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What's in the water?

Calculating the number of T4 bacteriophages in a suspension using serial dilution

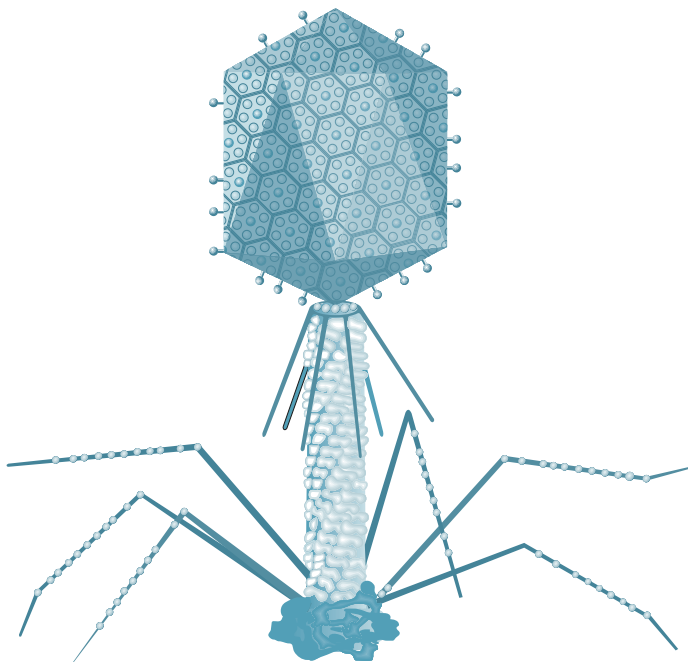
Aim

The aim of this investigation is to calculate the number of bacteriophages ('phages) in a culture. Because the culture will contain many millions of 'phages it is necessary to dilute the culture. Because several dilutions take place in succession, this technique is called 'serial dilution'.

Introduction

Before the 2016 Olympic games in Brazil, concern was expressed about high levels of pollution at several of the locations to be used for water sports [1]. Were there high levels of bacteria and viruses in the water, and would they pose a health risk to the athletes participating in the games?

Estimating the number of bacteria in a water sample is relatively simple — you can place the sample on a Petri dish of suitable agar growth medium as see what, if anything, grows. Each bacterial colony



*The structure of a T4 bacteriophage.
After Yap and Rossmann (2014)*

on the plate corresponds to an individual bacterium in the sample. Where the population of bacteria in the sample is likely to be high, it may be necessary to dilute the sample, perhaps several times, so that the number of colonies is easier to count. Since the dilution and the volume of liquid used to inoculate the plate is known, it is possible to multiply up the numbers and so calculate the number of bacteria that must have been present in the original, undiluted, sample. This type of repeated dilution is called 'serial dilution' and it is an important technique in microbiology, where you might routinely deal with many thousands or even millions of organisms.

A population of viruses is slightly more difficult to count because they need to grow and replicate inside another organism. This practical procedure shows how this can be done for a certain type of virus — bacteriophages.

Bacteriophages are viruses that infect bacteria. The bacteriophage T₄ infects the 'B' strain the bacterium *E. coli*. The technique described below involves mixing dilute solutions of bacteriophage with a culture of *E. coli* in soft (half-strength), molten agar. This mixture is then poured onto the surface of solid agar and left to set. After incubation, clear zones will be seen on the surface of the agar plate in areas where the bacteriophage has killed the *E. coli*. Each clear zone on the plate corresponds to one infection by a bacteriophage. Because you know the dilution of the bacteriophage used, it is possible to calculate the number of 'phages in the original culture.

Safety guidelines

Good microbiology laboratory practice should be followed at all stages during the preparation of cultures and in the subsequent practical exercise.

Equipment and materials

Needed by each student or group

Equipment

- Facilities for basic microbiology *e.g.*, impervious work surface, wire, inoculation loop, Bunsen burner, lab coat
- Access to a water bath at 50 °C
- Access to an incubator at 35 °C
- Access to a microbial spills kit
- A fine-tipped permanent marker pen

