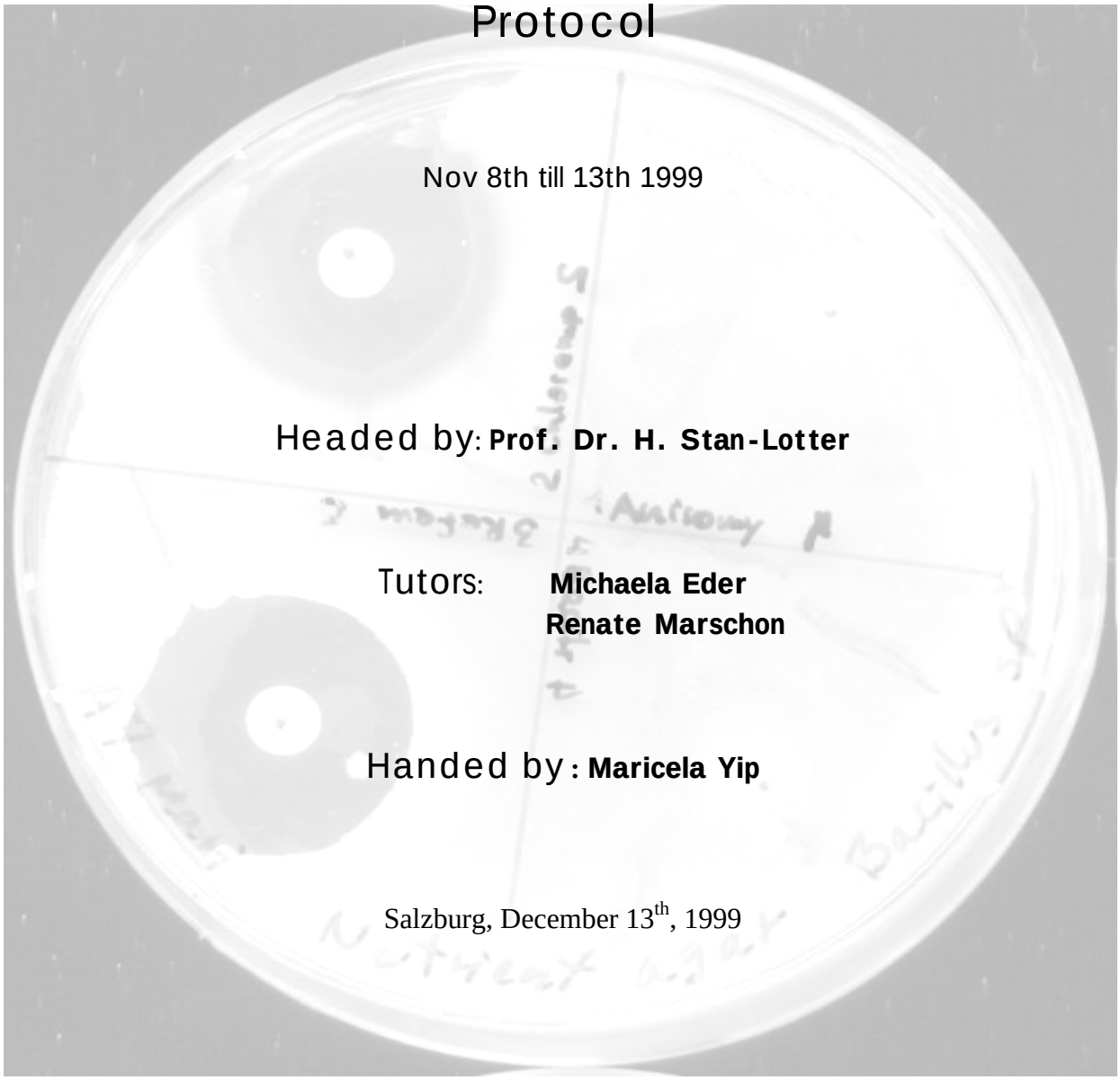
Microbiology II-99

Selected experiments for advanced Microbiology

Protocol



Nov 8th till 13th 1999

Headed by: **Prof. Dr. H. Stan-Lotter**

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Salzburg, December 13th, 1999

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Introduction

We conducted several experiments of different types of bacterial from different sources such as from natural biotopes, in untreated milk samples, in water samples, and in extremophiles archaebacteria. The bacteria found were also subjected to several treatments; for example, to identify the species with the gram-stain, to test their reactions by using different culture media, to find about their antibiotics resistance, etc. Finally, we tried to estimate the bacterial growth with one or two bacterial species.

Table 1 Schedule of experiments during the second week of November, 1999

	MON Nov. 8th		TUE Nov. 9th		WED Nov. 10th		THU Nov. 11th		FRI Nov. 12th		TUE Nov. 16th	
Lectures Hours	9-11	13-14	9-10		9-11			13-14	9-11			
Experiment 1												
Experiment 2												
Experiment 3												
Experiment 4												
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Experiment 8												

Sterilization & Aseptic techniques:

Many of the laboratory studies that be made with pure cultures (single species of microorganisms) must be sterilized and maintain them in sterile condition (free of living forms), and we must be able to inoculate sterile medium with a pure (axenic) culture of microorganisms without contaminating it.

The time to apply some aseptic techniques in the laboratory are: before proceeding with the culture of microorganisms, we must first consider how to exclude contaminants. Microorganisms are everywhere; because of their small size, they are easily dispersed in the air and on the surfaces. Therefore, we must sterilize the culture medium soon after its preparation to eliminate microorganisms already contaminating it. It is of essential importance to take precautions during the subsequent handling of a sterile culture medium to exclude from it all but the desired organisms. Thus other materials that come into contact with a sterile culture must themselves be sterilized.

Heat sterilization: death form heating is an exponential (1st order function), it will take longer at lower temperatures than at higher ones. The nature of the heating is also important, in moist heating has better penetrating power than dry heating. Furthermore dry cells (endospore) are more heat-resistant than moist ones. Killing of heat-resistant endospores requires heating at temperatures above boiling point and the use of steam under pressure. The autoclave achieves these parameters because it is a sealed device that allows the entrance of steam under pressure.

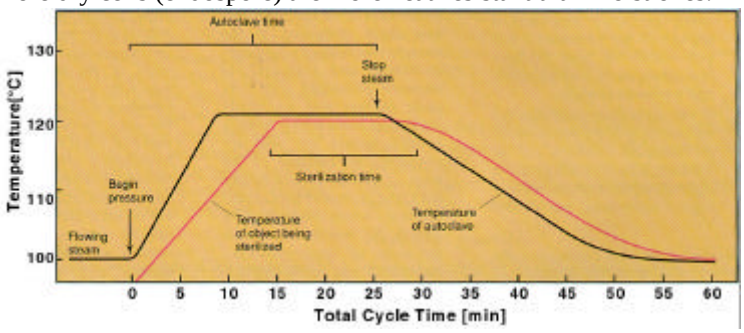


Fig 1 A typical autoclave cycle showing the sterilization path of bulky objects. The temperature of the objects rises more slowly than the temperature of the autoclave.

Pasteurization: is a process that reduces the microbial population in milk and other heat-sensitive foods. Pasteurization is not synonymous with sterilization because not all organisms are killed. Instead, pasteurization of milk is usually achieved by passing the milk through a heat exchanger. Careful control of the milk flow rate and the temperature of the heat source raises the temperature of the milk to 71°C for 15 sec. The milk is then rapidly cooled.

Radiation sterilization: an effective way to sterilize or reduce the microbial burden is the use of electromagnetic radiation (EMR). Microwaves, UV radiation, X-rays, gamma-rays, and electrons can be used for radiation sterilization. The antimicrobial effects of microwaves are due to the thermal effects. UV radiation have sufficient Energy to cause breaks in DNA leading to the death of the exposed organisms although UV cannot penetrate solid, opaque, light-absorbing surfaces, its usefulness is limited to disinfecting of exposed surfaces. Ionizing radiation is EMR of sufficient energy to produce ions and other reactive molecular species; i.e. electrons (e-) hydroxyl radicals (OH•), and hydride radicals (H•). Each of these reactive molecules is capable of degrading and altering biopolymers such as DNA and proteins.

Filter sterlization: Heat sterilization cannot be used for sterilization of heat-sensitive liquids as gases. A filter is a device with pores too small for the passage of microorganisms but large enough to allow the passage of liquids and gases, it has a similar sterilizing effect as the methods already described above.

1. Bacteria from natural Biotopes

1.1 Bacterial sample from the Human Body (Day 1 Nov. 8th)

Purpose: Collection of bacteria from various locations of the human skin and subsequently cultivation on agar plates. In this case, the bacterial colony was obtained from inside the nose.

Procedure:

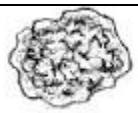
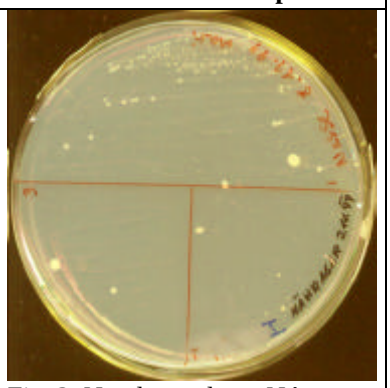
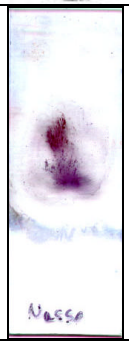
- Obtain the bacterial sample by first dipping a sterile Q-tip into the demineralized sterile water; make sure not to touch the cotton part with the fingers;
- Extract some biogenic material from the nose and roll on the mucus gently onto the agar dish.
- Sterilize the inoculation needle by holding it into the Bunsen burner until glowing red hot; cool it down by dipping it into the agar closely at the edge of the dish (do not touch the inoculum yet).
- Use the spread plate technique to distribute the deposited accumulation over the agar dish (s. appendix - handling techniques). **Note**: Take advantage of the thermal effect by keeping the Bunsen burner in operation for the entire experimentation to limit additional contamination by airborne contaminants.
- Mark the petri dish properly with date and sample ID before incubating it for 24 hours at 37°C (storage of dishes with the agar up-side down).

