how fast the cells grow during the experiment.

In contrast, the growth rate  $\mu$  that is reached in a steady state of exponential growth is independent of the history of the system. It is well reproducible for a given bacterium and controlled environmental conditions. For example,  $\mu$  depends on temperature and, for *E. coli*, has a maximum near 37°C. Importantly, the growth rate  $\mu$  depends on the nutrient environment. In particular, *E. coli* can metabolize

different sugars which are used as a source of carbon and energy. For each sugar, bacteria grow with a characteristic growth rate  $\mu$ . *E. coli* grows fastest on glucose, where it can reach generation times as short as  $\sim 20$ min, and slower on other sugars like lactose.

### Bacterial growth in batch culture

Quantitative experiments are often performed in a steady state of exponential growth, well after the end of lag phase and before the onset of entry into stationary phase; this ensures that a defined cell state is reached, making any measurement that is performed reproducible. **Batch culture** is a common way of performing experiments that require bacteria in exponential growth. Here, a small volume of bacterial culture is transferred into a larger volume of fresh medium. This culture is then incubated at controlled temperature with shaking; the bacteria grow and divide until nutrients are depleted.

### Measuring cell numbers

There are several different techniques for following the number of cells in a growing culture. Usually, a small controlled sample volume is taken from the well-mixed culture at regular time intervals and the number of cells in this sample is determined using one of the following techniques:

- **Colony forming units (CFUs).** In this classical technique, a dilution series is prepared (e.g. 10-fold, 100-fold, etc.) and a controlled volume is spread on an agar plate containing a growth medium. These plates are then incubated overnight. The next day, bacterial colonies can then be observed with the naked eye and counted directly (Figure 2). If the culture was sufficiently diluted, each colony originated from a single bacterium. Thus, the number of bacteria that was in the original sample can be reconstructed from this measurement. An advantage of this technique is that it does not require any sophisticated equipment.

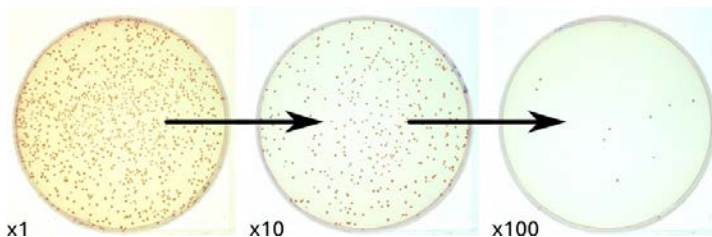


Figure 2. Pictures of colonies on agar plates at different dilutions. Source: Wikipedia.

- **Direct cell counting.** The number of cells in the sample volume is determined by directly counting the cells. This can be done by putting the cells in a counting chamber (hemocytometer) and observing them under the microscope. Another technique uses a cell counter which pumps a controlled volume through a narrow channel where the cells pass a detector one-by-one and are counted (see below).
- **Optical density.** Here, the sample volume is placed in a cuvette and the absorbance of a light beam is measured in a spectrophotometer. This instrument shines light (usually at 600nm) through the sample, measures the decrease in light intensity after passing through the sample (Figure 3), and determines the absorbance based on the Beer-Lambert law. The absorbance is approximately proportional to the biomass density in the culture, which in turn is a proxy for the cell density. This technique is fast and easy but measures the number of cells only indirectly, i.e. a calibration curve

must be measured for the conversion of optical density into cell numbers.

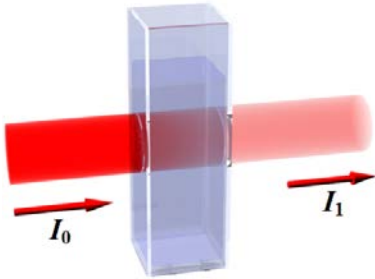


Figure 3. Schematic of an optical density measurement in a cuvette.  $I_0$  and  $I_1$  are the intensities of the light beam before and after passing through the cuvette, respectively. Modified from <http://www.deltaopticalthinfilm.com/optical-density/>.

