Learning Outcomes

When you have completed this module, you should be able to:

- Define dilution and dilution factor
- Explain why dilutions are necessary for microbial counts
- Prepare dilutions and calculate total dilution of serial dilutions
- Set up an appropriate dilution scheme to result in a countable plate
- Evaluate and interpret the results of plate counts, including selecting the appropriate plate for counting and scaling up from the diluted sample to the original population

Why we need to dilute

For the remainder of this module, we're going to stick with the Viable Plate Count method -- the only method that can tell a live cell from a dead one. But we still have another problem -- the possibility of too many colonies to count. For example, if there are 500 Vibrio per mL and we put 1mL onto a plate, 500 colonies on a Petri dish would look something like this:

![Image of a Petri dish with colonies]

It is so crowded on the plate, that it would not be possible to obtain an accurate count of the number of colonies. And the problem would be even worse if you were trying to count a population in the thousands or millions! You could literally have a carpet of colonies (also known as a 'confluent lawn') growing on your Petri dish. Not only would it be impossible to count the colonies, but not all colony-forming units would be able to form colonies because of the overcrowding and competition for nutrients. This is where dilution saves the day. Not just dilution, but serial dilution... meaning dilution over and over again.
**Why do we dilute?** To have fewer colonies to count and to obtain a more accurate count.

**Why do we do it repeatedly?** Because we don't know how much dilution we need. Every time we dilute, we'll also make a new plate to incubate. So we might do 5 dilutions and grow up 5 plates. Then we'll end up throwing away 4 of them. Sound wasteful? Well, dilution and plating is quick and easy compared to the pain of starting your experiment all over again just because your plate has too many or too few colonies.

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**Setting the stage: Diluting Cold Press Coffee**

Of course, lots of things can be diluted, not just samples containing bacteria……...

So let's see what the process of "diluting" looks like. Here's an everyday example:

Cold press coffee is a super strong form of coffee made without heating the water. The brew starts out with about a zillion molecules of caffeine per cup. In order to actually be able to drink the stuff, you have to dilute it. For example, you could put one cup of cold press coffee into a pot and add 9 cups of water.

![Diagram of diluting cold press coffee](image)

How many molecules of caffeine are in the pot? **One zillion**

How many cups of coffee are in the pot? **10** (1 cup of cold press + 9 cups of water)

If you pour 1 cup from the pot, how many molecules of caffeine will it have? **A tenth of a zillion**
### Working out the dilution

When you're thinking about dilution, it helps to simplify your actions into dilution factors. When we said the diluted coffee was

“1/10\textsuperscript{th} as strong as the original”

We were explaining how much the coffee was diluted. We could also have said:

“The coffee was diluted 1/10”, or

“The coffee was diluted 10\textsuperscript{-1}”, or less frequently,

“The coffee was diluted 0.1”

Here are some more examples for you to try:

1. 1 mL coffee + 4 mL water = \textbf{1 in 5 or 5\textsuperscript{-1}}
2. 1 mL coffee + 9 mL water = \textbf{1 in 10 or 10\textsuperscript{-1}}
3. 1 mL + 99 mL water = \textbf{1 in 100 or 10\textsuperscript{-2}}

As you've probably guessed, this works exactly the same whether you're talking about caffeine or bacterial cells. Here's what a dilution of 1/100 or 10\textsuperscript{-2} looks like on the lab bench:

Notice that it really doesn't matter how much of the original stock you started with, as long as you had at least 1 mL to put into the new container. What matters is how much you transfer and how much water you add. The dilution is then defined as:

\[
\text{Volume transferred} / \text{total volume}
\]

Where the total volume is the volume transferred + volume of water added

\[
1 / (1+99) = 1/100 = 10\textsuperscript{-2} = 0.01.
\]
As shown above, the dilution can be expressed as a fraction (1/100), in scientific notation ($10^{-2}$) or less commonly, as a decimal (0.01). In this example, water is the diluent (a general term for the liquid used to dilute the sample).

**Overall dilution**

Now that you are very comfortable with dilution factors…..what happens if you dilute and then dilute again? For example, we could take super-strong coffee, dilute it 1/5, and then dilute THAT by 1/10. So:

Here’s the rule:

To find the overall (or total) dilution, simply multiply the dilution at each step. In this example, to work out how much the original coffee was diluted, multiply 1/5 by 1/10. This works regardless of how many dilutions you do.