Material: Water perm. Marker
Bunsen burner w/ igniter
Inoculation needle
Glass spatula (hockey stick)
Air sampler MAS-100
Microscope (40x) Wild 38 w/ ext. cold light source
Microscope (1000x) Leitz
10 Object slides
Incubator (37°C)
200µL Gilson-Pipette w/ sterile tips (yellow)
some sterile Q-tips
3 NA-Agardishes*
2 Endo-Agardishes*
1 Test tube w/ 4mL of sterile demin. water plus alu cap
≈50mL EtOH in beaker w/ lid

Samples for Inoculation:
Bacillus subtilis (G^+)-Blue
Escherichia coli (G^-)-Red
Human Samples: Nose
Other Samples: Air and Water

(*) provided by the tutors; see also appendix - Agar preparation

Diagram 1: **Results** & **Discussion** (Day 4 Nov. 11th):

Description	Draw	Overview of streak plate
1 colony found, with irregular edge, convex elevation, rough surface, white in color; 0.5mm in diameter;		 Fig. 2 Nasal sample on NA-agar (streak plate)
3 colonies found, with smooth edge, pulvinate elevation, smooth surface, white-yellow in color; 1mm in diameter		
42 colonies found, with irregular edge, raised elevation, smooth surface (center slightly elevated but not umbonate), white in color; 1mm in diameter;		
1 colony found of each, both with smooth edge, upper raised - lower pulminate elevation, both with smooth surface, upper grayish – lower white in color; overall diameter: 3mm;		
1 fungi found – not described		
Further examination under the microscopy, most of the sample look blue (G^{Pos}), cocci shaped, some tend to aggregate and other are isolated from one another. However, there are some red cocci (G^{Neg}) as well. According to the literature, the upper respiratory tract can house a verity of bacterial strains (*Staphylococcus, streptococcus,* and *Corynebacteria*). Although harmful, they do not exceed dangerous levels (restricted nutrients) and are part of a healthy individual.		Fig. 3 Gram-stain of a nasal bacteria

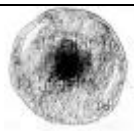
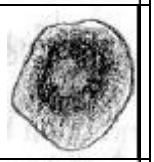
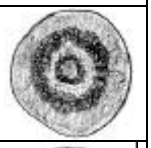
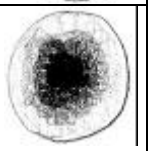
1.2. Bacterial samples from water (Day 1; Nov. 8th)

Purpose: Collection of bacteria from water sources of the environment and cultivating them on agar plates. In this particular case the sample was obtained from a draining canal along Alpenstrasse Avenue.

Procedure: The water sample was not extremely turbid, thus, no dilution series was executed:
Day 1: 8.11.99

- Extract 100µL from the sample container and pipet it onto the center of the agar dish.
- Heat-sterilize the hockey stick by dipping it into ethanol and ignite it with the burner already in operation.
- Cool down the stick by placing it for a few seconds onto the agar next to the plastic rim of the petri dish.
- Gently distribute the pipet sample smoothly over the entire agar.
- Mark the petri dish properly with date and sample ID before incubating it for 24 hours at 37°C (storage of dishes with the agar up-side down).
  Note: Take advantage of the thermal effect by keeping the Bunsen burner in operation for the entire experimentation to limit additional contamination by airborne contaminants.

Diagram 2: **Results** & **Discussion** (Day 4 Nov. 11th):

Description	Draw
3 colonies found, with smooth edge, convex elevation, smooth surface, pink in color; 6mm in diameter;	
3 colonies found, with smooth edge, convex to slightly umbonate elevation, smooth surface, center pink, 1st inner ring pink to violet, 2nd outer ring weakly pink, outer edge white in color; 3mm in diameter;	
8 colonies found, with smooth edge, raised elevation, smooth surface; center slightly white to pink, 1st ring pink, edge pink to white in color – all over appearance metallic glance; 6mm in diameter **suspected** to be *E.coli* from fecal material	
6 colonies found, with smooth edge, raised elevation, smooth surface, center white, 1st inner ring red, 2nd middle ring white, 3rd outer ring pink, outer edge white; overall diameter: 6mm;	
7 colonies found, with smooth edge, raised elevation, smooth surface, center pink, 1st inner ring white to pink, 2nd outer ring white, overall diameter: 4mm	

Overview of spread plates

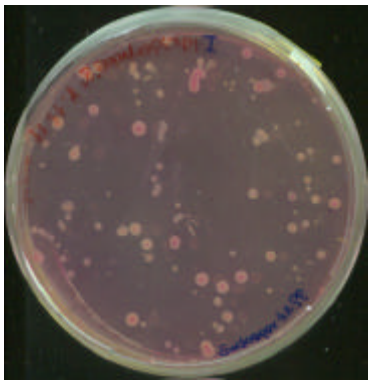


Fig 4 Water sample on endoagar 1

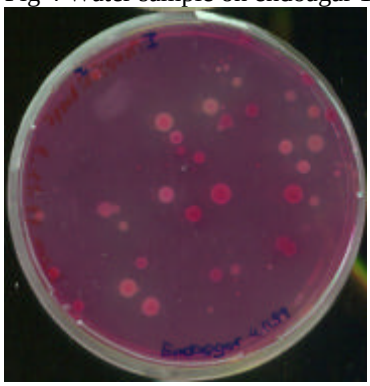
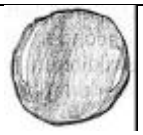
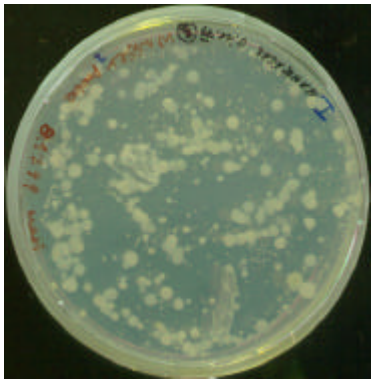
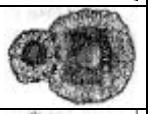
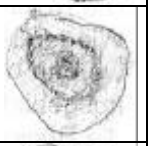
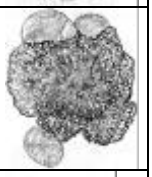
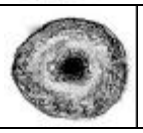


Fig 5 Water sample on endogar 2

Furthermore, the Endo agar test of the water sample revealed the presence of lactose positive strains (color turned red) and some colonies with a shiny reddish metallic glance, indicating the presence of coliform groups which included organisms such as *E.coli*. It is likely that the water under investigation was contaminated with fecal material (either from humans or other warm blooded animals) and, thus, unsafe to drink.

Diagram 3: **Results** & **Discussion** (Day 4 Nov. 11th):

Description	Draw	Overview of spread plates
16 colonies found, with smooth edge, pulvinate elevation, smooth surface, white in color; 6mm in diameter;		 Fig 6 Water sample on nutrient agar
8 colonies found, with toothed edge, flat elevation, rough surface, overall appearance white, edge more or less transparent; 5mm in diameter;		
6 colonies found, with smooth edge, flat elevation, smooth surface; allover appearance yellowish to greenish with white edge; 5mm in diameter		
1 colony found, with rough edge, flat elevation, rough surface, white in color; overall diameter: 8mm; outer colonies belong to the uppermost colony		
5 colonies found, with smooth edge, pulvinate elevation, smooth surface, center reddish to brownish, outer edge white, overall diameter: 0.5mm		
3 colonies found, with smooth edge, pulvinate elevation, smooth surface, center yellow, outer edge transparent, overall diameter: 0.5mm		
28 colonies found, with smooth edge, umbonate elevation, smooth surface, center white, outer edge transparent, overall diameter: 4mm		

1.3. Bacteria air sample

Purpose: Collection of bacteria from gaseous solutions and cultivation onto agar plates. In this particular case the sample was obtained from the basement housing the animals (i.e.mouse, rats) belonging to the institute of zoology.

Procedure: Professional sampling of air uses an automated air sampler; another simpler method is achieved by exposing an uncovered agar dish for two hours in a room.

- **1st sample** using the air-sampler: Load the air-sampler with a sterile NA-agar dish (upper compartment).
- Place sampler firmly on the ground or other safe surface and execute the programmed suction cycle (aspiration should last 1 minute at a rate of 100L/min).
- Unload dish, mark properly with date and name and incubate for 24hr at 37°C.
- **2nd sample** - simple air exposure: Uncover an agar dish and place it for approx. two hours in, or sweep it briefly through the air of a room. In this case a sleeping room was selected.
- Return sample in the incubator for at least 24h at 37°C (storage of dishes with the agar up-side down).

Diagram 4: **Results** & **Discussion** (Day 4 Nov. 11th):

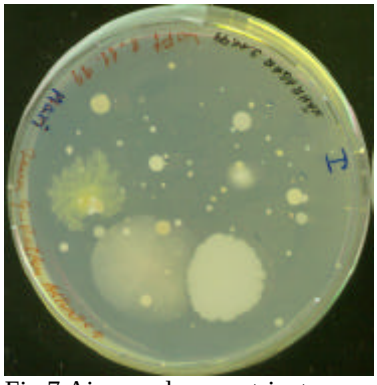
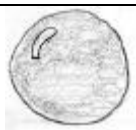
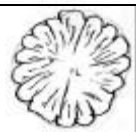
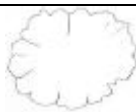
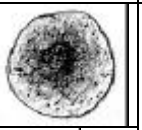
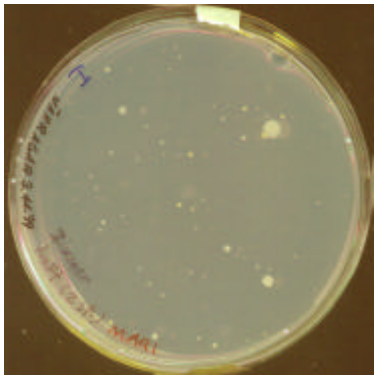
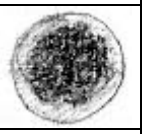
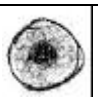
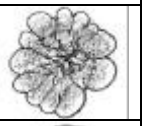
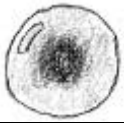
Description	Draw	Overview of suction plate
6 colonies found, with smooth edge, pyramid-like elevation, smooth surface, white to gray in color; 2mm in diameter;		 Fig 7 Air sample on nutrient agar
5 colonies found, with smooth edge, pulvinate elevation, smooth surface, overall appearance white, 4mm in diameter;		
1 colony found, with smooth edge, umbonate elevation, smooth surface; center yellow, outer edge white; 2.5mm in diameter;		
1 colony found, with undulate edge, flat elevation, rough surface, white in color; overall diameter: 4mm;		
2 colonies found, with smooth edge, convex elevation, smooth surface, center gray, 1st inner ring transparent, 2nd outer ring white, outer edge transparent, overall diameter: 5mm;		
1 colony found, with lobed edge, flat elevation, rough surface, white in color, overall diameter: 25mm!		
3 colonies found, with smooth edge, pulvinate elevation, rough surface, yellowish in color, overall diameter: 2mm;		
4 colonies found, with smooth edge, pulvinate elevation, smooth surface, reddish in color, 1mm in diameter;		
2 colonies found, with smooth edge, pulvinate elevation, smooth surface, yellowish in color, 1mm in diameter;		
2 large fungal colonies present		

