Determination of the microbial count (Item No.: P4140400)

Curricular Relevance

Area of Expertise: Biology
Education Level: University
Topic: Microbiology
Subtopic: Microbiology
Experiment: Determination of the microbial count

Difficulty	Preparation Time	Execution Time	Recommended Group Size
Intermediate	30 Minutes	1 Hour	2 Students

Additional Requirements:

Experiment Variations:

Keywords:

Microbial count, Microorganisms, Analysis of drinking water, Contaminants

Overview

Short description

Principle

The microbial count is the number of viable microorganisms in one millilitre or gramme of the material to be examined, e.g. water, soil, milk, ice cream, etc. It is of high practical importance for the analysis of drinking water, soil, and foodstuffs. The microbial count can only be determined exactly if all of the related tasks are performed in a way that prevents the material that is to be examined from being contaminated with foreign microorganisms.

Fig. 1: Diaphragm filter.

Equipment

Position No.	Material	Order No.	Quantity
1	Erlenmeyer flask, narrow neck, 500 ml	36121-00	2
2	Graduated cylinder 100 ml	36629-00	1
3	Test tube, 160 x 16 mm, 100 pcs	37656-10	1
4	Petri dish, d 100 mm	64705-00	12
5	Watch glass, dia.60 mm	34570-00	4
6	Test tube rack for 12 tubes, holes d= 22 mm, wood	37686-10	1
7	Glass beaker DURAN®, tall, 600 ml	36006-00	1
8	Universal oven, 32 liters, 230 V	49559-93	1
9	Compact Balance, OHAUS TA 302, 300 g / 0.01 g	49241-93	1
10	Autoclave with insert	04431-93	1
11	Spatula, double blade, 150 mm	33460-00	1
12	Graduated pipette, 1 ml	36595-00	2
13	Tripod,ring d=140 mm, h=240 mm	33302-00	1
14	Wire gauze with ceramic, 160 x 160 mm	33287-01	1
15	Labor pen, waterproof	38711-00	1
16	Safety gas tubing, DVGW, sold by metre	39281-10	1
17	Bunsen burner, natural gas,w.cock	32167-05	1
18	Microscopic slides, 50 pcs	64691-00	1
19	Cover glasses 18x18 mm, 50 pcs.	64685-00	10
20	Pipettor	36592-00	1
21	Glass rod,boro 3.3,l=300mm, d=7mm	40485-05	1
22	Sterile stoppers f. id 15mm, 250	39266-00	1
23	Sterile stoppers f. id 29mm, 100	39267-00	1
24	Bottle,nar.mouth,250ml,clear,p.st	41102-01	4
25	Stopper, IGJ 19/26,glass,clear	41252-10	4
26	Filter flask, 1000ml, PN 45	34421-01	1
27	Rub.stop.d=49/41,1 hole 15mm	39263-15	1
28	Membrane filter apparatus, 250 ml	64906-00	1
29	Membrane filters, d = 50 mm, 0.45 µm, 100	64907-00	1
30	Tweezers,straight,pointed,120mm	64607-00	1
31	Rubber tubing,vacuum,i.d.6mm	39286-00	1
32	Water jet pump, plastic	02728-00	1
33	Graduated pipette 10 ml	36600-00	1
34	Circular filter,d 90 mm,100 pcs	32977-03	1
35	Nutrient discs,standard, 10	64908-03	1
36	Heating + cooking hotplate,230V	04025-93	1
37	Ethyl alcohol, absolute 500 ml	30008-50	1
38	Water, distilled 5 l	31246-81	1
39	Immersion oil, 50 ml	31381-05	1
40	Formaldehyde sol. ca.35% 500 ml	48146-50	1
41	Methylene-blue B, for microscopy,25g	31567-04	1
42	Glycerol, 250 ml	30084-25	1
43	Meat extract 10 g	31521-03	1
44	Peptone,dry,from meat 50 g	31708-05	1
45	pH test sticks 6.5-10, 100 sticks	30301-04	1
46	Sodium hydroxide, pellets, 500 g	30157-50	1
47	Agar-agar, powdered 100 g	31083-10	1
48	SWIFT 120 student microscope (M3602C-3)	63021-99	1

Robert-Bosch-Breite 10
D - 37079 Göttingen
Tel: +49 551 604 - 0
Fax: +49 551 604 - 107
info@phywe.de
www.phywe.com

Task

Determine the microbial count of a sample.