### Measuring cell size and number with a Coulter counter

A Coulter counter is an instrument that pumps a controlled volume of an electrolyte solution containing particles (here: bacteria) through an orifice and detects each event when a particle passes through the orifice (Figure 4). It can thus be used to determine the number of particles per volume. It is further used to precisely measure the size (volume) of each particle. The measurement is based on the **Coulter principle** which states that a particle moving through an orifice, together with an electric current, produces a change in impedance that is proportional to the volume of the particle traversing the orifice. This increase in impedance results from the displacement of electrolytes by the particle.

#### *Instrument setup*

In a Coulter counter, a tube with a small aperture on the wall is immersed in a container with particles suspended in an electrolyte solution (Figure 4). Two electrodes, one located inside the aperture tube and one outside create a current through the electrolyte when a voltage is applied. The impedance between the two electrodes is then measured. When a particle passes through the aperture, it displaces a volume of the electrolyte solution from the aperture that is equal to the volume of the particle. As a result, the impedance across the aperture increases for a short period of time. This increase can be detected as a voltage or current pulse. The pulse height is proportional to the volume of the particle. Absolute volumes can be determined by calibrating the instrument using spherical beads of known volume.

Thus, the volume of each particle that passes through the aperture can be determined. From many such measurements, the particle size distribution is obtained. Additional parameters that characterize the shape of the pulse (e.g. its width) can also be recorded. While the technique measures particle volume, the results are often represented in terms of equivalent spherical diameter (i.e. the diameter of the particle assuming that it is a perfect sphere). Note that this is not a good approximation for rod-shaped bacteria like *E. coli*.

State-of-the-art cell counters such as the Multisizer™3 used in this experiment precisely control the volume of liquid that passes through the aperture. Consequently, apart from particle size distributions, they enable measurements of the particle concentration.

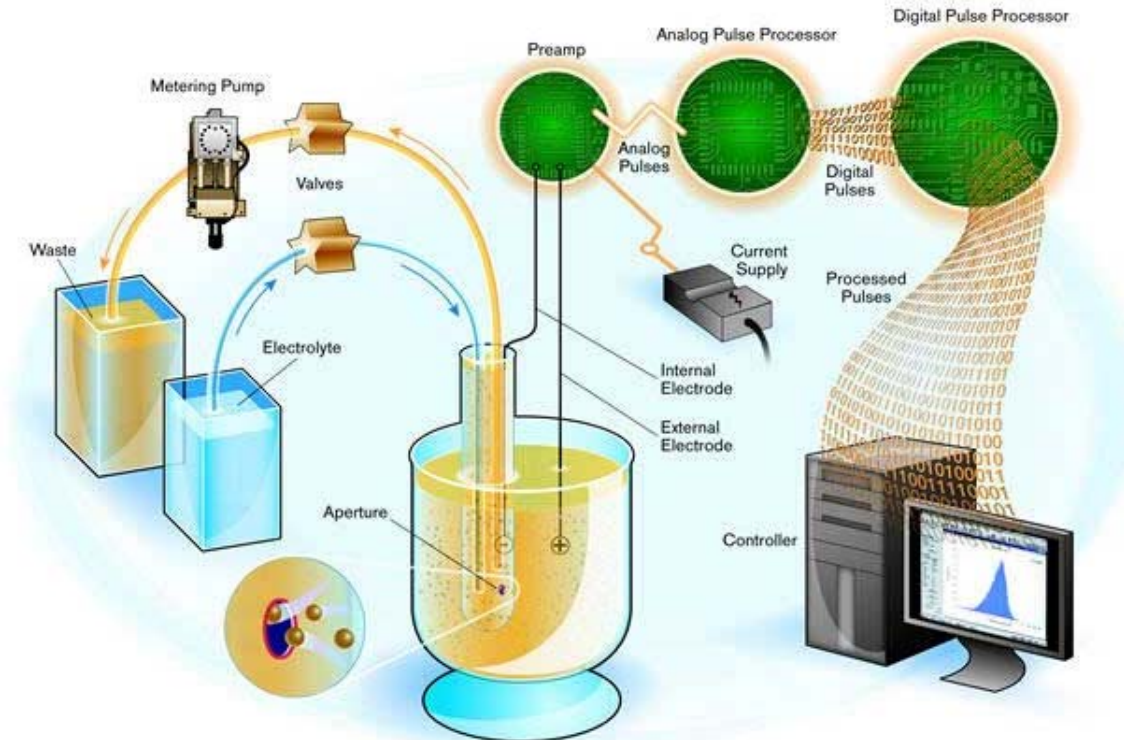


Figure 4. Schematic of a Coulter counter. Source: [www.beckman.com](http://www.beckman.com).