Diagram 5: **Results** & **Discussion**:

	Description	**Draw**	**Overview of exposure plate**
	6 colonies found, with rough edge, convex elevation, punctiform surface, yellowish in color, 2mm in diameter;		 Fig 8 Air-sample of private room
	26 colonies found, with smooth edge, pulvinate elevation, smooth surface, white in color, 0.5mm in diameter;		
	2 colonies found, with smooth edge, convex elevation, smooth surface, yellowish in color, outer edge transparent, 2mm in diameter;		
	7 colonies found, with smooth edge, flat elevation, smooth surface, center white, 1st inner ring transparent, outer edge white in color, 0.5mm in diameter;		
	2 colonies found, with smooth edge, umbonate elevation, smooth surface, center yellowish, outer edge white in color, 0.5mm in diameter;		
	1 colony found, with smooth edge, pulvinate elevation, smooth surface, reddish-brown in color, 1mm in diameter;		
	1 colony found, with undulate edge, flat elevation, punctiform surface, white in color, 3mm in diameter;		
	1 colony found, with smooth edge, pulvinate elevation, smooth surface, white in color, 6mm in diameter;		
	19 rather tiny fungal hyphae found		

2. Microbes in an untreated sample of Milk - I

Material: perm. Marker
- 500mL sealable glass container
- 200µL Gilson-pipette w/ sterile tips (yellow)
- 1mL Gilson pipette w/ sterile tips (blue)
- 20mL sterile glass pipett w/ bulb
- Test-tube rack
- 3 empty Petridishes w/ lid
- Incubator (45°C)
- Hiclave Wolf HA-300MII w/ indicator tape
- Spatula
- Digital flat pan balance w/ plastic tray
- Vortex Mixer Meolab 7-2020
- 400mL bottle w/ screw caps of demineralized water
- 3 test tubes w/ Morton caps containing 9.9mL sterilized demineralized water.
- ≈15g Chinablue medium (Merck)

Samples: 0.1L of fresh, raw milk in sterile Greiner tube w/screw caps (vending machine - Shopping center Herrnau)

2.1 Medium broth to promote bacterial growth (Day 1 Nov. 8th)

Purpose: Chinablue medium enables easy differentiation of lactose fermenting (lactose^{pos}) and non-lactose fermenting microorganisms (lactose^{neg}). The change into a blue color of the incorporated indicator is caused by the presence of acids of the metabolic end products of the lactose fermenting pathway.

Procedure: Prepation of the Chinabue medium:
- Weigh 28.4g of Chinablue granulate into a sealable 800mL bottle.
- According to the instructions labeled by Merck, add the demineralized water to the end.
- Autoclave the broth for 15mins at 121°C (see appendix - Technical procedures and handling details).
  Note: Do not close tied the tab of the bottle containing the broth once it is placed in the autoclave to allow pressure equalization during the process of sterilization.
- Place sterilized broth into incubator at a temperature of 45°C to avoid solidification of agar.

2.2 Dilution series of raw milk sample (Day 1 Nov. 8th)

Purpose: To obtain the appropriate colony number, the sample to be counted must be diluted. Since one rarely knows the approximate viable count ahead of time, it is necessary to make more than one dilution. It is recommended to execute at least 3 dilutive steps.

Procedure:
- Extract 100µL from the Greiner tube containing the fresh raw milk sample and pipet it into the 1st sterilized test tube containing 9.9mL of sterilized and demineralized water (dilution is equivalent to $1E^{-2}$).
- Shake thoroughly on vortex mixer before extracting 1mL into the 1st empty petri dish; properly mark both the test tube and the pretri dish.
- Extract 100µL from the 1st test tube and pipet it into the 2nd test tube containing 9.9mL sterilized water (dilution is equivalent to E^{-4}).
- Shake thoroughly on vortex mixer before extracting 1mL into the 2nd empty petri dish; properly mark both the test tube and the petri dish.
- Extract 100µL from the 2nd test tube and pipet it into the 3rd test tube containing 9.9mL sterilized water (dilution is equivalent to E^{-6}).
- Shake thoroughly on vortex before extracting 1mL into the 3rd empty petri dish; properly mark both test tube and dish.
- Add 20mL of liquefied Chinablue agar into each dish. Do not forget to shake all dishes gently to obtain a homogenous mixture of the agar and the inoculum.
  Note: Chinablue agar requires quick handling to prevent solidification while pipetting is in progress.
- Allow dishes to cool down at room temperature before placing them into an incubator for two days at 37°C (storage of dishes with the agar up-side down).
- Store raw-milk sample in incubator at 30°C, since this sample will be used in the fermentation experiment 3 as well.

Diagram 6: **Results** (Day 4 Nov. 11th): Raw-milk sample (Anif)

Petri dish #	**Colony Count** Blue Lactose-Pos	White Lactose-Neg	Total
1	215	166	385
2	3	1	4
3	6	7	13

dF	**tdF**	**Initial concentration** colonies / mL
10^{-2}	10^{-2}	$38.5 \cdot 10^{3}$
10^{-2}	10^{-4}	$40 \cdot 10^{3}$
10^{-2}	10^{-6}	$13 \cdot 10^{6}$ *

(*) see discussion

$$\text{Dilution Factor (}\mathbf{dF}\text{)} = \frac{\text{pipetted volume of sample [mL]}}{\text{volume of sample added [mL] + volume to which it was added [mL]}}$$

Total Dilution Factor (**tdF**) = product of all intermediate dF's

$$\text{Initial concentration} = \frac{\text{cells on plate}}{\text{plated volume [mL]}}$$

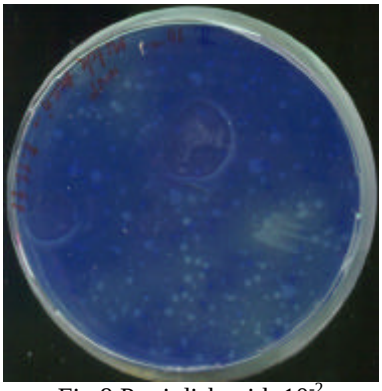


Fig 9 Petri dish with 10^{-2}
Colonies appear round and smooth and are distributed throughout the plate

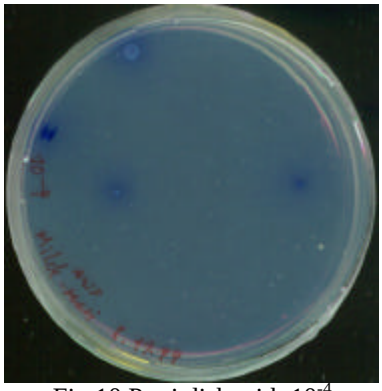


Fig 10 Petri dish with 10^{-4}
The number of colonies were less.

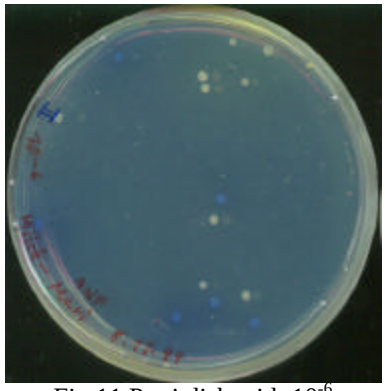


Fig 11 Petri dish with 10^{-6}
Although the concentration was less (E^{-6}), there was more growth of colonies.

Evaluation & Discussion:
The scanned fresh raw milk sample in its original conditions (neutral pH and the presence of milk sugar lactose) favored the rapid multiplication of lactose fermenting bacteria [*Lactococcus lactis* (=*Streptococcus lactis*, Logan) and other bacteria like *E.coli*, *Staphylococcus*, etc.]. Their existence of *S. lactis* was easily recognized as bluish shaded colonies stained by the CBC-agar after an incubation period of two days at 30°C. The colorless colonies most likely are manifestations of lactose^{neg} bacteria such as *Serratia, Salmonella, Proteus*, etc. (see fig. 9-11).
Although the series of dilution did not show a falling pattern of the colony numbers, it provided us with a useful estimate of the number of microbes present in a fresh raw milk sample. In accordance with the colony count 3 in fig. 11, is probably due to improper handling of the spread plate method. It is likely that hot CBC agar was poured onto the raw milk; therefore, killing the bacteria present. Another reason could have been an improperly executed dilution series in which homogenous distribution of microbes did not take place, causing the microorganisms to settle down at the bottom.

Why is the CBC-agar cooled down at 45°C? In order to avoid chemical changes, protein denaturation, or other unexpected damages to the structure of CBC-agar. Second, the agar should not solidify during the pipetting process. And third to avoid overheating the milk with very hot agar; consequently, avoid killing the bacteria present on the raw and unpausterized milk.

3. Microbes in an untreated sample of Milk – II

Material: perm. Marker
Bunsen burner w/ igniter
Inoculation needle
Pasteur pipette - 230mm w/ rubber bulb
20µL Gilson-pipette w/ sterile tips (yellow)
3 empty Petri-dishes w/ lid
10mL bottle containing Xylol
Pair of latex gloves
6 object slides
Incubator (30°C)
pH indicator paper (Merck)
Samples: 100mL sample of fresh, untreated milk in sterile Greiner w/ screw cap (from experiment 2)

3.1 Bacterial proliferation in a sample of raw milk over a period of several days (Day 1 through 5 Nov. 8th - 12th)

Purpose: The activity of bacterial growth makes the milk sample more acidic as the days go by. In order to observe successive trends, the pH requires monitoring throughout the week. The same raw milk sample as in exp.2 will be used. To observe the biocenosis taking place in the sample, the milk has to be stored in the incubator at 30°C and examined every day for changes in pH and structure of the milk

Procedure: Shake milk sample thoroughly before proceeding:

- Extract approx. 5µL from the Greiner tube containing the sample of raw milk, place it onto an object slide, disperse the droplet with the pipette thoroughly, and dry at room temperature.
- Removal of fat in milk is achieved by adding few drops of Xylol onto the dried slide (use Pasteur pipette).
  Note: Xylol is toxic!!! Work under an aspirator and wear protective gloves.
- To increase fast volatilization, gently sweep the slide through the flame of the Bunsen burner a couple of times - avoid excessive heating which would otherwise destroy both bacteria and the protein structure of the milk.
- Mark the slide properly with date and store it for later use at a safe place.
- Repeat slide procedure and pH evaluation every day; do not forget to place the Greiner tube with the raw milk sample back into the incubator (at 30°C).