Robert-Bosch-Breite 10
D - 37079 Göttingen

Tel: +49 551 604 - 0
Fax: +49 551 604 - 107

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www.phywe.com

Set-up and procedure

Procedure

Sterile withdrawal of the examination material
For the sterile withdrawal of a sample it is absolutely necessary that all of the tools and equipment parts are sterile and that any foreign infection during the withdrawal is absolutely avoided.
96% ethyl alcohol, material to be examined (soil samples, water samples, etc.)
A **soil sample** can be taken in a sterile manner by filling a sufficient amount of soil with a sterile spatula, spoon, or putty knife into a wide-mouth bottle with a ground-in stopper (see 1.5) that has been sterilised in a hot-air steriliser. Ensure that the inner surfaces of the bottle or stopper are not contaminated with foreign microorganisms during the filling process. Sterilise the spatula, spoon, or putty knife directly at the sampling location immediately prior to sampling by plunging it into 96% ethyl alcohol for approximately one minute and by passing it through the flame of a gas burner (Fig. 2). If several different soil samples are taken, ensure that no microorganisms are transferred from one sample to another.
Proceed in a corresponding manner for taking other, non-liquid material samples (e.g. ice cream).
For the sterile withdrawal of a **water sample**, dip a sterile wide-mouth bottle with a ground-in stopper into the water at the sampling location, remove the stopper, let the desired quantity of water flow into the bottle, and re-insert the stopper under water.
Other liquid material can be transferred into a sterile bottle of a suitable size and with a ground-in stopper by way of a sterile pipette, or it can be poured into the bottle.
The determination of the microbial count should be performed as quickly as possible after the sampling process.

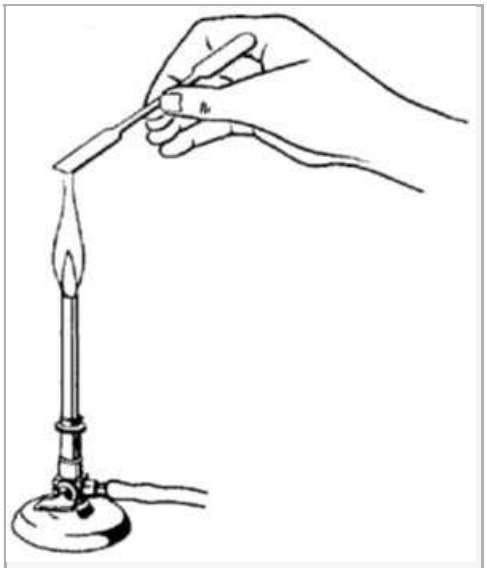

Fig. 2: Flaming of the spatula.

Plate pouring method according to KOCH
This method was introduced into the bacteriological technology by ROBERT KOCH. It is the oldest, yet still most common, method for determining the microbial count.
Withdraw - under sterile conditions - 1 g or 1 ml from the material to be examined that has been collected in a sterile vessel and mix it with 9 ml of sterile, distilled water. If the material to be examined is solid, e.g. a soil sample, weigh 1 g of it on a tared watch glass that has been sterilised in the flame of a gas burner beforehand, and fill this quantity into a test tube with 9 ml of sterile, distilled water. Transfer the soil sample with the aid of a metal spatula that has been sterilised in the flame of a gas burner and then allowed to cool.
If the material to be examined is liquid, e.g. a water sample, milk, or lemonade, transfer 1 ml of the sample into a test tube with 9 ml of sterile, distilled water by way of a sterile, graduated pipette (see P4140100).
Based on this 1:10 dilution, prepare further 1:10 dilutions by repeatedly pipetting 1 ml of the most recent dilution in a new test tube with 9 ml of sterile, distilled water under sterile conditions (Fig. 3).

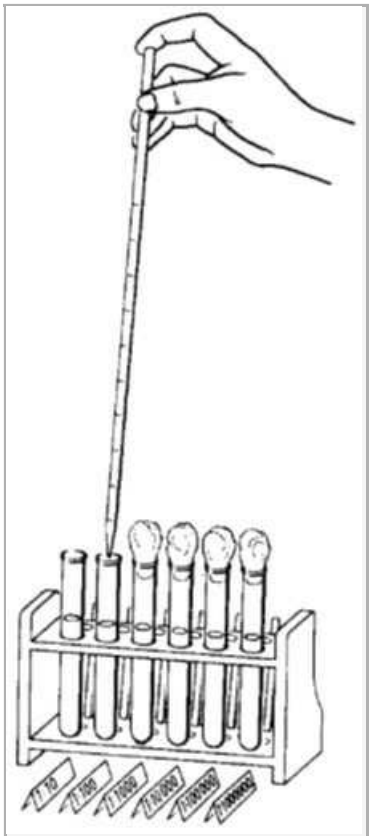

Fig. 3: Diluting the samples.