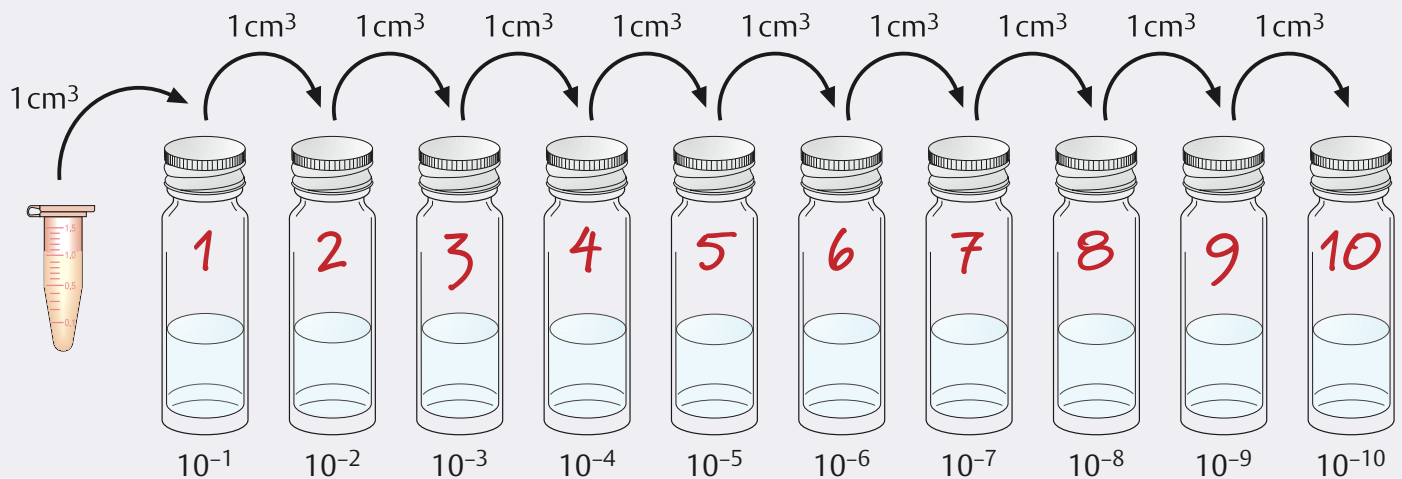
Materials

- Broth culture of *E. coli* B strain, 2 cm³
- T₄ bacteriophage suspension, 2 cm³
- Nutrient or LB agar plates, 6
- Tubes containing 3–4 cm³ each of soft nutrient or LB agar, 6
The tubes of soft agar should be kept in the water bath at 50 °C before use so that the agar remains molten.
- Universal bottles, each containing 9 cm³ of sterile distilled or deionised water.
- 1 cm³ sterile syringes without needles, 19, OR suitable pipettes with sterile disposable tips
- Waste container of freshly-made disinfectant solution (*e.g.*, a 500 cm³ container of 1% (w/v) *Virkon* solution of 5% (v/v) *Biocleanse* solution)
- Paper towels

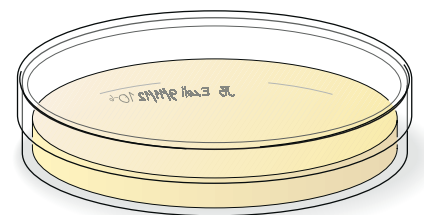
Procedure

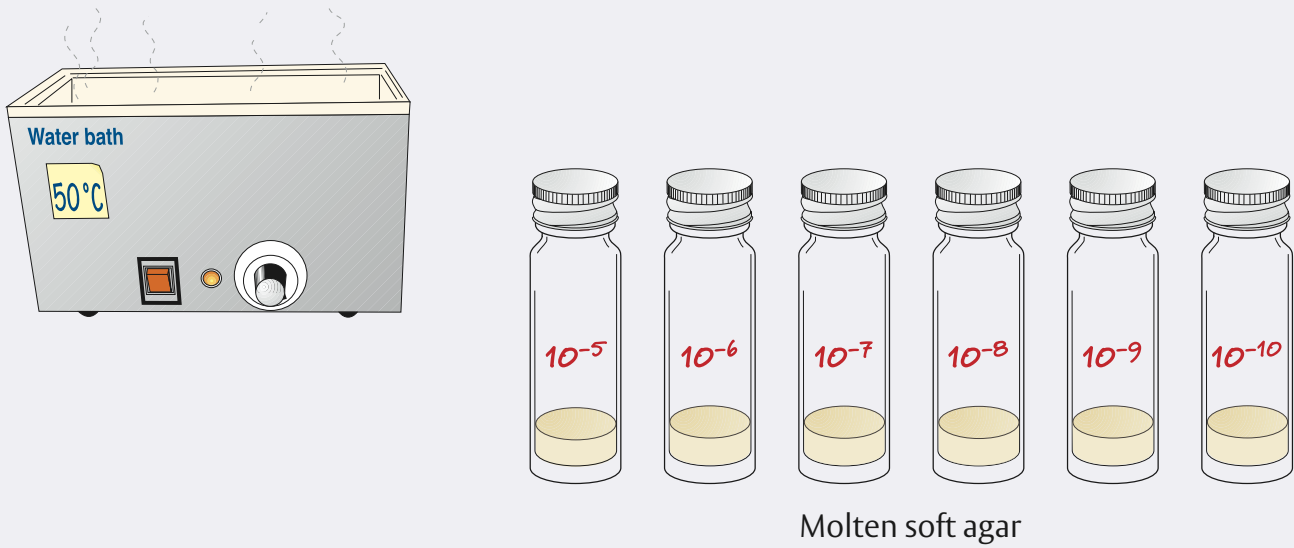
It is essential to use aseptic techniques when carrying out the following procedure. These should be demonstrated to you before you start.

