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ICBM

METHODS SCRIPT

AG CYPIONKA v0.1

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Table of contents

Version history

v0.1 ─ 16.04.2010

- translation of all methods completed
- overview of R-/S- statements given*
- overview of hazard codes given**
- safety advices regarding individual chemicals added***
- disposal guideline added

* retrieved 01.04.2010 (http://www.sigmaaldrich.com/sigma-aldrich/help/help-welcome/risk-and-safety-statements.html)
** retrieved 01.04.2010 (http://www.chemie.de/tools/sicherheit/Gefahrensymbole.php3)
*** according to the Merck ChemDat

Disposal guidelines

Different classes of waste are usual products of even standard laboratory procedures. Resulting wastes are separated based on individual characteristics and collected in properly labelled containers. Classes of waste and respective coded can be found in the table below. Pieces of information regarding individual chemicals can be found in respective material and safety data sheets (MSDS). If there are any questions get in touch with responsible members of staff.

Code	Class
18710	Paper filters including harmful impurities (organic) ─ Papierfilter mit schädlichen Verunreinigungen (organisch)
18711	Paper filters including harmful impurities (inorganic) ─ Papierfilter mit schädlichen Verunreinigungen (anorganisch)
31434	Used filters and soaked up material (charcoal etc.) ─ Verbrauchte Filter und Aufsaugmassen (Aktivkohle)
31435	Used filters and soaked up material with harmful impurities ─ Verbrauchte Filter und Aufsaugmassen mit schädlichen Verunreinigungen
52102	Acids, acidic solutions, etchants (acidic) ─ Säuren, Säuregemische, Beizen (sauer)
52402	Bases, alkaline solutions, etchants (alkaline) ─ Laugen, Laugengemische, Beizen (basisch)
52403	Ammonia solution ─ Ammoniak-Lösung
52713	Concentrates and semi concetrates containing cyanide ─ Konzentrate und Halbkonzentrate (cyanidhaltig)
55220	Solvent mixtures including halogenated organic solvents ─ Lösemittelgemische inklusive halogenierte organische Lösungsmittel
55370	Solvent mixtures without halogenated organic solvents ─ Lösemittelgemische ohne halogenierte organische Lösungsmittel
57124	Ion exchange resins ─ Ionenaustauscherharze
57127	Plastic containers with harmful leftovers ─ Kunststoffbehältnisse mit schädlichem Restinhalt
59301	Fine chemicals ─ Feinchemikalien
59302	Chemicals leftovers (organic) ─ Laborchemikalienreste (organisch)
59303	Chemicals leftovers (inorganic) ─ Laborchemikalienreste (anorgansich)

R-/S- Statements

Indication of particular risks

R 1:	Explosive when dry
2:	Risk of explosion by shock, friction, fire or other sources of ignition
3:	Extreme risk of explosion by shock, friction, fire or other sources of ignition
4:	Forms very sensitive explosive metallic compounds
5:	Heating may cause an explosion
6:	Explosive with or without contact with air
7:	May cause fire
8:	Contact with combustible material may cause fire
9:	Explosive when mixed with combustible material
10:	Flammable
11:	Highly Flammable
12:	Extremely Flammable
14:	Reacts violently with water
15:	Contact with water liberates extremely flammable gases
16:	Explosive when mixed with oxidizing substances
17:	Spontaneously flammable in air
18:	In use may form flammable/explosive vapour-air mixture
19:	May form explosive peroxides
20:	Harmful by inhalation
21:	Harmful in contact with skin
22:	Harmful if swallowed
23:	Toxic by inhalation
24:	Toxic in contact with skin
25:	Toxic if swallowed
26:	Very Toxic by inhalation
27:	Very Toxic in contact with skin
28:	Very Toxic if swallowed
29:	Contact with water liberates toxic gas
30:	Can become highly flammable in use
31:	Contact with acids liberates toxic gas
32:	Contact with acids liberates very toxic gas
33:	Danger of cumulative effects
34:	Causes burns
35:	Causes severe burns
36:	Irritating to the eyes
37:	Irritating to the respiratory system
38:	Irritating to the skin
39:	Danger of very serious irreversible effects
40:	Limited evidence of a carcinogenic effect
41:	Risk of serious damage to eyes
42:	May cause sensitization by inhalation
43:	May cause sensitization by skin contact
44:	Risk of explosion if heated under confinement
45:	May cause cancer
46:	May cause heritable genetic damage
48:	Danger of serious damage to health by prolonged exposure
49:	May cause cancer by inhalation
50:	Very Toxic to aquatic organisms
51:	Toxic to aquatic organisms
52:	Harmful to aquatic organisms
53:	May cause long-term adverse effects in the aquatic environment
54:	Toxic to flora
55:	Toxic to fauna
56:	Toxic to soil organisms
57:	Toxic to bees
58:	May cause long-term adverse effects in the environment
59:	Dangerous for the ozone layer
60:	May impair fertility
61:	May cause harm to the unborn child
62:	Possible risk of impaired fertility
63:	Possible risk of harm to the unborn child
64:	May cause harm to breast-fed babies
65:	Harmful: May cause lung damage if swallowed
66:	Repeated exposure may cause skin dryness or cracking
67:	Vapours may cause drowsiness and dizziness
68:	Possible risk of irreversible effects