The question as to how far the material should be diluted depends on the type of material. Soil samples for which a rather high microbial count can be expected, e.g. garden mould, are diluted up to 1:100000 or 1:1000000, sandy soil, on the hand, only up to 1:1000 or 1:10000.
After the material has been diluted to the required extent, liquefy the content of four tubes with standard nutrient agar for bacteria by boiling it in a water bath and letting it cool to 60°C (hand-hot). A suitable water bath for this purpose is a 600 ml beaker that is half full with water. Remove the cotton plug from the tube and transfer 1 ml of the second to last dilution level of the material under sterile conditions into the still liquid nutrient medium with the aid of a pipette. Mix the content of the tube by rolling the tube between your palms (Fig. 4).

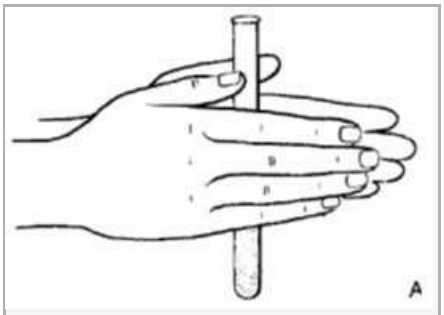

Fig. 4: Mixing the samples.

Flame the mouth of the tube in the flame of a gas burner and pour its content into a sterile Petri dish.
Inoculate a second test tube with standard nutrient agar for bacteria with the same dilution level and the two other tubes with the last dilution level of the material to be examined in the same manner. Pour their contents into separate Petri dishes.
Label the plates (type of material, dilution level, date) and incubate them at 30°C in an incubator.

Diaphragm filter method
The determination of the microbial count in accordance with the diaphragm filter method is based on an enrichment of the microorganisms of a known quantity of sample material, e.g. water or soil, on the surface of a filter, followed by their microscopic or culture-based identification. The diaphragm filter that is used for this purpose consists of a skeleton substance based on cellulose esters and regenerated cellulosic fibres. These substances form a multi-layered system of cavities and their pore size ensures that microorganisms are retained. In order to filter the sample material, place the diaphragm filter into the diaphragm filter apparatus. A filter flask that is connected to a water jet pump via a vacuum hose is used for collecting the filtrate (Fig. 1).
Push a rubber stopper that fits into the neck of the filter flask (Fig. 5, 1) and that has a suitable hole (Fig. 5, 2) over the discharge pipe of the lower part of the diaphragm filter apparatus (Fig. 5, 3). Before doing so, apply some glycerine to the discharge pipe and the hole of the rubber stopper in order to lubricate them. Insert the rubber stopper with the lower part of the diaphragm filter apparatus into the neck of the filter flask and add the porcelain frit (Fig. 5, 4) with the integrated blue gasket facing downwards. Then, lay a diaphragm filter (Fig. 5, 5) with the chequered side up on the porcelain frit. Attach the upper part of the diaphragm filter apparatus (Fig. 5, 6) that is to hold the sample material and connect the upper part to the lower part by way of the metal clamp (Fig. 5, 7). Finally, connect the filter flask to a water jet pump via a vacuum hose.
Pour the sample material, e.g. a water sample, into the upper part of the diaphragm filter apparatus, start the water jet pump, and filter the sample. The quantity to be filtered depends on the suspected microbial concentration of the sample. Usually, quantities such as 1000 ml in the case of tap water and 100 ml of medium-contaminated river water are filtered.