Day 4 & 5, Nov. 11 & 12th:
Execution of Gram-staining (as described in the appendix –Handling techniques) of all slides prepared.

Diagram 7: **Results** & **Discussion** :

Day	1	2	3	4	5	6
pH-Values	6-5	5-4	4-3	3	3-2	3
Rate of Succession	**First stage**	**Second stage**				**Third stage**
Description	Gram^{pos} cocci in chains	Gram-Positive rods, often in chains				Gram^{pos} + occurrence of yeast cells
Fig. 12 - 16: **Gram-stain Slides**						Not performed
Biocenosis	*L.lactis*	*Lactobacillus*				*Yeast*

Discussion:
Fermentation of lactose by *Lactococcus lactis* (= *Streptococcus lactis*, Logan) to lactic acids lowers the pH and causes coagulation of the proteins. The lowered pH eventually inhibits the reproduction of *L.lactis* which is then succeeded by the lactose fermenting species *Lactobacillus*, which tolerate the lowered pH and thus can complete the lactose fermentation, lowering the pH even further. Eventually, these species are followed by yeasts that will reproduce at a lower pH and utilize the lactic acid – this raises the pH again. Since the entire cycle of biocenosis takes about 10 days at a incubation temperature of 30°C, the third stage (yeast phase) was not observed at all because the total process took only 5 days.

4. Oxygen Relation in microbial Growth I

4.1 Relation of oxygen concentration in a solid medium and their types of microbial growth (Day 1 and 3; Nov. 8th, 10th)

Purpose: Few microbes are strictly aerobic or anaerobic; most are somewhere in between. An agar-shake culture was used to distinguish amongst these extremes. With this method it was possible to observe the location of the area of growth in the tube as the index of the oxygen requirements of microorganisms.

Procedure: (See appendix for YTA agar preparation):

- When the agar is not in liquid form, melt YTA agar by placing it for 10mins into an incubator (expels any dissolved oxygen) and cool down to a temperature of 42-45°C.
- Pipette 15µL of the *E.coli* medium into a deplugged test tube
- Pipette 15µL of *P.fluorescens* into the 2nd test tube.
- Pipette 100µL of water sample into the 3rd test tube (tube #4 is used as a control tube, thus not to be inoculated!!!).
- Add 5mL of YTA agar to each of the four test tubes and whirl gently to avoid incorporation of air in the agar.
  Note: YTA agar requires quick handling to prevent solidification while pipetting and avoid making bubbles.
- Solidify the agar filled tubes at room temperature and incubate at 37°C for 2 days (in the case of *P. florescens.* at 30°C).

Material: perm. Marker
20µL Gilson-pipette w/ sterile tips (yellow)
25mL sterile glass pipette w/ bulb
4 sterilized test tubes w/ rubber caps
Test tube rack
2 Incubators (30°, 37°C)
pH-Meter*
Magnetic stirrer w/ rod*

Samples: 2 100mL Erlenmeyer flask w/ cotton stopper, each containing:
10mL *E.coli* in liquid medium
10mL *P.fluorescens* in liquid medium
≈25mL YTA agar*
100mL Greiner tube with a water sample
100mL 1M HCl to modify pH of YTA agar*

(*) see appendix - Agar preparation

Diagram 8: **Results** & **Discussion** (Day 3; Nov. 10th):

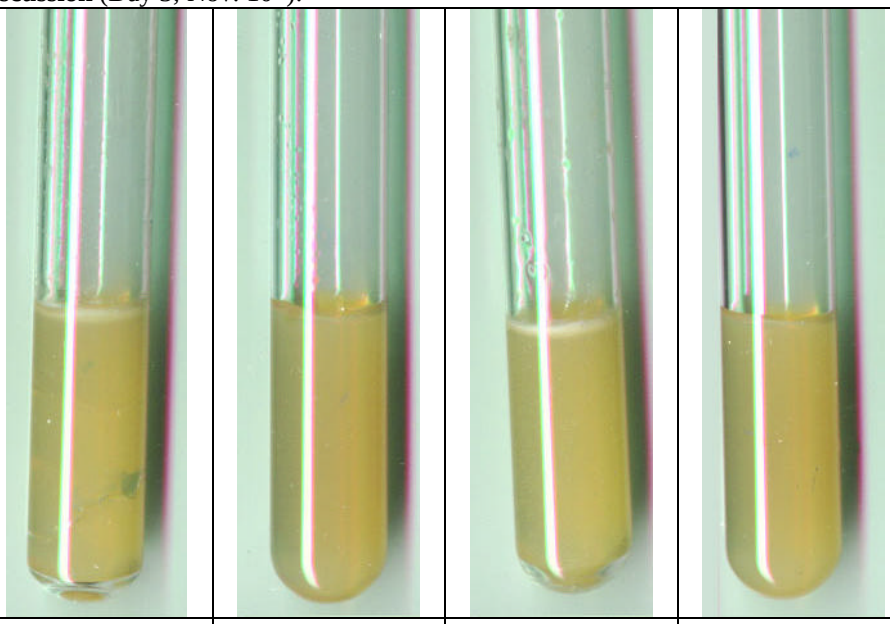


Fig.17 - 20: Results of the Agar-Shake Culture based on YTA

Inoculated strain	*E.coli*	*P.fluorescens*	Water sample	Control tube
Inoculated Vol.	15µL	15µL	100µL	-
Incubation temp.	37°C	30°C	37°C	37°C
Appearance	chambered appearance w/ many cracks in the lower 2/3 section of tube	no abnormalities; looks like the control sample	bacteria was able to grow throughout the tube	no abnormalities observed
Bacterial growth	aerobic	no growth	facultative aerobe	no growth

Discussion: (See experiment 5, p.14)

5. Oxygen Relation in microbial Growth II

Material: perm. Marker
20µL Gilson-pipette w/ sterile tips (yellow)
25mL sterile glass pipette w/ bulb
4 sterilized test tubes w/ rubber caps
Testtube rack
2 Incubators (30°, 37°C)
pH-Meter*
Magnetic stirrer w/ rod*
Samples: 2 100mL Erlenmeyer flask w/ cotton stopper, each containing:
10mL *E.coli* in liquid medium
10mL *P.fluorescens* in liquid medium
≈25mL BCP agar*
100mL Greiner tube with a water sample
100mL 1M HCl to modify pH of BCP agar*

(*) see appendix - Agar preparation

5.1 Relation of oxygen concentration in a solid medium and their types of microbial growth (Day 1 and 3; Nov. 8th , 10th)

Purpose: Few microbes are strictly aerobic or anaerobic; most are somewhere in between. An agar-shake culture was used to distinguish amongst these extremes. With this method it was possible to observe the location of the area of growth in the tube as the index of the oxygen requirements of microorganisms.

Procedure: (See appendix for BCP agar preparation):

- If the agar becomes solid, melt BCP agar by placing it for 10mins into an incubator (expels any dissolved oxygen) and cool down to a temperature of 42-45°C.
- Pipette 15µL of the *E.coli* medium into a deplugged test tube
- Pipette 15µL of *P.fluorescens* into the 2nd test tube.
- Pipette 100µL of water sample into the 3rd test tube (tube #4 is used as a control tube and will not be inoculated!!!).
- Add 5mL of BCP agar to each of the four test tubes and whirl gently to avoid incorporation of air in the agar.
  Note: BCP agar requires quick handling to prevent solidification while pipetting and avoid making bubbles while shaking.
- Solidify the agar filled tubes at room temperature and incubate at 37°C for 2 days (in the case of *P. florescens* at 30°C).

Diagram 9: **Results** & **Discussion** (Day 3; Nov. 10th):

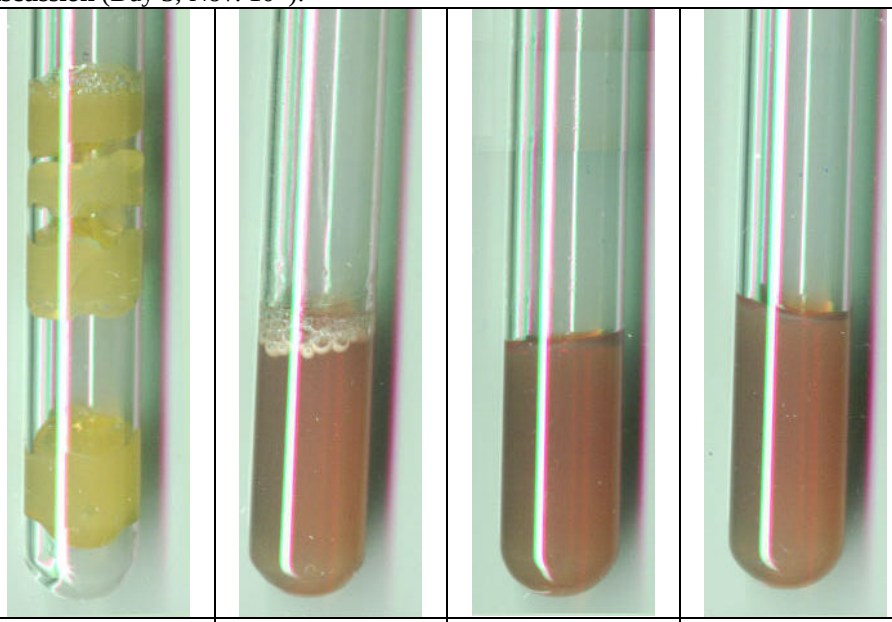


Fig.21 - 24: Results of the Agar-Shake Culture based on BCP

Inoculated strain	*E.coli*	*P.fluorescenc*	Water sample	Control tube
Inoculated Vol.	15µL	15µL	100µL	-
Incubation temp.	37°C	30°C	37°C	37°C
Appearance	Sliced agar was due to the intense anaerobic activity; lower section is more perforated than the upper part.	growth seems to have taken place only at the surface of agar;	growth seem to be concentrated in the upper 3/4 of the agar;	no abnormalities observed
Change in color and pH	Lighter in color than the control tube; pH << 6.8	light color change pH < 6.8	no color change	no color change;
Results	anaerobe	aerobe	microaerophilic	no growth

Discussion and evaluation of selected questions

Microorganisms vary in their need for, or tolerance of oxygen. **Aerobes** are species capable of growth at full oxygen tensions (pO_2 of air: 21%).
Microaerophiles, by contrast, are aerobes that can use O_2 only when it is present at levels reduced from that in air; usually because of their limited capacity to respire or because they contain oxygen-sensitive molecules.
Organisms that lack a respiratory system cannot use oxygen as their terminal electron acceptor, thus, termed are **anaerobes**.
Anaerobes are further classified into **aerotolerant anaerobes** (can tolerate oxygen without using it) and **obligate anaerobes** (stricter), which are killed by O_2. Consequently, none of the samples examined in experiment 4 and 5 can be considered strictly anaerobic; instead, all strains seem to cover the spectrum of aerobe, facultative anaerobic and anaerobic.