- 1 Label the Universal bottles of sterile water from 1 to 10. This water will be used for the serial dilution of the bacteriophage suspension.
- 2 Use a sterile 1 cm³ syringe or pipette to aseptically transfer 1 cm³ of the bacteriophage suspension to the tube of water labelled '1'. Place the used syringe or pipette tip in the disinfectant solution. Close the tube and gently swirl the contents to mix.
- 3 Use a new sterile 1 cm³ syringe or pipette to transfer 1 cm³ of the contents of tube 1 to the tube labelled '2'. Place the used syringe in the disinfectant solution. Close tube 2 and gently swirl the contents to mix.
- 4 Repeat Step 3 with each of the numbered tubes in succession, using a new syringe or pipette tip each time. Tubes 1 to 10 now contain dilutions of 1 in 10 to 1 in 10,000,000,000 (that is, 10⁻¹ to 10⁻¹⁰).

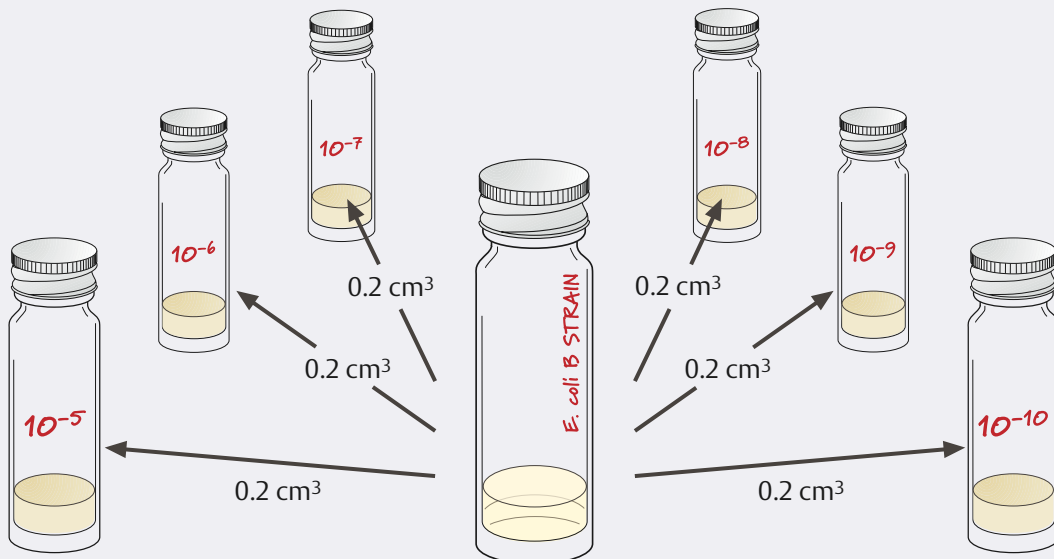


- 5 Label the six tubes in the water bath containing the molten soft agar: 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ and 10⁻¹⁰. You will probably need to dry the tube with a paper towel before you can label them.
- 6 Also label the bases of the six agar plates with the date, *E. coli*, student initials and with one of the following bacteriophage dilutions: 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ or 10⁻¹⁰.