Combination of particular risks

14/15:	Reacts violently with water, liberating extremely flammable gases
15/29:	Contact with water liberates toxic, extremely flammable gas
20/21:	Harmful by inhalation and in contact with skin
20/21/22:	Harmful by inhalation, in contact with skin and if swallowed
20/22:	Harmful by inhalation and if swallowed
21/22:	Harmful in contact with skin and if swallowed
23/24:	Toxic by inhalation and in contact with skin
23/24/25:	Toxic by inhalation, in contact with skin and if swallowed
23/25:	Toxic by inhalation and if swallowed
24/25:	Toxic in contact with skin and if swallowed
26/27:	Very Toxic by inhalation and in contact with skin
39/27/28:	Very Toxic: danger of very serious irreversible effects in contact with skin and if swallowed
39/28:	Very Toxic: danger of very serious irreversible effects if swallowed
42/43:	May cause sensitization by inhalation and skin contact
48/20:	Harmful: danger of serious damage to health by prolonged exposure through inhalation
48/20/21:	Harmful: danger of serious damage to health by prolonged exposure through inhalation and in contact with skin
48/20/21/22:	Harmful: danger of serious damage to health by prolonged exposure through inhalation, and in contact with skin and if swallowed
48/20/22:	Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
48/21:	Harmful: danger of serious damage to health by prolonged exposure in contact with skin
48/21/22:	Harmful: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed
48/22:	Harmful: danger of serious damage to health by prolonged exposure if swallowed
48/23:	Toxic: danger of serious damage to health by prolonged exposure through inhalation