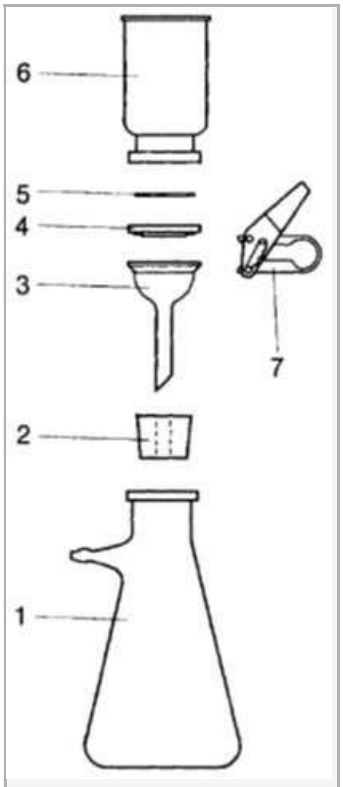

Fig. 5: Components of the diaphragm filter

In order to stain the microorganisms that were filtered out, remove the diaphragm filter from the apparatus and let it dry in the air. Place three circular filters with a diameter of 90 mm into a Petri dish with a diameter of 100 mm. Flatten them so that they are in close contact with the bottom of the dish and impregnate them with an aqueous methylene-blue solution. The circular filters should be moist, but it must be ensured that the staining solution does not form any puddles on the paper.
Place the air-dried diaphragm filter with the coated side up for 10 minutes onto the circular filters that are impregnated with the staining solution (Fig. 6). During this time, the staining solution penetrates the diaphragm filter and stains the filter, as well as the collected microorganisms, dark blue.
Since only the microorganisms should be stained, transfer the diaphragm filter thereafter into a second Petri dish on three layers of circular filters that are impregnated with distilled water. This way, the dye will be washed out of the diaphragm filter. Continue removing the dye by replacing the circular filters several times until no further dye is extracted from the diaphragm filter. Then, let it dry in the air.
After everything has passed through the apparatus, fix the microorganisms that were retained on the filter by pouring and filtering 10 ml of a 10% formaldehyde solution into the upper part of the diaphragm filter apparatus. Then, wash the entire equipment by filtering 50-100 ml of distilled water.

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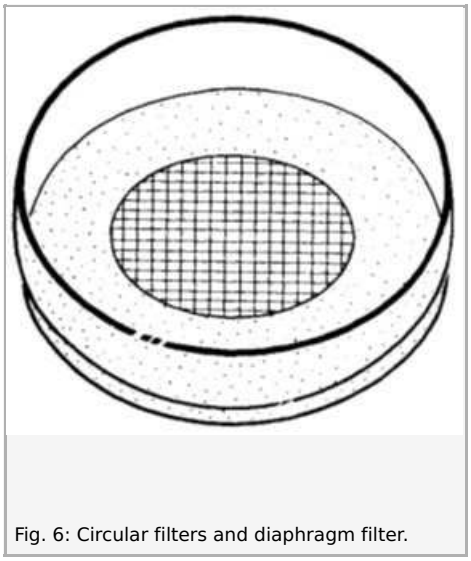

Fig. 6: Circular filters and diaphragm filter.

Microscopic analysis of the microorganisms
The microscopic analysis enables only a rough orientation concerning the microbial count of the sample material in terms of its magnitude. For student experiments, however, this method is often sufficient depending on the problem at hand.
In order to perform the microscopic analysis, cut small pieces with an edge length of approximately 5 mm out of the diaphragm filter. Transfer them to a drop of immersion oil or glycerine on a microscope slide, thereby making them transparent, cover them with a cover glass, and examine them with 400x and 1000x microscopic magnification (objective 40x or 100x, eyepiece 10x). Through the microscope, dark blue microorganisms can be seen on a whitish, light or slightly blue background.
For the evaluation, count the microorganisms per field of vision in several areas of the preparation and calculate the mean value. With this method, the microbial concentration of different types of sample material, e.g. of several water samples, can be compared in a relative manner. The absolute determination of the microbial count by way of a microscope is not possible, however. In addition, it also has the disadvantage that not only living microorganisms that are important from a hygienic point of view, but also dead microorganisms that are also stained are taken into consideration as well.

Culture-based analysis of the microorganisms
If the microbial count is to be determined and compared not only in terms of its magnitude, but rather in a precise manner, evidence concerning the microorganisms must be provided in a culture-based manner, i.e. by developing them into bacterial or fungal colonies. This way, only viable microorganisms, i.e. only those that are of importance from a hygienic point of view, will be taken into consideration. Sterile conditions must be strictly maintained for this purpose. This means that all parts of the diaphragm filter apparatus that come into contact with the sample material must be sterilised. Otherwise, any microorganisms that adhere to it will be flushed onto the filter, thereby falsifying the test result.
At first, proceed in the same manner as for the microscopic analysis of the microorganisms. However, after you have placed the porcelain frit onto the lower part of the diaphragm filter apparatus, flame the frit repeatedly and thoroughly with the flame of a gas burner.
For the culture-based analysis of the microorganisms, the diaphragm filters come supplied together with the nutrient discs, packaged in a sterile manner in polyethylene bags. Use a flame-sterilised pair of tweezers to take a diaphragm filter out and place it on the sterilised porcelain frit. Then, flame the inside of the upper part of the diaphragm filter apparatus thoroughly, attach it to the lower part with the porcelain frit, and connect the two parts with the aid of the metal clamp. Pour the sample material, e.g. a water sample, into the diaphragm filter apparatus and withdraw it with the aid of the water jet pump. Depending on the suspected microbial concentration, it may be necessary to dilute the sample material under sterile conditions as for plate pouring in accordance with KOCH.
After everything has passed through the apparatus, rinse the inside of the upper part of the diaphragm apparatus with sterile, distilled water in order to transfer any microorganisms that adhere to the walls to the diaphragm filter. This water must also be filtered by withdrawing it with the water jet pump.
Use a flame-sterilised pair of tweezers to take a nutrient disc out of the sterile packaging and place it in a sterile Petri dish with a diameter of 100 mm that is filled with 5 ml of sterile, distilled water that has been transferred to the dish by way of a sterile pipette. The filter paper disc that is joined to the nutrient disc must face downwards. Petri dishes are sterilised in a hot-air steriliser. The same applies to pipettes or they are sterilised in alcohol and flamed afterwards. Distilled water is filled into test tubes that are sealed with cotton plugs or into Erlenmeyer flasks. Then, it is sterilised in an autoclave.
Take the diaphragm filter out of the diaphragm filter apparatus with the aid of a flame-sterilised pair of tweezers and place it on the nutrient disc that is impregnated with sterile, distilled water (Fig. 7).