The figure 25 illustrates the distribution of aerobe and anaerobe extremes within an agar shake culture tube.

By using Morton caps on test tubes rather than tightly fitting stoppers, prevents bacterial contamination during the incubation period while allowing the free exchange of respiratory gasses of any aerobic microorganisms from/to the airspace contained within.

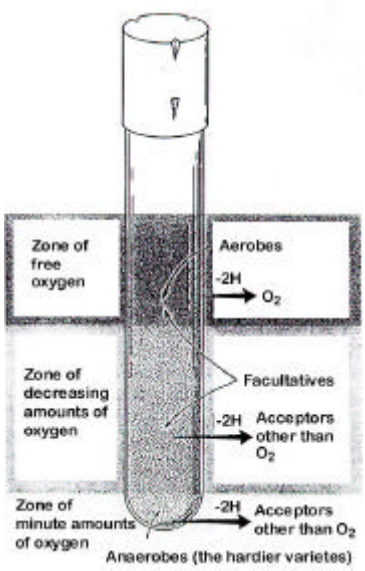


Fig.25 Typical distribution of microorganisms according to their oxygen requirements

Answers to selected questions:

What is <u>oxidation</u>:
A process by which a compound gives up electrons (or H-atoms) and becomes oxidized.

<u>Respiration</u>: The process in which a compound is oxidized with O_2 or an O_2 substitute functioning as the terminal electron acceptor, usually accompanied by ATP production by oxidative phosphorylation.

<u>Biological oxidation</u>: The electron donor of the final respiratory steps in cellular respiration (bucket brigade of the electron transport chain) is Oxygen: $3H^+ + ½O_2^- \rightarrow H_2O$

<u>Fermentation</u>: Anaerobic catabolism of an organic compound in which the compound serves as both an electron donor and an electron acceptor and in which ATP is produced by substrate-level phosphorylation.

<u>Anaerobic respiration</u>: in which some substance such as SO_4^{-2} or NO_3^- serves as terminal electron acceptor instead of O_2.

6. Extremophile Bacteria (Archaea-Bacteria)

6.1 Cultivation, growth, and examination of a thermo-acidophilic archae-bacterium (Day 1 and 2; Nov. 8th, 9th)

Material: perm. Marker
20µL Gilson-pipette w/ sterile tips (yellow)
6x 20mL ster. pipettes w/ bulb
6 sterilized test tubes w/ Morton caps
5 empty petri dishes w/ lid
Bunsen burner w/ igniter
Inoculation needle
Testtube rack
3 Incubators (37°, 50°, 65°C)
Vortex mixer Meolab 7-2020
pH-Meter*
Magnetic stirrer w/ rod
30mL Acidianus medium*
5x 20mL M2-medium w/ increasing NaCL content: 0.5, 5, 10, 15, and 20%
Samples: 5x 100mL Erlenmeyer flasks + cotton stopper each w/:
10mL *Acidianus brierleyi*
10mL *E.coli*;
10mL *H. sp. EDH 10*;
10mL *H. medeterranei*;
10mL *H. volcanii* in liquid medium

(*) see appendix - Agar preparation

Purpose: Proliferation of *Acidianus brierleyi* not only requires a suitable culture medium but works best at a lower pH. Preparation of the medium and subsequently inoculation with the strain enables monitoring of the ongoing progression on a daily basis.

Procedure: (See appendix for *Acidianus* medium preparation):

- Pipett 4mL of *Acidianus* medium (pH ≈ 6) into each of the six sterilized test tubes making sure that any pipetted sample do not contain some granules of solid sulfur.
- Add 100µL of *Acidianus* liquid broth to the three test tubes (the other remaining three tubes are kept as control tubes), and label the tubes according to their incubation temperature, name of student, experiment number, etc.
- Use Gilson pipette to apply a tiny amount onto the pH-indicator tape in order to monitor the pH value of all of the six tubes.
- Place pairs of tubes into each of the three (one pair consists of an inoculated tube and a control tube) and incubate them at 37°C, 50°C, and ≈65°C.
- Finally, monitor the pH values every day for the rest of the week.

Table 1: **Results** (Day 3, 4, and 5; Nov. 10th, 11th, 12th):

Test tube set	Date	pH of inoculated tube	pH of control tubes	Comments
37°C	8th	3	4 5	gradual but slow decrease in the pH of the inoculated tube
	9th	3	5	
	10th	2 3	5	
	11th	2 3	6	
	12th	2 3	5 6	
50°C	8th	3	4 5	moderate decrease in the pH of the inoculated tube
	9th	2 3	5	
	10th	2 3	5	
	11th	2 3	6	
	12th	2	5 6	
65°C	8th	3	4 5	progressive decrease in the pH of the inoculated tube
	9th	2 3	5	
	10th	2 3	5	
	11th	1 2	6	
	12th	1	5 6	

Discussion: The Archea-Bacteria *Acidianus brierleyi* is a facultative anaerobe and thermophilic capable of lithotrophic growth by oxidation or reduction of elementar sulfur (S^0). The range of pH suitable for growth spreads from 1 to 5. Under aerobic conditions the organism uses S^0 as an electron donor, oxidizing S^0 to H_2SO_4. Anaerobically, *Acidianus* uses S^0 as an electron acceptor (with H_2 as electron donor) forming H_2S as the reduced product. Thus, the metabolic fate of S^0in cultures of *Acidianus* depends on the presence of O_2 and / or an electron donor. *Acidianus* is spherical in shape. It grows at temperatures from about 65°C up to a maximum of 95°C, with an optimum of about 90°C. It has an unusually low GC base ratio 31%, the DNA of 30-40% GC content would melt almost instantly at 90°C. Obviously hyperthermophiles have evolved protective mechanism to prevent DNA melting *in vivo*.

Aerobic pathway: $2H_2O + 2S^0 + O_2 \rightarrow H_2SO_4$
Anaerobic pathway: $H_2 + S^0 \rightarrow H_2S$

Table 2: General Characteristics of *Acidianus sp*. (Brock 1997)

Genus	Morphology	Number of Species	DNA (mol-% GC)	opt. temperature [°C] Min.	Opt.	Max.	Optimum pH
Acidianus	Sphere	2	31	65	85-90	95	2

6.2 Cultivation and growth of selected Eu- and Archaea-Bacteria at different salt concentrations (Day 1 and 2; Nov. 8th , 9th)

Purpose: Thermohalophilic archaebacteria and nonhalophilic eubacteria were cultivated on M2 agar media enriched with NaCL at gradually increasing concentrations. The strains used have different salt requirements; thus, growth should be adapted according to the most preferred substrate.

Procedure: See appendix for M2-NaCL agar preparation:

- Label dishes with proper NaCl concentrations (0.5, 5, 10, 15, and 20%) and divide each dish into 4 sectors. Each sector should be labeled according to the strain later on used for inoculation (*E.coli, Halobacterium EDH10, Haloferax mediterranei, Haloferax volcanii*).
- Pipette 20mL of the 0.5% NaCl-M2-agar into the first petri dish; repeat this procedure with the remaining four petri dishes using the 5, 10, 15, and 20% NaCl M2-agar respectively, and allow solidification at room temperature before drying all dishes in an incubator at 37°C for 1 to 2 hours.
  Note: All agar requires quick handling to prevent solidification while pipetting. Swap any used glass pipette against a new sterilized one when using different agar.
- Once dry, inoculated the outlined sectors with the labeled species, using an inoculation needle and the burner for sterilization.
- Place dishes into plastic bag and incubate at 37°C for 3 to 4 days (*E.coli* sector can be examined after 24hr) with the agar up-side down.

Diagram 10: **Results** (Day 6; Nov. 15th): Unfortunately, limited supply of M2 agar of 0.5% and 5% NaCl concentrations forced me to share plates with a fellow student.

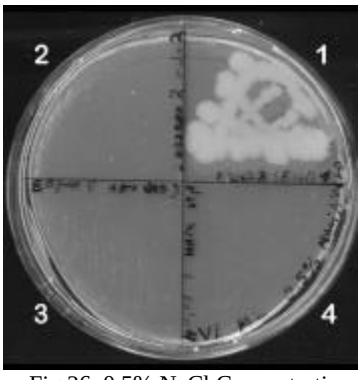


Fig 26 0.5% NaCl Concentration

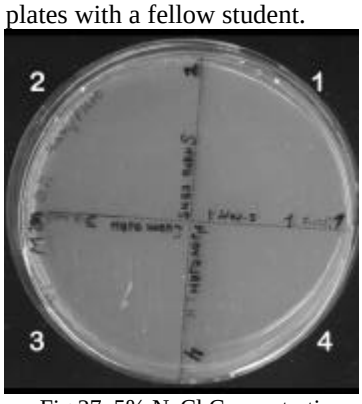


Fig 27 5% NaCl Concentration

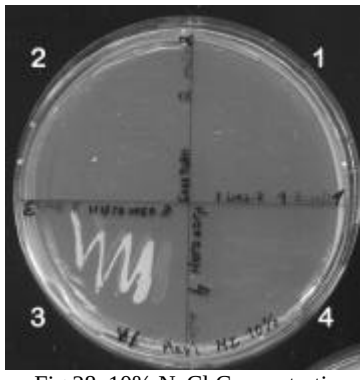


Fig 28 10% NaCl Concentration

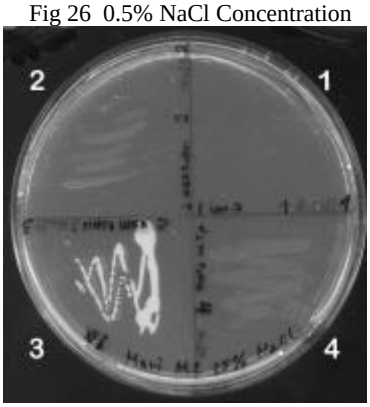


Fig 29 15% NaCl Concentration

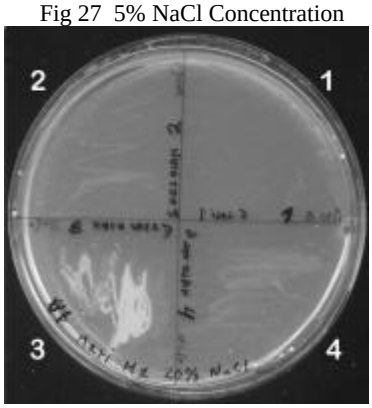


Fig 30 20% NaCl Concentration

Legend: Images taken on the 15th of November
Sector 1*: E.coli*:
Sector 2: *Halobact.*sp. EDH 10
Sector 3: *H. mediterranei*:
Sector 4: *H. volcanii*

Table 3: **Results** of bacterial growth according to their NaCl concentrations

NaCl	meso- and nonhalophilic				extreme halo- and thermophilic Archaea											
	1. ***E.coli*** (eubacterium)				2. ***H. sp. EDH 10***				3. ***H. mediterranei***				4. ***H. volcanii***			
Nov'99	9th	10th	12th	16th	9th	10th	12th	16th	9th	10th	12th	16th	9th	10th	12th	16th
0.5%	+	+	++	++	-	-	-	-	-	-	-	-	-	-	-	-
5%	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
10%	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	+
15%	-	-	-	-	-	-	-	+	-	-	+	++	-	-	-	++
20%	-	-	-	-	-	-	-	++	-	-	+	+++	-	-	+	+++

Discussion & evaluation of selected questions

Undoubtfully, the Archea-Bacteria favor salty and warm conditions. The extreme halophiles (grew best at salt concentrations ranging from 15-30%, whereas the non-halophile eubacterium was able to flourish at the lowest salt concentration of 0.5%.