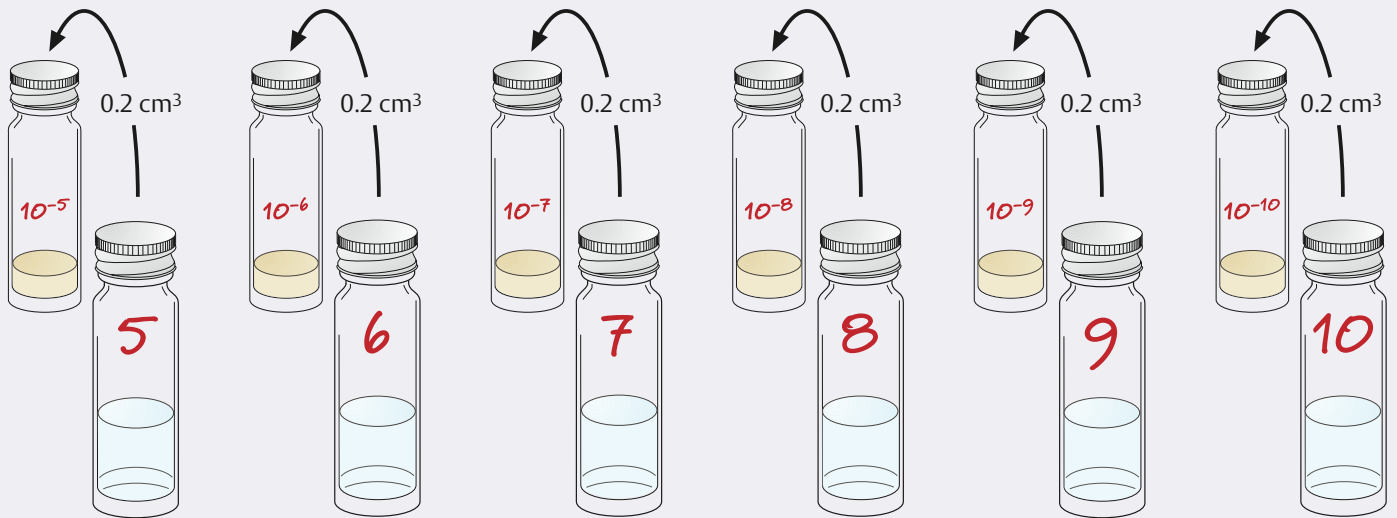


The next stage is to add the *E. coli* and the diluted bacteriophage suspensions to the soft agar. Normally only the last six dilutions of phage (from 10^{-5} to 10^{-10}) are used: the first four dilutions are not used as they are too concentrated. When you add the phage and *E. coli* to the soft agar, it is important to work quickly so that the agar does not set before you have finished. Plan the work carefully and make sure you have everything you need readily to hand. It may also help if you work with a partner, if possible.

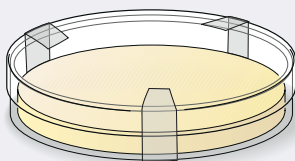
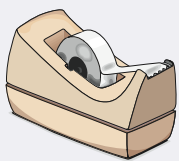


- Using a sterile syringe or pipette, aseptically transfer 0.2 cm³ (200 μL) of the *E. coli* culture into each of the tubes of soft agar.

- 8 Next, using a new sterile syringe or pipette tip for each dilution, aseptically add 0.2 cm^3 ($200\mu\text{L}$) of the appropriate diluted phage suspension to the tubes.



- 9 Once the *E. coli* culture and diluted phage have been added to the soft agar, ensure that the bottle tops are firmly screwed on, then gently roll each bottle to mix its contents.
- 10 Aseptically pour the contents of the bottle onto the agar in the appropriately-labelled agar plate and allow it to set.
- 11 Once the agar in the plates has set, seal with tape, invert them and incubate at 30°C .
- 12 After 24-48 hours examine the plates. Plaques (clear zones) should be visible where the bacteriophage has killed the *E. coli*.



Calculation of the number of phages

The total number of infective agents can be calculated per cm³ of bacteriophage suspension. Select an agar plate where there are between 20–200 plaques. Count the number of plaques and record the number. This number divided by the dilution factor multiplied by the volume of the diluted bacteriophage suspension used will give the number of infective agents per cm³.

$$\text{Infective agents per cm}^3 = \frac{\text{Number of plaques}}{\text{Dilution factor} \times 0.2}$$

For example, if 180 plaques are counted on the plate at the 10⁻⁸ dilution, and 0.2 cm³ of bacteriophage suspension has been used:

$$\frac{180}{0.0000001 \times 0.2} = 90,000,000,000 \text{ infective agents per cm}^3$$

Teacher's and technician's notes

Introduction

Bacteriophage T4 will only infect the B strain of *E. coli*, not the K-12 strains that are commonly used in schools, so it is necessary to obtain a B strain of *E. coli* for this work.

Either nutrient agar and broth or Luria-Bertani (LB) agar and broth can be used for this work.

Good microbiology laboratory practice should be observed throughout. Generally speaking, 30 °C should be regarded as the maximum incubation temperature used in schools, necessitating 24 hour incubations. However, in some educational institutions, incubation at 37 °C is permitted — where this is so, only overnight (~8 hour) incubation is required.