**Design a dilution scheme!**

So let’s put some of these skills together to design dilution schemes. To do this, you need to figure out the overall dilution you’re aiming for. In this case, just take the concentration you want to achieve, and divide it by the concentration you currently have. For example, if you want coffee with 5 caffeine molecules per cup, but you have coffee with 5000 molecules per cup, you have to prepare a dilution of 5/5000 or 1/1000.

The trickier part is figuring out a series of dilutions that will achieve this overall dilution. There is usually more than one way to achieve the same total dilution, and in deciding the best one to choose, you should avoid using volumes that are really large (this is wasteful and mixing may be a problem) or very small (sample taken may not be representative of the original sample). As any measurement or mixing errors are compounded when preparing a series of dilutions (or serial dilutions) it is generally advisable to minimise the number of steps.

The online version has examples in designing dilutions:
http://mathbench.org.au/microbiology/viable-plate-count-or-how-to-count-to-a-million/5-design-a-dilution/
How to scale up

So far we’ve figured out how to make a dilution, which we can then plate and count. But this means we are only counting a fraction of what was originally there. How do we account for that? By scaling up.

"Scaling up" means starting from the number or concentration in a sample and figuring out how many were in the original brew of coffee, or original sample of a chemical or a bacterial culture.

For example, we can start with a cup of weak coffee and figure out how much caffeine was in the original brew. All we need to know is what the overall dilution factor was. In the case of weak coffee, it was 1/50.

So, when we count the caffeine molecules in a cup of weak coffee, we know that we’ve got 1/50th of what was in a cup of the original brew -- or in other words, there was 50 times as much in a cup of the original. So we multiply the number we counted or measured by the inverse of the dilution – in this case, 50. **This inverse of the dilution is called the dilution factor.**

- **If the dilution is in the form of a fraction**, you can "flip" the fraction (i.e., 1/50 becomes multiply by 50/1).
- **If the dilution is written in scientific notation** (e.g. $10^{-5}$) multiply by 1 over the number (i.e., $1/10^{-5}$ becomes multiply by $10^5$).

Finally, notice that I'm telling you the total number of caffeine molecules in ONE CUP of the original brew. If I don't know the actual amount of original brew, I won't know the total amount of caffeine that there was in the whole brew.
Back to the lab

Here are some problems, ranging from easy to a bit hard...

I did a series of dilutions with an overall dilution factor of 1/20,000, and then plated a 1mL sample. After 1 day, I counted 27 CFUs on the petri dish. How many CFUs would there be per 1mL of the original stock?

- Inverse of 1/20,000 is 20,000
- 27 x 20,000 = ...

Answer: 540,000 or $5.4 \times 10^5$ CFU per mL

I did a series of dilutions, 1/10, 1/10, and 1/100. At the end I plated and grew a 1mL sample, and counted 48 CFUs. How many CFUs would there be per 1mL of the original stock?

- What's the overall dilution factor?
- Overall dilution = 1/10 x 1/10 x 1/100 = 1/10,000
- Multiply by the inverse...
- Overall dilution factor = 10 x 10 x 100 = 10,000

Answer: 48 x 10,000 = 480,000 or $4.8 \times 10^5$ CFUs per mL

I did a series of dilutions as follows:

* 1 mL added to 9 mL water
* 1 mL added to 99 mL water
* 1 mL added to 49 mL water

If the final 1mL sample had 152 colonies, what was the original concentration?

- The dilution were 1/10, 1/100, and 1/50
- The overall dilution was 1/50,000

Answer: 152 x 50,000 = 7,600,000 or $7.6 \times 10^6$ cfu/mL
Count a plate!

The online version of this module contains an interactive applet, which allows you to look at different serial dilutions. To find this applet go to: http://mathbench.org.au/microbiology/viable-plate-count-or-how-to-count-to-a-million/7-count-a-plate/.

Once again, let’s take a breather from the theory and do some practice. This is one of the do-it-in-your-sleep kind, and it has two steps:

1. Look at the dilution scheme to determine the total dilution
2. Look at the plate and multiply the number of colonies by the dilution factor (the inverse of the total dilution) to determine the total CFUs per mL in the original sample.

Why we plate more than one dilution

Remember the picture of the impossible-to-count Petri dish with 500 colonies? No one wants that to happen to them, especially late on a Friday afternoon. So how can we avoid it?