What other phenomena can be observed at different salt concentrations?
E.coli grew after the first day of incubation (0.5% NaCl) and it continued so till the 15th of Nov; the petri dish was almost full of *E.coli*.
With 5% of NaCl, none of the bacteria grew because either it was too much for *E.coli* or it was too little for the extreme halophilic archaebacteria.
At a level of 10% NaCl, significant growth for *Haloferax mediterranei* could be observed, and it was able to continued to do so in higher NaCl concentrations of 15-20% till the 15th of Nov (day of last observation made).
The *Halobacterium* sp. EDH10 and *Haloferax volcanii* grew even slower, but obviously with the same pace at 15% and 20% NaCl concentrations. These significant observations were made on the 12th of Nov.
However, the three extreme halophilic archaebacteria have been found to grow much slower even though they were subjected to higher concentrations (10-20% NaCl). It seemed to me that the temperature of 37°C was not within the optimal range; although, days later, an adaptive effect must have taken place, because their growth rate seemed to have improved drastically (16th of Nov), while *E.coli* stagnated.

Relation of growth and temperature: Temperature is one of the most important environmental factors influencing the growth and survival of organisms. It can affect living organism in either of two opposing ways. As the temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates and growth becomes faster. However, above a certain temperature, proteins, nucleic acids, and other cellular components may be irreversibly damaged. Thus, as the temperature is increased within a given range, growth and metabolic function increase up to a point where inactivation reactions set in. Above this point, cell functions fall sharply to zero. We find that for every organism there is a minimum temperate below which growth no longer occurs, an optimum temperature at which growth is most rapid, and a maximum temperature above which growth is not possible. The optimum temperature is always nearer the maximum than the minimum. These three temperatures, often called the **cardinal temperatures**, are generally characteristics of each type of organism but are not completely fixed, as they can be modified slightly by other factors of the environment in particular, the composition of growth medium. The cytoplasmic membrane of the organisms must be in a fluid state for proper functioning. Perhaps the minimum temperature of an organism results from freezing of the cytoplasmic membrane so it no longer functions properly in nutrient transport or proton gradient formation. It is possible to broadly distinguish four groups in relation to their temperature optima:

Psychrophiles (low temperature optima; in unusually cold environment < 15°C)
Mesophiles (midrange temperatures optima; in warm blooded animals and in terrestrial and aquatic environment. In temperate & tropical latitudes. 30-48°C)
Thermophiles (high temperatures optima; in unusually hot environment > 48°C)
Hyperthermophiles (very high temperatures optima; in extremely hot habitats such as hot springs, geysers, and deep-sea hydrothermal vents. > 80°C)

Thermophile bacteria cultivation for the purpose of heat-resistant protein extraction is used in the pharmaceutical industry; name some difficulties that might arise:
Apart from significant advantages like very compact protein filaments and sensitive structures of their Lipids, these bacteria challenge the manufacturing process in the following ways:

- Temperatures above 100°C cannot work under normal atmospheric pressure. Therefore, autoclave must be used.
- Difficulties with the medium in which thermophile bacteria could grow (success only in experimental locations) because the agar will melt at 90°C, so silicagel is used instead.
- Glass containers are resistant but only for small amounts of cultures.
- And for industrial use, rust free steel is subject to corrosion at very high temperatures.

7. Antibiotic Sensitivity

7.1 Sensitivity test of selected bacterial strains against the most common antibiotics (Day 2 and 5; Nov. 9th, 12th)

Purpose: Antimicrobial activitiy is measured by determining the smallest amount of agent needed to inhibit the growth of a test organism, a value called the minimum inhibitory concentration (MIC). The agar diffusion method was used for this experiment in order to study MIC. A petri plate containing an agar medium evenly inoculated with the test organism is prepared. Known amounts of the antimicrobial agent are added to filter paper discs, which are then placed on the surface of the agar.

Procedure: (see appendix for M2, LB, and NA agar preparation):

- Label dishes according to the agar and media required (2x M2; 3x LB; 2x NA); separate each dish into 4 sectors, and assign numbers to each sector corresponding to an antibiotic.
- Pipett 20mL of agar into the 7 petri dishes with the following:
  2 x M2 (20% NaCl); 3 x LB; 2 x NA.
  Note: All agar requires quick handling to prevent solidification while pipetting. Swap glass pipette when using different agar.
- Once solidified, place dishes in incubator for 2hr at 37°C.
- Pipette 100µL of each strain and use the spread plate method to inoculate with the following plates (see appendix - handling techniques):
  Halobacte sp. EDH 10: onto 2 M2 agar
  Bacillus subtilis: onto 2 NA-agar
  Escherichia coli: onto 2 LB agar
- Sterilized water: onto a third LB dish
  Note: Take advantage of the thermal effect by keeping the Bunsen burner in operation for the entire experimentation to limit additional infection by airborne contaminants. And placed all the plates with the agar layer downwards!!!
- Once dry (under tungsten lamp) use tweezers to apply 4 filter discs onto each dish (except for water dish) as shown in the scans below.
  Note: sterilize tweezers each and every time when picking up a new filter disc with ethanol and burner.
- Use the Gilson pipette to apply 5µL of the chosen antibiotics in accordance with the labeled sector.
- Incubate all dishes at 37°C for at least 4 to 5 days (in the case of *Bacillus subtilis* for 30°C).

Material: perm. Marker
- 200µL Gilson-pipette w/ sterile tips (yellow)
- 20µL Gilson-pipette w/ sterile tips (yellow)
- 3 25mL sterile glass pipette w/ bulb
- 7 empty petri dishes w/ lid
- Bunsen burner w/ igniter
- 100mL beaker w/ some EtOH
- Test tube w/ 4mL of sterile demin. water plus cap
- Inoculation needle
- Pair of tweezers
- 2 Incubator (30°, 37°C)
- pH-Meter*
- Magnetic stirrer w/ rod
- Tungsten lamp
- 35x 5mm sterile filter discs

Samples: 3x 100mL Erlenmeyer flask w/ cotton stopper, each containing:
- 10mL *Escherichia coli* in liquid medium
- 10mL *Bacillus subtilis* in liquid medium
- 10mL *Halobacter* EDH 10 in liquid medium
- 2x 20mL of M2 agar*
- 3x 20mL of LB agar*
- 2x 20mL of NA agar*
- 40µL antibiotics each of:
  1. Anisomycin 5mg/ml in DMSO
  2. Chloramphenicyl 2mg/ml in MtOH
  3. Rifampicin (10:1) in MtOH
  4. Bacitracin 1mg/mL in DMSO
  5. Novobiocin 0.5mg/mL in H_2O
  6. Ampicillin 2mg/mL in H_2O
  7. Streptomycin 2mg/mL in H_2O
  8. Tetracyclin 2mg/mL in H_2O

(*) see appendix – HSL Agar preparation

Diagram 11: **Results** (Day 5; Nov. 12th)

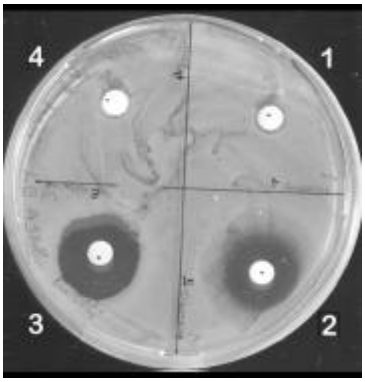


Fig 31 *E.coli* on LB-agar (1-4)

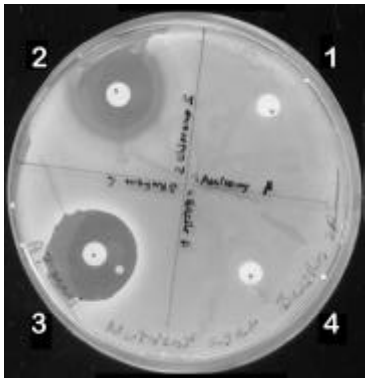


Fig 32 *B.subtilis* on NA-agar (1-4)

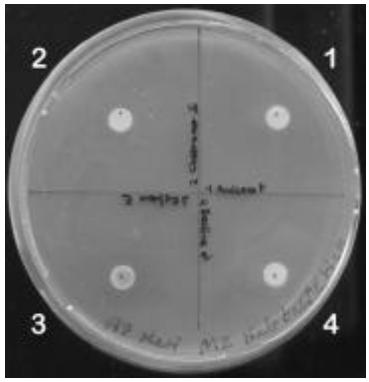


Fig 33 *Halobacter* on M2-agar (1-4)

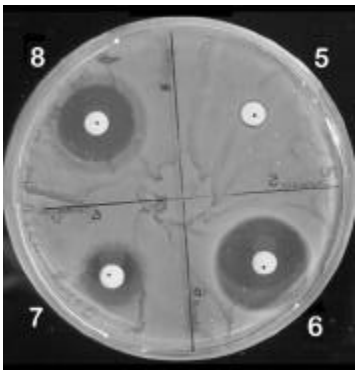


Fig 34 *E.coli* on LB-agar (5-8)

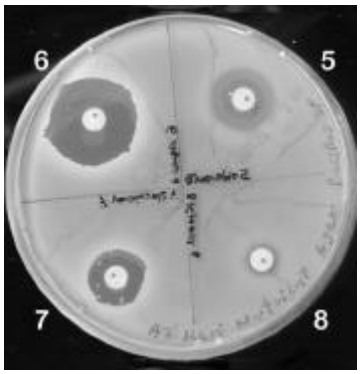


Fig 35 *B.subtilis* on NA-agar (5-8)