26/27/28:	Very Toxic by inhalation, in contact with skin and if swallowed
26/28:	Very Toxic by inhalation and if swallowed
27/28:	Very Toxic in contact with skin and if swallowed
36/37:	Irritating to eyes and respiratory system
36/37/38:	Irritating to eyes, respiratory system and skin
36/38:	Irritating to eyes and skin
37/38:	Irritating to respiratory system and skin
39/23:	Toxic: danger of very serious irreversible effects through inhalation
39/23/24:	Toxic: danger of very serious irreversible effects through inhalation in contact with skin
39/23/24/25:	Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed
39/23/25:	Toxic: danger of very serious irreversible effects through inhalation and if swallowed
39/24:	Toxic: danger of very serious irreversible effects in contact with skin
39/24/25:	Toxic: danger of very serious irreversible effects in contact with skin and if swallowed
39/25:	Toxic: danger of very serious irreversible effects if swallowed
39/26:	Very Toxic: danger of very serious irreversible effects through inhalation
39/26/27:	Very Toxic: danger of very serious irreversible effects through inhalation and in contact with skin
39/26/27/28:	Very Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed
39/26/28:	Very Toxic: danger of very serious irreversible effects through inhalation and if swallowed
39/27:	Very Toxic: danger of very serious irreversible effects in contact with skin
48/23/25:	Toxic: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
48/24:	Toxic: danger of serious damage to health by prolonged exposure in contact with skin
48/24/25:	Toxic: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed
48/25:	Toxic: danger of serious damage to health by prolonged exposure if swallowed
50/53:	Very Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
68/20/22:	Harmful: possible risk of irreversible effects through inhalation and if swallowed
68/22:	Harmful: possible risk of irreversible effects if swallowed
68/21:	Harmful: possible risk of irreversible effects in contact with skin
48/23/24:	Toxic: danger of serious damage to health by prolonged exposure through inhalation and in contact with skin
48/23/24/25:	Toxic: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed
48/23/25:	Toxic: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
48/24:	Toxic: danger of serious damage to health by prolonged exposure in contact with skin
48/24/25:	Toxic: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed
48/25:	Toxic: danger of serious damage to health by prolonged exposure if swallowed
50/53:	Very Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
51/53:	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
52/53:	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment
48/20:	Harmful: danger of serious damage to health by prolonged exposure through inhalation
48/20/21:	Harmful: danger of serious damage to health by prolonged exposure through inhalation and in contact with skin
48/20/21/22:	Harmful: danger of serious damage to health by prolonged exposure through inhalation, and in contact with skin and if swallowed
48/20/22:	Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
48/21:	Harmful: danger of serious damage to health by prolonged exposure in contact with skin
48/21/22:	Harmful: danger of serious damage to health by prolonged exposure in contact with skin and if swallowed
48/22:	Harmful: danger of serious damage to health by prolonged exposure if swallowed
48/23:	Toxic: danger of serious damage to health by prolonged exposure through inhalation
48/23/24:	Toxic: danger of serious damage to health by prolonged exposure through inhalation and in contact with skin
48/23/24/25:	Toxic: danger of serious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed
51/53:	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
52/53:	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment
68/20:	Harmful: possible risk of irreversible effects through inhalation
68/20/21:	Harmful: possible risk of irreversible effects through inhalation and in contact with skin
68/20/21/22:	Harmful: possible risk of irreversible effects through inhalation, in contact with skin and if swallowed
68/21/22:	Harmful: possible risk of irreversible effects in contact with skin and if swallowed
68/22:	Harmful: possible risk of irreversible effects if swallowed

Indication of safety precautions

S1:	Keep locked up
2:	Keep out of the reach of children
3:	Keep in a cool place
4:	Keep away from living quarters
5:	Keep contents under ... (appropriate liquid to be specified by the
37:	Wear suitable gloves
38:	In case of insufficient ventilation, wear suitable respiratory equipment
39:	Wear eye/face protection
40:	To clean the floor and all objects contaminated by this material use ... (to be specified by the manufacturer)
41:	In case of fire and/or explosion do not breathe fumes

manufacturer)

6: Keep under ... (inert gas to be specified by the manufacturer)

7: Keep container tightly closed

8: Keep container dry

9: Keep container in a well-ventilated place

12: Do not keep the container sealed

13: Keep away from food, drink and animal feeding stuffs

14: Keep away from ... (incompatible materials to be indicated by the manufacturer)

15: Keep away from heat

16: Keep away from sources of ignition - No smoking

17: Keep away from combustible material

18: Handle and open container with care

20: When using, do not eat or drink

21: When using, do not smoke

22: Do not breathe dust

23: Do not breathe gas/fumes/vapour/spray (appropriate wording to be specified by the manufacturer)

24: A void contact with skin

25: Avoid contact with eyes

26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

27: Take off immediately all contaminated clothing

28: After contact with skin, wash immediately with plenty of ... (to be specified by the manufacturer)

29: Do not empty into drains

30: Never add water to this product

33: Take precautionary measures against static discharges

35: This material and its container must be disposed of in a safe way

36: Wear suitable protective clothing

42: During fumigation/spraying wear suitable respiratory equipment (appropriate wording to be specified)

43: In case of fire, use ... (indicate in the space the precise type of fire-fighting equipment. If water increases the risk add - Never use water)

45: In case of accident or if you feel unwell, seek medical advice immediately (show label where possible)

46: If swallowed, seek medical advice immediately and show this container or label

47: Keep at temperature not exceeding ... E C (to be specified by the manufacturer)

48: Keep wetted with ... (appropriate material to be specified by the manufacturer)

49: Keep only in the original container

50: Do not mix with ... (to be specified by the manufacturer)