Very easily ... while we’re doing our dilutions, we just keep plating each intermediate step. Then the next day, we decide which plate looks the most reasonable for counting. Since we cleverly labelled each plate with its overall dilution, we also know how to scale back up to get the original concentration.

So, now we finally have all the pieces of serial dilution assembled:

- Determine which dilutions to prepare
- Prepare serial dilutions
- Prepare plates
- Count colonies

To make things easier, the standard operating procedure is to go by factors of 10, and to do about 5 or 6 plates altogether.

The online version of this module contains an interactive applet, which allows you to look at different serial dilutions. To find this applet go to: http://mathbench.org.au/microbiology/viable-plate-count-or-how-to-count-to-a-million/8-why-plate-more-than-one-dilution/

So, for example, if I believe that I have about 42 million CFUs per mL, I might want to start with a first dilution of about 1/1000 (or 10⁻³), and plate that. Then I make 4 more 1/10 (or 10⁻¹) dilutions, plating each one in turn. In the end, I have 5 plates, labeled: 1/1,000; 1/10,000; 11/100,000; 11/1,000,000; and 1/10,000,000. The plate counts I expect in my original guess (42 million) was correct.
Picking the plate count

For this, we have an easy rule of thumb: the plate you count should have between 30 and 300 colonies (or 250-250 if you are following Australian Standard methods).

This rule of thumb has the undeniable advantage of being easy to use. But why does it work?

Let's imagine that we have a sample with exactly 42 million cells per mL. And let's say that our technique is flawless. If we count a plate prepared with 1 mL of a 1:1,000,000 dilution, we should find exactly 42 CFUs (remember, we have magically flawless technique and the medium and growth conditions suit the bacteria in the sample). But we would get the same number of colonies if our sample started with 42,000,001 cells, or 42,000,100 cells. Or even 42,100,000 cells. In fact, even with our absolutely flawless technique, the best we can say is that there are between 41,500,000 and 42,500,000 cells per mL in the original sample. So our absolutely unavoidable error is plus or minus 500K/42 million -- about 1.1%. That's pretty good.

Let's look at some other ways we could count the same 42 million cell sample. The information in the table shows that the theoretical error would be less if we used samples from lower dilutions (for example, 10\(^{-4}\) or 10\(^{-5}\) dilutions), but the colonies would be so crowded, it would be impossible to count them accurately. Overcrowding also means that not all viable cells in the sample will form colonies because of the competition for space and nutrients. On the other hand, if we base our calculations on plates with very few colonies, the error is too great.

So you can see that the 25 to 250 guideline is really a compromise between countability and accuracy.

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>Plate count</th>
<th>Error</th>
<th>Ease of counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10,000 (or 10(^{-4}))</td>
<td>4200</td>
<td>41,005,000 to 42,005,000 +/- 5000 = 0.011% great!!</td>
<td>NO, can't do it, this is too much to count</td>
</tr>
<tr>
<td>1:100,000 (or 10(^{-5}))</td>
<td>420</td>
<td>41,050,000 to 42,050,000 +/- 50,000 = 0.11% still pretty good</td>
<td>Takes time but countable</td>
</tr>
<tr>
<td>1:1,000,000 (or 10(^{-6}))</td>
<td>42</td>
<td>41,500,000 to 42,500,000 +/- 500,000 = 1.1% not too good</td>
<td>Easy to count</td>
</tr>
<tr>
<td>1:10,000,000 (or 10(^{-7}))</td>
<td>4</td>
<td>35,000,000 to 45,000,000 +/- 5 million = 11% ACK!</td>
<td>A four year old can count this plate!</td>
</tr>
</tbody>
</table>
Putting it all together

The online version of this module contains an interactive applet, which allows you to quantify a bacterial population. To find this applet go to: http://mathbench.org.au/microbiology/viable-plate-count-or-how-to-count-to-a-million/10-putting-it-all-together/

Quickies

Below, you can practice a couple more times. Also included are a few unusual situations ... things do go wrong, of course, and you should be able to recognise that. If you think the dilution series is not valid, click on "re-do the dilution". Lastly, "TNTC" means "too numerous to count".