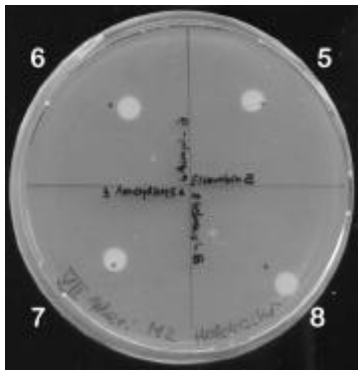


Fig 36 *Halobacter* on M2-agar (5-8)

Table 4: Results of Zones of Inhibition - Group 1

			Antibiotics - zone of inhibition [mm]							
			Dish 1				Dish 2			
Agar	**strain**	**Date**	**1. Ani**	**2. Chl**	**3. Rif**	**4. Bac**	**5. Nov**	**6. Amp**	**7. Str**	**8. Tet**
LB	*E. coli*	10th	7	20	23	7	8	21	15	18
NA	*B. subtilis*	10th	0	20	24	0	12	24	15	7
M2	*Halobacter*	10th	25	0	20	20	40	0	0	0

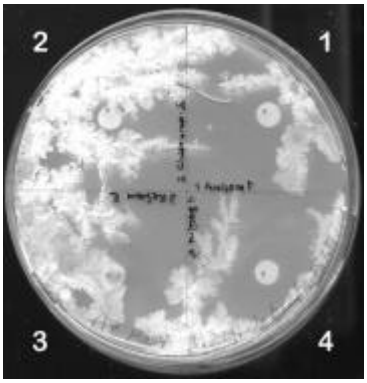


Fig 37 *Halobacter* on M2-agar (1-4)

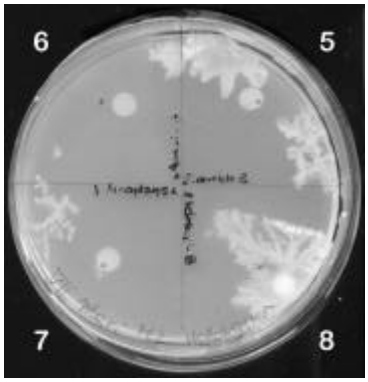


Fig 38 *Halobacter* on M2-agar (5-8)

Fig. 37 and 38 differ only with those of Fig 33 and 36 in that they have been incubated for an extra 10 days.

The control-dish (water) did show extensive contamination. Probably the sterile water sample or the hockey stick have not been microbe-free!

Table 5: Results of growth inhibition of the entire group with statistical evaluation of a representative antibiotic

Antibiotics Sensitivity Test: **Zone of Inhibition** [mm]									
Test Organisms	**Group**	**1. Ani**	**2. Chl**	**3. Bac**	**4. Rif**	**5. Nov**	**6. Amp**	**7. Str**	**8. Tet**
E.coli	Gr 4	0	21	0	27	6	19	0	17
Bacillus subtilis.	Gr 4	0	17	0	15	17	25	9	9
Bacillus subtilis	Gr 4	0	25	0	25	15	23	13	7
Halobacter EDH 10	Gr 4	8	11	7	15	----	----	----	----
Halobacter EDH 10	Gr 4	7	0	9	17	----	----	----	----
E.coli	Gr 1[1]	7	20	23	7	8	21	15	18
Bacillus subtilis	Gr 1[1]	0	20	24	0	12	24	15	7
Halobacter EDH 10	Gr 1[1]	25	0	20	20	40	0	0	0
E.coli	Gr 1	7	20	22.2	8	7	25	15	21
Bacillus subtilis	Gr 1	0	26	25	0	10	25	16	8
Halobacter EDH 10	Gr 1	20	8	25	22	40	0	0	9
E.coli	AG	7	15	18	7	7	27	16	21
Bacillus subtilis	AG	0	19	28	0	18	27	17	10
Halobacter EDH 10	AG	0	0	0	0	----	----	----	----
E.coli	TW	6	16	18	7	7	27	18	17
Bacillus subtilis	TW	6	21	24	6	21	17	16	7
Halobacter EDH 10	TW	0	0	0	0	----	----	----	----
E.coli	Gr GW	0	22	21	0	0	20	17	18
Bacillus subtilis	Gr GW	0	9	21	10	25	0	0	10
Halobacter EDH 10	Gr GW	0	27	28	0	20	?	10	5
E.coli	Gr AP	0	20	22	0	0	23	20	22
E.coli	Gr AP	0	20	20	0	0	21	20	21
Bacillus subtilis	Gr AP	0	28	28	0	15	26	15	8
Bacillus subtilis	Gr AP	0	24	28	0	12	30	17	0
E.coli	Gr DS	0	20	25	0	0	18	13	20
E.coli	Gr DS	0	20	25	0	0	27	12	19
Bacillus subtilis	Gr DS	0	25	30	0	11	32	18	8
Bacillus subtilis	Gr DS	0	25	28	0	10	29	15	10
E.coli	Gr MD	0	5	17	7	7	22	18	20
E.coli	Gr MD	0	20	23	8	7	26	23	19
Bacillus subtilis	Gr MD	0	26	30	0	18	8	18	9
Bacillus subtilis	Gr MD	0	18	30	0	19	28	2	0
E.coli	Gr PB	0	15	20	0	0	20	15	20
E.coli	Gr PB	0	20	20	0	0	25	15	15
Bacillus subtilis	Gr PB	0	25	30	0	15	30	15	10
Bacillus subtilis	Gr PB	0	25	25	0	15	30	15	0
E.coli	Gr SS	0	19	23	0	0	25	17	16
Bacillus subtilis	Gr SS	0	21	17	0	19	29	18	10
Bacillus subtilis	Gr SS	0	23	29	0	14	30	15	10
E.coli	Gr F	0	30	2	0	0	20	15	15
Bacillus subtilis	Gr F	0	25	30	0	20	15	10	0
Halobacter EDH 10	Gr F	0	10	20	5	25	0	0	5
Shaded area represents the calculated arithmetical mean* of the zones of inhibition:									
n	Number of samples					18.00			
$\bar{x}$	Sample mean					15.89[2]			
(SD)	Standard deviation					4.10[2]			
SD [%]	Standard deviation (%)					25.81[2]			

([1]) Personally obtained results – (see table 4), ([2]) Results calculated with the software package as shown in the appendix

Discussion: the (SD) as a measure of the precision applies to small sets of data (n=18). Regardless of ist width of 68.3% of the area beneath a Gausian curve of data that lies within one standard deviation (25.8% = 4.10) of the mean ($\bar{x} \approx 16.00$) does not represent the true value because the (SD) is very wide, this suggests that there were systematic errors in all of the measurements made during the experiment. Some of the systematic errors were: inadequate amount of antibiotics pipetted; paper disks filter hat moved; the agar dishes were too wet and were not allow to dry properly; the zones of inhibition were not correctely interpreted and measured.

Discussion of experiments and evaluation of selected questions

Which differences are evident amongst Eubacteria (*E.coli B.subtilis*) and Archaea-Bacteria (Halophiles)? Eubacteria developed zones of inhibitions while halophilic Archaea-Bacteria do not. Most antibiotics that specifically affect protein synthesis in bacteria do not necessarily affect archaeal (or eukaryotic) protein synthesis (s. Brock, p.626, 1997). The *Halobacter* plates needed more time to grow that some zones of inhibition were (after 10 days) manifested.

Are there tiny colonies present within the inhibition zone and if so, why? Yes, there was evidence that the bacteria acquired the ability to grow in the presence of an antibiotic to which the microorganism is usually sensitive. There was development of antibiotic-resistant mutants within the inhibition zone of the antibiotic assay discs on the following plates (Brock, p.307, 1997).
The following antibiotics, Chloramphenicol on *E.coli* and *B.subtilis* as well as Ampicillin on *E.coli* revealed an intermediate sensitivity pattern. As shown in the scans 31, 32, and 34, the sensitive zone of inhibition is surrounded by a ring of semi-sensitive reaction. Consequently, growth did not came to a halt, but was more or less slowed down. One reason can be found in the specific modes of action regarding each antibiotic, its concentration and the permeability of the cell wall itself.

Ampicillin: A penicillin-derivative that blocks cell wall synthesis of the β-Lactamat-Ring.
Chloramphenicol: Blocks 50S-Ribosome-Units during protein synthesis.
The mode of action regarding the remaining antibiotics are:
Anisomycin: Blocks the transfer of Peptidyl of the amino acids during RNA-polymerase.
Rifampicin: Blocks the DNA-directed RNA-polymerase.
Bacitracin: Blocks cell wall synthesis.
Novobiocin: Blocks DNA-Gyrase; required to enable transcription of DNA into mRNA.
Streptomycin Blocks 30S-Ribosom-Subunit during protein synthesis.
Tetracyclin: Blocks 30S-Ribosom-Subunit during protein synthesis.

E.coli LB agar (#1-4): Mutant resistant against #2. Chloramphenicol
B.subtilis NA agar (#1-4): Bacteria did not form zone of inhibition on #4. Bacitracin because this bacteria produces this antibiotic (Brock, 1997). Mutant resistant against #3. Riffampicin
B.subtilis NA agar (#5-8): Mutant resistant against #5. Novobiocin & #7. Streptomycin

Describe any measures to ensure that sterility is maintained during the process of experimentation (use outcome of successful control media): The control media was full of bacteria after it was incubated for one day at 37°C because I didn't maintain sterilization procedures, I left the plate opened too long and some bacteria from the air already settled onto the agar. It is very important to maintain sterilization procedures in order to ensure good results and free from contaminants that might influenced the cultures media, some of these such procedures are:

- Prior to sterilization, a tube containing medium is usually loosely capped with one or a variety of fitted metal or plastic closures. This prevents the entry of more contaminants but permits free interchange of air or other gases.
- To start a bacterial culture, a number of cells (the inoculum) are transferred (inoculated) into a sterilized medium.
- In the inoculation procedure the needle or loop that is used to transfer microorganisms should be heated to redness by flaming immediately before and after the transfer. Flaming destroys living forms on the surface of the needle or loop.
- The mouth of the tubes from which cultures are taken and into which they are transferred should also be passed through the burner flame immediately before and after the needle is introduced and removed. In addition to destroying organisms on the lips of the tubes, flaming creates outward convection currents, which decrease the chance of contamination.