51: Use only in well-ventilated areas

52: Not recommended for interior use on large surface areas

53: Avoid exposure - obtain special instruction before use

56: Dispose of this material and its container to hazardous or special waste collection point

57: Use appropriate container to avoid environmental contamination

59: Refer to manufacturer/supplier for information on recovery/recycling

60: This material and/or its container must be disposed of as hazardous waste

61: Avoid release to the environment. Refer to special instructions safety data sheet

62: If swallowed, do not induce vomiting: seek medical advice immediately and show this container or label

63: In case of accident by inhalation, remove casualty to fresh air and keep at rest

64: If swallowed, rinse mouth with water (only if the person is conscious)

Combination of safety precautions

1/2: Keep locked up and out of the reach of children

3/7: Keep container tightly closed in a cool place

3/9/14: Keep in a cool well-ventilated place away from ... (incompatible materials to be indicated by manufacturer)

3/9/14/49: Keep only in the original container in a cool well-ventilated place away from ... (incompatible materials to be indicated by manufacturer)

3/9/49: Keep only in the original container in a cool well-ventilated place

3/14: Keep in a cool place away from ... (incompatible materials to be indicated by the manufacturer)

7/8: Keep container tightly closed and dry

7/9: Keep container tightly closed and in a well-ventilated place

7/47: Keep container tightly closed and at a temperature not e exceeding ... °C (to be specified by manufacturer)

20/21: When using, do not eat, drink or smoke

24/25: Avoid contact with skin and eyes

27/28: After contact with skin, take off immediately all contaminated clothing and wash immediately with plenty of.... (to be specified by the manufacturer)

29/35: Do not empty into drains, dispose of this container and it's material in a safe way

29/56: Do not empty into drains, dispose of this material and its container to hazardous or special waste-collection point

36/37: Wear suitable protective clothing and gloves

36/37/39: Wear suitable protective clothing, gloves and eye/face protection

36/39: Wear suitable protective clothing and eye/face protection

37/39: Wear suitable gloves and eye/face protection

47/49: Keep only in the original container at temperature not exceeding ... °C (to be specified by manufacturer)

24/25: Avoid contact with skin and eyes

Hazard codes

E explosive

F+ highly flammable

F flammable

O oxidising

T+ very toxic

T toxic

Xn harmful

C corrosive

Xi irritant

N dangerous for the environment

1. Media preparation

Preparation of growth media

Practical advices

Regarding all methods associated with making up media it is essential to work sterile.

The growth media are prepared in special glass vessels according to Widdel (1980, Figure 1). The chemicals are weighed in and dissolved in the below described succession (see schemata). The bottling tube is covered with tinfoil and the connecting piece for gas inflow offering a cotton filter is closed by a rubber stopper. The lateral screw caps are also closed but for autoclaving one of them is screwed on a little in order to prevent the vessel from bursting during heating. The medium is autoclaved 30 minutes at 121° C. After autoclaving, supplementary solutions are added through one of the lateral connecting pieces.

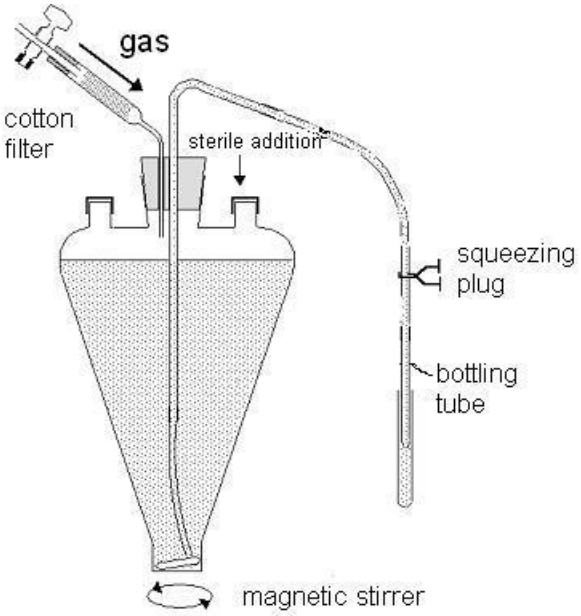


Figure 1: Glass vessel for preparing anaerobic medium (Widdel, 1980).