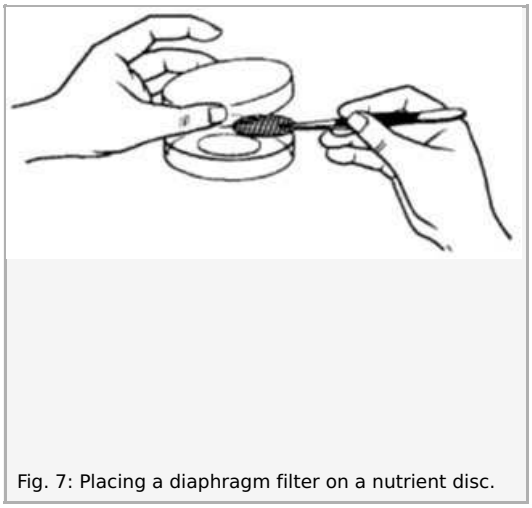

Fig. 7: Placing a diaphragm filter on a nutrient disc.

Ensure that no air bubbles are trapped between the diaphragm filter and the nutrient disc. Open the Petri dish only briefly for the various working steps and keep it otherwise closed so that the culture cannot be contaminated with microorganisms from the air. When opening the Petri dish, lift the cover only slightly up and hold it over the surface of the dish at all times.
Incubate the Petri dish with the nutrient disc and diaphragm filter at 30°C in an incubator.
The nutrients of the nutrient disc are dissolved by the added distilled water. They reach the surface of the diaphragm filter by diffusion. The microorganisms that have collected there will multiply, provided that the nutrient supply is suitable for them. Within three to five days, they will form bacterial and fungal colonies that can be seen with the naked eye. The number of viable microorganisms in the filtered sample material is then determined by performing a count of the colonies.

Robert-Bosch-Breite 10
D - 37079 Göttingen
Tel: +49 551 604 - 0
Fax: +49 551 604 - 107
info@phywe.de
www.phywe.com

Theory and evaluation

Results

Within three to four days, numerous colonies of microorganisms develop on the nutrient media. Perform a count of the colonies on each disc, multiply this value with the reciprocal value of the associated dilution factor, and determine the microbial count, i.e. the number of viable microorganisms in one gramme or millilitre of the sample material.
The sample material is diluted in order to obtain a quantity of colonies on the nutrient media that is ideal for performing a count. If there are not enough due to a too strong dilution, the results are not sufficiently statistically firm for comparative studies. If, on the other hand, there are too many colonies due to insufficient dilution, they cannot be counted sufficiently well. This is why plates of two neighbouring dilution levels are prepared. In order to better ascertain the results, at least two plates for every level. Plates with a dense microbial growth can be counted better by dividing them into several sectors at the bottom on the outside of the dish with the aid of a grease pencil and by counting the sectors individually.
KOCH's plate pouring method is based on two conditions: firstly, that all of the microorganisms are present as separate entities in the dilutions that are used and, secondly, that every viable microorganism actually grows on the nutrient medium that is used, thereby forming a colony that can be counted. Since this is not the case for every existing viable microorganism, the results often differ rather considerably during parallel experiments. The values that are determined in this manner are usually slightly too low. Regardless of these disadvantages, the plate pouring method in accordance with KOCH is still one of the most common methods for determining microbial counts since there are currently no determination methods that are absolutely precise.

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