#### Experimental considerations

**Aperture size.** Particles with a size of 2 to 60% of the aperture diameter can be measured with an accuracy better than 1%. For example, the 30  $\mu\text{m}$  aperture used in this experiment is ideal for particles from about 0.6 to 18  $\mu\text{m}$  in diameter. *E. coli* falls in the lower end of this size range. Greater apertures become necessary for measuring larger cells.

**Coincidence.** Anomalous electrical pulses can occur if the concentration of sample is so high that multiple particles often enter the aperture simultaneously. There is no way to distinguish if a large pulse is caused by a single large particle or by multiple particles that entered the aperture at once. To avoid this problem, it is crucial to use a well-mixed and sufficiently diluted sample.

**Debris.** The electrolyte solution inevitably contains small debris particles that can be of a similar size as the bacteria that are measured. This problem is reduced by passing the electrolyte solution through a 0.2  $\mu\text{m}$  filter which removes any larger debris. Still, bacteria are sufficiently small that it can be difficult to clearly separate them from the debris.

#### Bacterial growth laws

Certain aspects of the physiology and composition of the bacterial cell depend only on its growth rate. For example, the concentration of ribosomes in the cell increases linearly with growth rate. Such phenomenological “growth laws” are conceptually analogous to laws like Ohm’s law in physics: they enable quantitative predictions about the behavior of the system even if the microscopic origins of the laws are not understood. Key examples of bacterial growth laws are summarized below. Importantly, these laws are only valid in steady state exponential growth.

### Ribosome concentration

The ribosome concentration in *E. coli* increases with the growth rate. Growth media with higher nutrient quality enable faster growth and lead to ribosomes occupying a greater fraction of the total cell mass. In the fastest growing *E. coli* cells, which double every ~20 minutes, ~50% of the total dry mass of the cell (i.e. its mass excluding water) is in ribosomes! The ribosome concentration  $r$  is related to the growth rate  $\mu$  via

$$r = r_0 + \mu/\kappa_t$$

where  $r_0$  is a  $y$ -axis intercept and  $\kappa_t$  is proportional to the rate of protein synthesis per ribosome. This relation holds quite generally (Figure 5) for diverse nutrient conditions and across different *E. coli* strains. For more details on this law and its various implications, see (Scott et al. 2010; Scott & Hwa 2011; Scott et al. 2014).

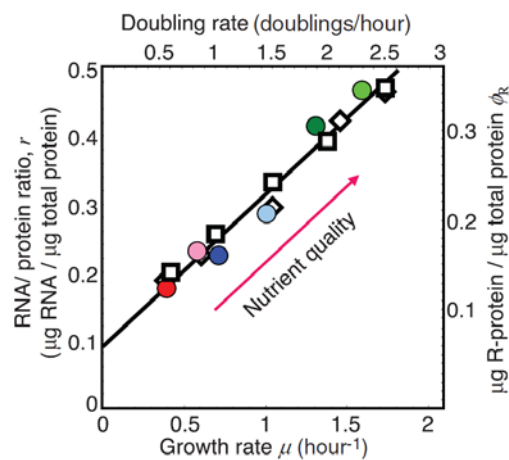


Figure 5. Ribosome concentration at different growth rates. RNA/protein ratio on the  $y$ -axis is a proxy for the ribosome concentration. The data points show measurements of this quantity for different *E. coli* strains and growth media that result in different growth rates. Modified from (Scott et al. 2010).