Oxic media

After autoclaving, the lateral screw caps are leak-proofed closed and the rubber stopper is removed from the connecting piece for gas inflow. Now, air can only enter the vessel through the cotton filter. To add the supplementary solutions to the cooled down medium, the vessel is connected to a N_2 gas inflow (5 kPa). The vessel with the completed medium remains connected to the nitrogen influx, because the excess pressure is needed for bottling. The completed medium is bottled to sterile containers.

Anoxic media

After autoclaving, the head space above the medium is flushed with N_2/CO_2 (80/20, v/v) immediately. The screw caps are leak-proof closed and the medium is cooled down while being stirred under N_2/CO_2 (80/20, v/v) (5 kPa) exposure. The supplement solutions are aseptically added to the cooled down medium. The pH value of the medium is adjusted (if necessary) to 7.2 – 7.4 using sterile 1 M Na_2CO_3 or 1 M HCl. The completed medium is bottled to sterile containers.

Mineral base medium for many anaerobic bacteria (Cypionka and Pfennig, 1986)

Table 1: Mineral base medium for many anaerobic bacteria.

		(F => fresh water, B => brackwater, M => marine)					
		F	B	M	F	B	M
Substance	MW	mM			g/l		
KH_2PO_4	136.09	1.5	1.5	1.5	0.20	0.20	0.20
NH_4Cl	53.49	5.0	5.0	5.0	0.25	0.25	0.25
KCl	74.55	4.0	4.0	4.0	0.30	0.30	0.30
$CaCl_2 * 2 H_2O$	147.02	1.0	1.0	1.0	0.15	0.15	0.15
$MgCl_2 * 6 H_2O$	203.30	2.5	10	15	0.50	2.00	3.00
NaCl	58.44	---	222	342	---	13	20
resazurine (0.5 mg/ ml)					ml 0.50	0.50	0.50

After autoclaving and cooling down, add from sterile stock solutions under N_2 exposure:

Trace element solution (SL10)	1.0 ml
Vitamin solution (V 7)	1.0 ml
Se + W-solution [1] (0.1 mM) $2 * 10^{-8}$ M	0.2 ml
$NaHCO_3$ [2] (1 M => 30 mM)	30 ml
Dithionite (crystalline) a little until decolouration	< 17 mg
pH [3] adjust with	sterile 1 M HCl or Na_2CO_3

[1] not required for all strains
[2] Attention! Protective vessel; do not touch hot containers!
[3] 6.8 - 7.0 for medium F, 7.0 - 7.3 for medium B and medium M

Annotation: The only difference between media F, B and M is the concentration of NaCl and $MgCl_2$. By adding concentrated salts (NaCl, 5 M, + $MgCl_2$, 0.2 M, ≈ 30 % salt) one is able to create medium B and M from a medium with a lower salt content:

45 ml/ l F-medium for	F => B
68 ml/ l F-medium for	F => M
23 ml/ l B-medium for	B => M

Often used electron donors and electron acceptors (mM):

H_2 (80 %) + CO_2 (20 %) + acetate (2 mM), lactate (20 mM), Na_2SO_4 (10 mM)
$Na_2S_2O_3$ (10 mM), $Na_2S_2O_5$ (5mM), $NaNO_3$ (10 mM)

Mineral base medium for marine aerobic bacteria

→ before autoclaving

Table 2: Mineral base medium for marine aerobic bacteria.

distilled H_2O	1000 ml	HEPES	2.38 g
		KBr (0.84 M)	1 ml
NaCl	24.32 g	H_3BO_3 (0.4 M)	1 ml
$MgCl_2$ * 6 H_2O	10 g	$SrCl_2$ (0.15 M)	1 ml
$CaCl_2$ * 2 H_2O	1.5 g	NH_4Cl (0.4 M)	1 ml
KCl	0.66 g	KH_2PO_4 (0.04 M)	1 ml
Na_2SO_4	4 g	NaF (0.07 M)	1 ml
Trace element solution SL 10	1 ml		
Selenite-Wolframate-Solution	0.2 ml		

KBr, H_3BO_3, $SrCl_2$, NH_4Cl, KH_2PO_4, NaF are added from sterile stock solutions. Before autoclaving, the pH of the medium is adjusted to 7.2-7.4 using 4 M NaOH.