During incubation, the agent diffuses from the filter paper into the agar; the further it gets from the filter paper, the smaller the concentration of the agent. At some distance from the disc, the MIC is reached. Past this point growth occurs, but closer to the disc growth is absent. A zone of inhibition is thus created; the diameter of the zone is proportional to the amount of antimicrobial agent added to the disc and to the overall effectiveness of the agent. The concentration gradient is inversely linked to the mathematical relation of the surface area of a circle ($A = d^2 \cdot \pi/4$); i.e. the larger the circumference the greater the area of coverage; thus, the lesser the amount of antiobotic agent available per unit area. This method is routinely used to test for antibiotic sensitivity in pathogens.

8. Turbimetric Estimation of Bacterial Growth

8.1 Optimum bacterial growth (Day 5 and 6; Nov. 11th, 12th)

Purpose: The increase in cellular number is observed by inoculating a small amount of culture medium into a suitable nutrient broth. Periodical optic density measurements should be performed to monitor growth. Some cell counts should be done once the OD-readout exceeds 0.1.

Procedure: (See appendix for nutrient broth preparation):

- Pipett 19mL of nutrient broth into the first two nephelometer flask.
- Add 1mL of the *Pseudomonas* colony into the 1st nephelometer flask.
- As a reference for the spectro-photometer, pipett 20mL of agar into the remaining nephelometer flask.
- Once marked and sealed properly, register the first optical readout by inserting the reference solution into the test chamber of the spectro-photometer; set to zero and run the preliminary scan with both the control flask and the culture flasks (both scans should be done at a wavelength of 600nm).
- Incubate both flasks for the next 30mins in the shaker at 30°C before executing another cycle of measurements with the photometer.
- Transfer the reading onto semi-logarithmic paper (abscissa = t; ordinate = optical density).
  Keep repeating the cycle of incubation and measurements until growth displayed a sigmoidal pattern and has definitely entered into the stationary phase. Once the display exceeds the level of OD>0.1, extract 2µL from the culture flask and evaluate the average density using a microscope operated in phase-contrast and the Thoma counting chamber; if counting can't be performed right away, pipette 5µL into a plastic bullet, seal it, mark it, and store in ice-bath to bring cell division to a halt.

Material: perm. Marker
- 200µL Gilson-pipette w/ sterile tips (yellow)
- 1mL Gilson-pipette w/ sterile tips (blue)
- 25mL sterile glass pipette w/ bulb
- 2 nephelometer flasks w/ cap and stopper
- several plastic bullets
- container w/ shredded ice
- Incubator shaker (30°C) Innova 4080
- Spectro-photometer Novaspec II
- Electronic pH-meter*
- Thoma counting chamber w/ cover slide
- Microscope (1000x) w/ phase-contrast module

Samples: 2x 100mL Erlenmeyer flask w/ cotton stopper, each containing:
- 20mL *Pseudomonas fluorescens* in liquid medium
- 2x 20mL of NB*

(*) see appendix – HSL Agar preparation

Results (Day 5 and 6; Nov. 11th, 12th):

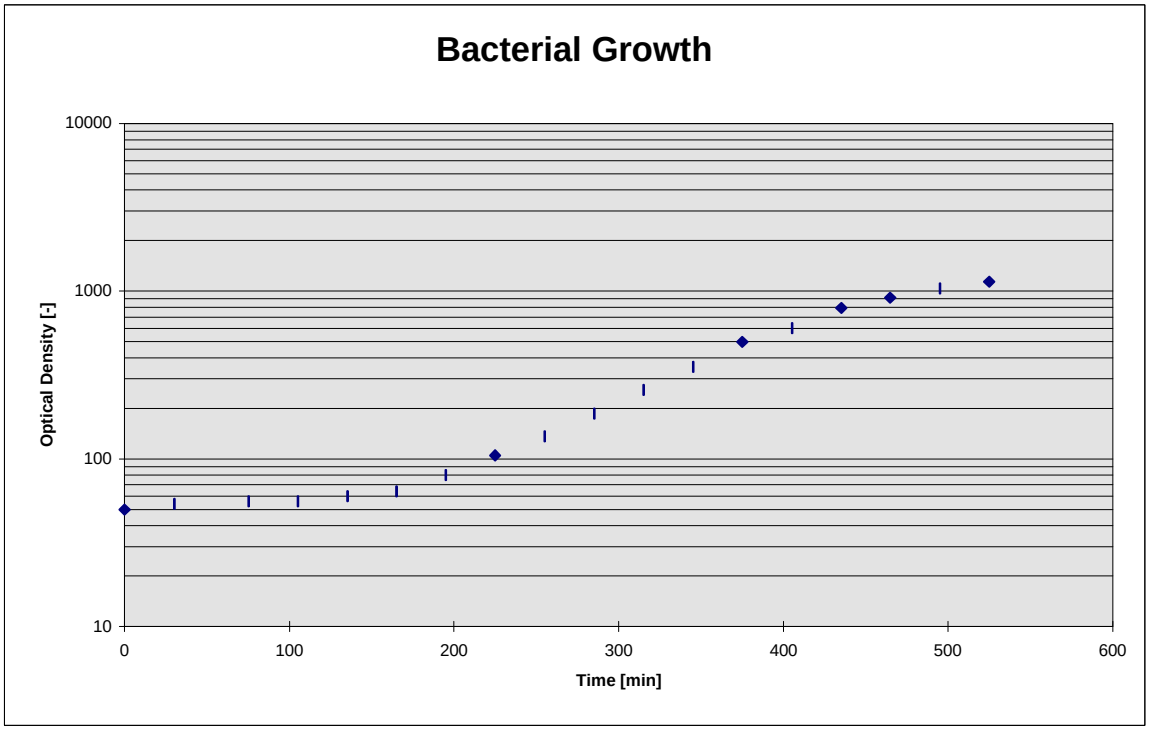


Fig. 39 Exponential growth of *Pseudomonas fluorescens*

Table 6: OD Measurements

time [hr:min]			*P.fluorescens*	
absolute	**relative**	**OD**	**mean[1]**	**count/mL**
9:15	00	0,050		
9:45	30	0,054		
10:00	45			
10:30	75	0,056		
11:00	105	0,056		
11:30	135	0,060		
12:00	165	0,064		
13:00	195	0,080		
13:30	225	0,105	$\bar{x}$: 8,75	$35 \cdot 10^6$
14:00	255	0,136	$\bar{x}$: 11,3	$45{,}3 \cdot 10^6$

time [hr:min]			*P.fluorescens*	
absolute	**relative**	**OD**	**mean[1]**	**count/mL**
14:30	285	0,186	$\bar{x}$: 15,5	$62 \cdot 10^6$
15:00	315	0,257[3]	$\bar{x}$: 21,4	$85{,}7 \cdot 10^6$
15:30	345	0,353[3]	$\bar{x}$: 41.6[2a]	$117{,}6 \cdot 10^6$
16:00	375	0,497	$\bar{x}$: 50.75[2b]	$165{,}7 \cdot 10^6$
16:30	405	0,602		
17:00	435	0,793		
17:30	465	0,915		
18:00	495	1,036		
18:30	525	1,135		
11:30	1020	1,296	(Not displayed on the graph)	

LAG Phase LOG-Phase STATIONARY Phase

(1) Average mean was obtained after evaluating four sub-squares of the counting chamber

(2) Motility of microorganism affected cell count; thus, number of cells given is likely to be exaggerated; these values have been corrected, according to the respective OD to 29,4 (2a) and 41,4 (2b) to calculate the initial count;

(3) Section of the steepest slope of the LOG-phase

cell population (n) =	$\frac{\log(OD) - \log(OD_0)}{\log(2)}$	OD_0, initial OD of LOG-phase OD, consecutive OD of LOG-phase

generation time (g) =	$\frac{t}{n}$	t, time intervall between the OD's of LOG-phase n, cell population

Calculation of the generation time using the section of the steepest slope -as outlined in the table above with the index (3) – results in a net doubling of the cell population in about 65.5mins (n = 0.458 generations every 30mins) which can be easily confirmed graphically in the chart of the previous page.

Determination of initial count is obtained by using the following formula:

$$n_i = \bar{x} \times \frac{\text{cell}}{\text{1 sub-square}} \times \frac{\text{16 sub-squares}}{\text{1 main square}} \times \frac{\text{1 main square}}{16 \cdot 250E^{-6}\ [mm^3]} \times \frac{1000\ [mm^3]}{1\ [mL]}$$ ....in [cells/mL]

Evaluation and Discussion:

The growth of the experiment doubled almost every 30 min (see Table 6), this pattern is called exponential growth, it can be easily observed by the graph on Fig. 23. One of the characteristics of this growth is that the rate of increase is initially slow but, once this increase starts, the growth rate is faster. In the first 30 min, the OD values increase by 4 (from 0.050 to 0.054) (see Table 6), after 4 or 5hrs, the OD values doubled (0.091), which means that the flask became more turbid.

Exponential growth displays a typical pattern in that certain section can be diferentiated:

- **LAG PHASE**: Growth is slow at first, while the "bugs" acclimate to the food and nutrients in their new habitat. It was between 0.050 & 0.060
- **LOG PHASE**: Once the metabolic machinery is running, they start multiplying exponentially, doubling in number every few minutes. This phase was between 0.064 & 0.0602.
- **STATIONARY PHASE**: As more and more bugs are competing for dwindling food and nutrients, booming growth stops and the number of bacteria stabilizes. This phase was between 0.0793 & 1.296.
- **DEATH PHASE**: Toxic waste products build up, food is depleted and the bugs begin to die. This phase was **not** observed because *Pseudomonas* grew slowly and can only be determined by viable cell count.

The population growth was also observed by the direct counting of the cells. It was observed in the log phase that the cells double every 30 min (from $\bar{x}$: 15.5 to $\bar{x}$: 21.4). Furthermore, we also obtained a sigmoidal growth path which it is valid for many populations, especially for bacteria that are replication strategists by executing binary fission as their main reproductive process.

The culture medium used contains meat extract which it is readily used by *P.fluorescens* - known to inhabit soil and animal body alike (Brock, 1997). *P.fluorescens* is a facultative aerobe microorganism able to fix elementar nitrogen, and is chemoorganotrophic. It stains Gram-negative and can be seen under the microscope as single rods.

P.fluorescens is an ecologically important in soil and in water; these microorganisms are responsible for the degradation of many soluble compounds derived from the breakdown of plant and animal materials in oxic habitats (Brock, 1997). *P.fluorescens* is not capable of forming spores but is quite motile by polarly arranged flagella. The temperature of the incubator (30°C) turned out to match with their optimal thermal requirements.

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Web related sites:

- Growth pattern: http://www.cellsalive.com/gallery.htm
- German Collection of Microorganisms and Cell Cultures: http://www.dsmz.de/species/strains.htm
- American Society for Microbiology: http://intl-aem.asm.org/search.shtml
- American Type Culture Collection: http://phage.atcc.org/searchengine/ba.html