Production of *E. coli* cultures for classroom use

Initially, you will need to grow a fresh plate of *E. coli* from the stock slope culture you have obtained from a school supplier, such as the NCBE. The plate should be incubated overnight at 37 °C or for 24 hours at 30 °C.

After incubation, aseptically transfer a loopful of *E. coli* culture from the Petri dish culture to a Universal bottle containing 10 cm³ of sterile broth. Incubate overnight at 37 °C or for 24 hours at 30 °C. After incubation, the broth culture should look very turbid. Normally each student or working group of students will require one broth culture of the *E. coli*.

If you intend to produce the phage culture at the same time, you will also need similar cultures of *E. coli* for this. Two or three cultures will be needed to prepare bacteriophage for a class with 10–12 students or working groups of students.

Production of bacteriophage suspension for classroom use

Inoculate an overnight or a 24 hour-old broth culture of *E. coli*, prepared as described above, with 100 µL (0.1 cm³) of the bacteriophage suspension. Incubate again either overnight at 37 °C or for 24 hours at 30 °C.

After incubation, either centrifuge the culture or allow it to stand in a fridge overnight. The clear supernatant contains the bacteriophage. Aseptically transfer the supernatant into sterile containers for class use (such as sterile 1.5 cm³ microcentrifuge tubes). If the suspension has not cleared well, aliquot the top layer. Each group will require 1 cm³ of suspension — although for ease of handling it is advisable to give each group 2 cm³ (e.g., 1 cm³ in each of two sterile 1.5 cm³ microcentrifuge tubes).

If necessary, the bacteriophage suspension can be kept in the fridge for 24–48 hours before use by the students. The suspension can also be stored frozen at –18 to –20 °C until required, but if you intend to store the phage preparation for more than a few weeks, it is advisable to add an equal volume of glycerol to the supernatant before freezing it.

Timing

The cultures of *E. coli* and bacteriophage for this practical activity must be prepared *at least four days* in advance.

Day 1

Obtain an *E. coli* B strain slope culture. Produce a streak plate from this culture. Incubate for 24 hours at 30 °C or overnight at 37 °C.

Day 2

Produce broth culture(s) from the agar plate culture. Incubate for 24 hours at 30 °C or overnight at 37 °C.

Day 3

Inoculate broth cultures with bacteriophage suspension. Incubate for 24 hours at 30 °C or overnight at 37 °C. Store in a fridge for up to 48 hours or freeze if not required immediately (add an equal volume of glycerol if stocks are to be frozen).

Day 4

Produce fresh *E. coli* broth cultures for students to use. Incubate for 24 hours at 30 °C or overnight at 37 °C.

Safety

Good microbiology laboratory practice should be observed throughout.

Equipment and materials

Needed by each student or group

Equipment

- Facilities for basic microbiology *e.g.*, impervious work surface, wire, inoculation loop, Bunsen burner, lab coat
- Access to a water bath at 50 °C
- Access to an incubator at 35 °C
- Access to a microbial spills kit
- A fine-tipped permanent marker pen

Materials

- Broth culture of *E. coli* B strain, 2 cm³
- T4 bacteriophage suspension, 2 cm³
- Nutrient or LB agar plates, 6
- Tubes containing 3–4 cm³ each of soft nutrient or LB agar*, 6
The tubes of soft agar should be kept in the water bath at 50 °C before use so that the agar remains molten.
- Universal bottles, each containing 9 cm³ of sterile distilled or deionised water.
- 1 cm³ sterile syringes without needles, 17, OR suitable pipettes with sterile disposable tips
- Waste container of freshly-made disinfectant solution (*e.g.*, a 500 cm³ container of 1% (w/v) *Virkon* solution of 5% (v/v) *BioCleanse* solution)
- Paper towels

* *Note: Soft agar is half-strength nutrient agar or LB agar and is used as an overlay on normal (full-strength) agar; this softer agar allows the bacteriophage to infect the nearby bacterial cells more easily.*

Suppliers

Escherichia coli B Strain and T4 bacteriophage

Both of these can be obtained from the NCBE:
www.ncbe.reading.ac.uk

References

1. *What's in the water? Pollution fears taint Rio's picturesque bay ahead of Olympics* by Jonathon Watts and John Vidal. *The Guardian* 3 August 2016. <https://www.theguardian.com/sport/2016/aug/03/pollution-fears-taint-rio-bay-olympic-games>
2. M.L. Yap and M.G. Rossmann (2014) Structure and function of bacteriophage T4 *Future microbiology* **9** (12) 1319–1327. Available online at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4275845/>