→ after autoclaving, the medium is allowed to cool down before the following solutions are added

$NaHCO_3$ solution	0.2 g in 10 ml H_2O
10-Vitamine solution (5-times conc.)	2 ml

Preparation of trace element solutions (Tschech and Pfennig, 1984)

Table 3: Trace element solutions.

	SL10[1)]	**SL11**	**SL12**
distilled H_2O	1000 ml	1000 ml[2)]	1000 ml[2)]
HCl (25 %)	10 ml	–	–
EDTA-Di-Na salt	–	5.2 g	3.0 g
$FeSO_4$ * 7 H_2O	–	–	1.1 g
$FeCl_2$ * 4 H_2O	1.5 g	1.5 g	–
$CoCl_2$ * 6 H_2O	190 mg	190 mg	190 mg
$MnCl_2$ * 2 H_2O	100 mg	100 mg	50 mg
$ZnCl_2$	70 mg	70 mg	42 mg
$NiCl_2$ * 6 H_2O	24 mg	24 mg	24 mg
Na_2MoO_4 * 2 H_2O	36 mg	36 mg	18 mg
H_3BO_3	6 mg	6 mg	300 mg
$CuCl_2$ * 2 H_2O	2 mg	2 mg	2 mg

[1)] first dissolve $FeCl_2$ in HCl

[2] before filling up with water, adjust pH to ~ 6.0

Application: use 1 ml of the respective solution per litre of medium

SL9

like SL11, instead of EDTA-Di-Na: Nitrilotacetic acid (NTA): 12.8 g

Preparation of Selenite-Wolframate-Solution (Widdel, 1980)

Table 4: Selenite-Wolframate-Solution.

distilled H_2O	1000 ml
NaOH	0.4 g
$Na_2SeO_3 \cdot 5\ H_2O$	6 mg
$Na_2WO_4 \cdot 2\ H_2O$	8 mg

Preparation of vitamin solutions

Table 5: Vitamin solutions.

	7-Vitamins solution[1)]	**10-Vitamins solution[2)]**
distilled H_2O	180 ml	1000 ml
Biotin solution [3)]	20 ml	–
Biotin	–	10 mg
Nicotinic acid	20 mg	25 mg
Thiamine-Dichloride	10 mg	25 mg
p-Aminobenzoic acid	10 mg	25 mg
Ca-D(+)-Pantothenic acid	5 mg	25 mg
Pyridoxamine -Dihydrochloride	50 mg	50 mg
Cyanocobalamine (Vit. B_{12})	10 mg	5 mg
Folic acid	–	10 mg
Riboflavin	–	25 mg
Lipoic acid (Thioctic acid)	–	25 mg

[1)] according to Pfennig, 1978
[2)] 5-times concentrated, according to Balch et. al., 1979
[3)] 10 mg Biotin in 100 ml H_2O (slight heat supply facilitates dissolving)

After sterile filtration, prepared solutions are filled in sterile screw cap vials. Vials are stored in the dark under cool conditions.

Application: 1 ml per litre medium (7-Vitamins solution), 2 ml per litre medium (10-Vitamins solution)

Setting up HPG agar plates

Preparing the medium
The recipe of the HPG medium is based on the mineral base medium for marine aerobic bacteria. As the medium is mixed with agar immediately before pouring the plates, containing salts are only dissolved in 700 ml water.

→ before autoclaving

Table 6: HPG medium.

distilled H_2O	700 ml	HEPES	2.38 g
		KBr (0.84 M)	1 ml
NaCl	24.32 g	H_3BO_3 (0.4 M)	1 ml
$MgCl_2$ * 6 H_2O	10 g	$SrCl_2$ (0.15 M)	1 ml
$CaCl_2$ * 2 H_2O	1.5 g	NH_4Cl (0.4 M)	1 ml
KCl	0.66 g	KH_2PO_4 (0.04 M)	1 ml
Na_2SO_4	4 g	NaF (0.07 M)	1 ml
Trace element solution SL 10	1 ml	Na-Lactate (1 M)	5 ml
Selenit-Wolframat-Solution	0.2 ml		
Yeast extract	0.03 g		
Peptone	0.06 g		

Before autoclaving, the pH of the medium is adjusted to 7.2-7.4 using 4 M NaOH. The medium is filled in Schott-bottles (blue caps) and autoclaved. Supplementary solutions are added under sterile conditions (clean bench) after autoclaving and allowing the medium to cool down:

$NaHCO_3$ solution	0.2 g in 10 ml H_2O
10-Vitamin solution (5-times concentrated)	2 ml
Glucose solution (0,5 M)	0.2 ml
Na-Thiosulphate (1 M)	1 ml

Keep an eye on working carefully <u>and</u> aseptically!