### DNA content

Similarly, the DNA content of bacterial cells in exponential growth depends only on the growth rate. Almost all DNA in *E. coli* is contained in its single circular chromosome. The replication of this chromosome is initiated in discrete events where two **replication forks** are started at the **origin of replication**; these two forks then move at approximately constant velocity in opposite directions along the chromosome and produce a copy of the DNA along the way. When they reach the terminus, which is located approximately halfway around the circular chromosome, replication is complete and two separate chromosomes are present in the cell. Copying the chromosome once in this way takes a time  $C \approx 40$  min (for historical reasons this time is called the “C period”). Completion of chromosome replication is followed by a delay of  $D \approx 20$  min (called the “D period”) before the cell divides. Importantly, multiple rounds of replication can run in parallel, enabling the cell to divide with a generation time shorter than 60 min (Figure 6).

Based on this information, the growth law for the average DNA content per cell  $G_{\text{cell}}$  as a function of the doubling time  $\tau$  can be derived:

$$G_{\text{cell}} = \frac{\tau}{c \ln(2)} (2^{(C+D)/\tau} - 2^{D/\tau}) \quad (2)$$

Here,  $G_{\text{cell}}$  is measured in genome equivalents. For details of this derivation, see (Bremer & Churchward 1977; Cooper & Helmstetter 1968).

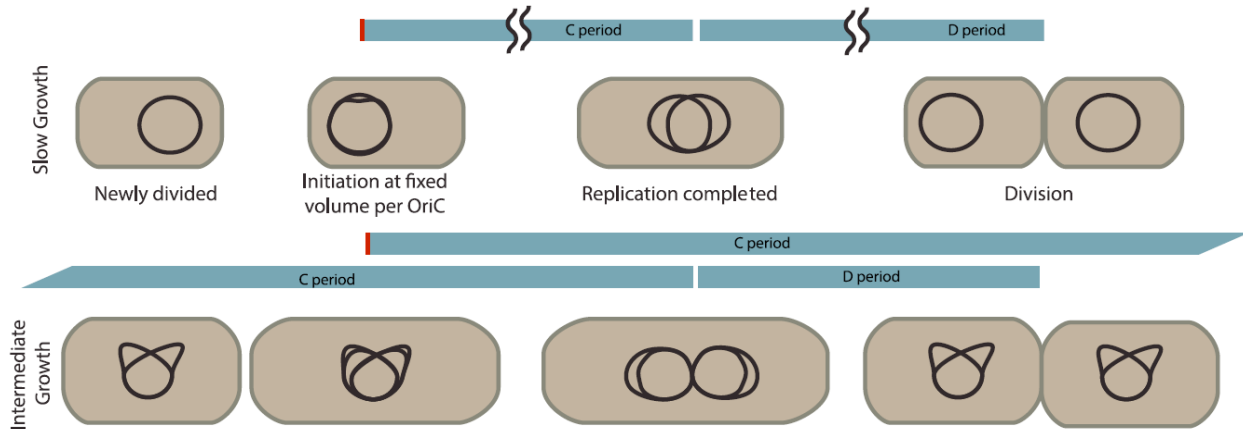


Figure 6. Schematic of chromosome replication in *E. coli* at different growth rates. Top: slow growth where only one replication round occurs at any given time. Bottom: faster growth where multiple replication rounds run in parallel. Modified from (Wallden et al. 2016).

### Cell size

There is a closely related law for the dependence of the average cell size on the growth rate. The cell size can be quantified as the dry mass of the cell. In general, proteins are the dominant fraction of this dry mass. Thus, we can approximately equate the dry mass with the total mass of all proteins in the cell. Further, the mass concentration of proteins in the cell is approximately constant (the cell volume is always densely packed with proteins). Thus, to a good approximation, the mass of the cell is directly proportional to its volume.

It was observed decades ago that the cell mass per origin of replication is approximately constant when a new round of DNA replication is triggered (Donachie 1968); this observation was recently also confirmed in single cells (Wallden et al. 2016). As a result, the dependence of cell size on growth rate is closely related to that of the DNA content. While the microscopic mechanism underlying this empirical observation is still not understood, it implies that the cell mass  $M_{\text{cell}}$  has the following dependence on the doubling time:

$$M_{\text{cell}} = M_0 2^{(C+D)/\tau}.$$

Here,  $M_0$  is a constant (namely, the constant cell mass per origin of replication). Since  $V_{\text{cell}} \propto M_{\text{cell}}$ , this implies

$$V_{\text{cell}} = V_0 2^{(C+D)/\tau}. \quad (3)$$

### Goals and tasks

The main goal of this experiment is to validate the dependence of cell size on growth rate. Specific tasks:

- I. Inoculate *E. coli* bacteria in four different growth media (i.e. different nutrient environments), quantify the growth rate and validate its dependence on the nutrient quality of the growth medium.