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>1:100,000</th>
<th>1:1,000,000</th>
<th>1:10,000,000</th>
<th>1:100,000,000</th>
<th>Re-do dilutions?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>TNTC</td>
<td>674</td>
<td>68</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Answer</td>
<td>Nope! TNTC means &quot;too numerous to count&quot;!</td>
<td>Nope! 674 is definitely more than 250!</td>
<td>Yes -- And the answer is ... about 680,000,000 cells/mL</td>
<td>Nope! 7 is too few to be valid</td>
<td>No. each count is about 10th of the count before it, so the dilution looks good.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>1:100,000</th>
<th>1:1,000,000</th>
<th>1:10,000,000</th>
<th>1:100,000,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>375</td>
<td>105</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Answer</td>
<td>Nope! 375 is too many to count</td>
<td>Something is wrong! The second count dropped only by a factor of 3, rather than a factor of 10</td>
<td>Nope! 11 is too few to be valid</td>
<td>Nope! 1 is too few to be valid</td>
</tr>
</tbody>
</table>
Dilution factor | 1:100 | 1:1000 | 1:10,000 | 1:100,000 |
---|---|---|---|---|
Count | TNTC | 249 | 24 | 0 |
Answer | Nope! TNTC means "too numerous to count"! | Yes -- and the answer is 249,000 cells/mL | No -- 24 is too few to be valid | Nope! 0 is not valid for scaling up |

Dilution factor | 1:10 | 1:100 | 1:1000 | 1:10,000 |
---|---|---|---|---|
Count | 954 | 96 | 10 | 0 |
Answer | Nope! 954 is definitely more than 250! | Yes -- The answer is 9,600 cells/mL | Nope! This is too few to be valid | Nope! 0 is not valid |

Dilution factor | 1:10,000 | 1:100,000 | 1:1,000,000 | 1:10,000,000 | 1:100,000,000 |
---|---|---|---|---|---|
Count | TNTC | 567 | 58 | 12 | 1 |
Answer | Nope! TNTC means "too numerous to count"! | Nope! 567 is definitely more than 250! | Yes -- although it looks like there was a problem with the next dilution. The answer is ... about 58,000,000 cells/mL | Nope! 12 is too few to be valid | Nope! 1 is not valid |

No, although it looks like something happened to the 4th plate, it doesn't affect the plate you will use.

Is the water safe?

Remember Matt? He's still lying in the hospital bed waiting to hear what caused his illness and the local oyster industry is keen to get back into business.

What counting method will you use?
Unfortunately, a direct microscopic count or a count using a spectrophotometer would not tell us what we need to know – how many live *Vibrio* are in the water. So we choose a viable plate count.

**What dilution scheme will you use?**

We need to get plate counts between 25 and 250 (note that some countries use 30-300), and we think that the concentration maybe as high as 1 million \((10^6)\) per mL, but it could be much lower. So, we should go ahead and do the first plate without even diluting! After that we could do standard serial ten-fold \((1/10)\) dilutions.

**How many dilutions do you need?**

You definitely need at least 1 dilution, in case the number is somewhere in the 250 or 500 per mL range. Without a dilution, that wouldn't be countable. If there are >250 colonies in a 1/10 dilution, there are >2,500 actual cells per mL. As the count may well be much higher than this, it would be sensible to make more dilutions and not waste much time or energy. That way you should end up with a plate that has between 25 and 250 colonies. It might be more work in the short term, but in the long run it may save you having to repeat the experiment.

So, how many dilutions? Let’s assume the count might be as high as \(10^6\) bacteria per mL. If you prepare a \(10^{-4}\) \((1/10,000)\) dilution of the water, and plate 1mL, there should be 100 colonies on the plate after incubation. As the count may be lower, it would be advisable to plate the undiluted water, as well as samples of the \(10^{-1}\), \(10^{-2}\), \(10^{-3}\) and \(10^{-4}\) dilutions.

**Summary**

Make sure you understand the following concepts – go back to the applets if you need to:

- Dilution (stated as a fraction or in scientific notation)
- Overall dilution (product of individual dilutions)
- Choosing the plate to count (25 to 250 CFUs)
- Scaling up from the plate to the population size (multiplying by the dilution factor)
- Setting up an appropriate dilution scheme to result in a countable plate

**Learning Outcomes**

Now that you have completed this module, you should be able to:

- Define dilution and dilution factor
- Explain why dilutions are necessary for microbial counts
- Prepare dilutions and calculate total dilution of serial dilutions
- Set up an appropriate dilution scheme to result in a countable plate
- Evaluate and interpret the results of plate counts, including selecting the appropriate plate for counting and scaling up from the diluted sample to the original population