2. Find out how optical density (biomass) is converted into cell numbers at different growth rates.
3. Determine the average cell size at the four different growth rates and compare it to the corresponding growth law, Eq. (3).
4. Measure the distribution of cell sizes in exponential growth and in stationary phase and compare it to the distribution expected from the theoretical cell age distribution.

### Experimental procedure

1. Fill four Erlenmeyer flasks with different growth media (20 mL) and inoculate each flask from an overnight culture of *E. coli* strain MG1655 (1,000-fold dilution). As growth media, the rich LB (lysogeny broth) medium and M9 defined minimal medium are used. To achieve different growth rates in the M9 medium, one culture will be supplemented with amino acids and glucose, another culture will be supplemented with amino acids and glycerol, and a third culture will be supplemented with glycerol as the only carbon source.
2. Put the flasks in a shaking incubator at 37°C. Start a timer.
3. Right away and then every 30-45 min: Take a sample (0.5 mL) from each flask, transfer to a cuvette, and **measure optical density at 600 nm** ( $OD_{600}$ ). Make sure that the culture is well mixed before the transfer and before measuring  $OD_{600}$ . Initially, a lag-phase is expected (cf. Figure 1).
4. In the meantime (i.e. between optical density measurements during the lag phase), **count cells and measure cell volumes of a culture in stationary phase:** Perform  $OD_{600}$  measurement for a stationary phase culture (i.e. the overnight culture) and practice using the cell counter with the stationary phase cells. Depending on the linear range of the spectrophotometer used with the cuvette, a dilution (e.g. 10 or 20-fold) of the stationary phase culture is necessary for an accurate reading. Next, dilute the stationary phase culture 5,000-fold in saline solution, then measure cell numbers and volumes in the cell counter. The Multisizer™3 accepts volumes of 10 mL of those saline solutions. Also, measure the particle size distribution of the debris in the saline solution alone.
5. **Count cells and measure cell volumes of exponentially growing culture:** When the fastest-growing culture is approaching  $OD_{600} = 0.1$ , prepare a suitable dilution (e.g. 500-fold) of this culture in saline solution. Then measure the cell numbers and volumes in the cell counter.
6. In the same way, measure cell numbers and volumes for the remaining three, slower growing, cultures. Start with the fastest-growing one and do the slowest-growing one last (but do not necessarily wait till  $OD_{600}=0.1$  is reached if it takes too long). Follow the growth curve of the cultures through OD measurements for the rest of the day.

### Analysis

Important: include error estimates for all quantities that are calculated.

1. Use a fit or linear regression to extract the growth rates from the  $OD_{600}$  measurements. Estimate the error of the growth rate. Calculate the corresponding doubling times and compare them to published values for similar growth media (Scott et al. 2010; Bremer & Dennis 2008).
2. Plot  $OD_{600}$  versus number of cells per culture volume and extract the conversion factor at the different growth rates and in stationary phase. Which different sources of error affect the number of cells per culture volume?



3. Calculate the average cell volume from the cell counter measurements and plot OD<sub>600</sub> per cell versus cell volume at the different growth rates and in stationary phase. Do your data support the view that OD<sub>600</sub> essentially measures biomass? Also, compare the cell volume you measured to literature values (e.g. [www.bionumbers.org](http://www.bionumbers.org)).
4. Plot the distributions of cell volumes at the different growth rates and in stationary phase. Correct these distributions for the debris in the saline solution. Quantify the skewness of these distributions (i.e. calculate Pearson's moment coefficient of skewness). Comment on the skewness for exponentially growing cells versus cells in stationary phase.
5. Plot the average cell volume as a function of the doubling time and fit Eq. (3) to these data. Compare the resulting values for  $C + D$  to literature values (Bremer & Dennis 2008).

## References

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**The reference typeset in bold is particularly relevant for the exam.**

T. Bollenbach, August 18, 2017