It is not necessary to check/adjust the pH of the finished medium.

Pouring the plates
Before the medium is mixed with liquid agar (4 %, at least 5-times washed), it is preheated to approximately 50 °C (water bath). After adding the agar, the medium is thoroughly mixed (no cords should be visible) before pouring the plates under sterile conditions using a clean bench.

Oxic medium and agar plates

Oxic medium

The components of the medium (Table 7) are weighed into 400 ml distilled H_2O using a 1 litre cylinder and dissolved under stirring (magnetic stirrer). The provided solutions are added using a sterile graduated pipette or one-way syringes/-cannulae.

Table 7: Oxic medium for the cultivation of marine aerobes.

Substances	Net weight per litre	Sterile solutions	Supply per litre
NaCl	24.32 g	Trace elements solution SL 10	1 ml
$MgCl_2 * 6 H_2O$	10 g	Tungsten/Selenite-solution	1 ml
$CaCl_2 * 2 H_2O$	1.5 g	KBr (0.84 M)	1 ml
KCl	0.66 g	H_3BO_3 (0.4 M)	1 ml
Na_2SO_4	4 g	$SrCl_2$ (0.15 M)	1 ml
HEPES	2.38 g	NaF (0.07 M)	1 ml
Glucose	5 g		

The medium used for the plates is filled up to 700 ml using distilled H_2O. The medium used for the liquid cultures is filled up to 1 l. The media is transferred into 1 l Schott-bottles. The pH of the medium is adjusted to 7.2 – 7.4 using NaOH. The media is autoclaved for 25 min at 121°C.

After cooling down, the following substances are added:

Ammonium-/Phosphate-solution	10 ml
NaHCO3 solution	0.2 g in 10 ml H_2O
10-Vitamins solution (5-times concentrated)	2 ml

The pH of the medium is checked and if necessary adjusted under sterile solutions.

Casting the plates

The agar solution (4% → 12 g in 300 ml distilled H_2O, at least 5-times washed) is autoclaved for 15 min at 121°C and kept liquid using a water bath (90°C). The medium is preheated at 60°C using another water bath. Bubble free agar solution is mixed with medium (Don`t shake! No cords!). After cooling down, the medium is transferred into sterile Erlenmeyer-flasks and the plates can be poured (sterile conditions, clean bench).

Agar dilution series

Needed material/equipment

- per series, 7 properly labelled, sterile test tubes and suitable rubber stoppers (keep some backup stoppers ready)
- 5-times washed, sterile agar (4%)
- water bath preheated to 42 °C
- water bath preheated to 65 °C
- water bath (icy water)
- 2 Bunsen burner
- 1 50 ml bottle (completed medium)
- sterile 1ml, 10ml pipettes

Procedure

The agar is liquidated and kept at 65 °C. 3 ml of agar are added to the single test tubes, which are kept at 42 °C. Next, 6 ml completed medium are added to each test tube. One series of test tubes is removed from the water bath and placed in a rack at room temperature. From the culture of interest, one drop (~ 0.5 ml) is added to the first test tube (slew the tube slightly). One drop is transferred (~ 0.5 ml) from the first tube to the second one. The first tube is placed cold water bath (icy water) and so on. Before each transfer, the corresponding tube is wiped to prevent water drops from being transferred (unwanted dilution). Before incubating the tubes at designated temperatures, N_2/CO_2 (80/20, v/v) is introduced.

Obtained single colonies can be collected for microscopic investigation and for getting pure cultures using a 1ml syringe and a proper cannula.

2. Analysis of nitrogen compounds

Detection of ammonium (Chaney and Marbach, 1962)

Solutions

A) Dissolve 3 g Phenol + 3 mg Na-nitroprusside (Na-pentacyanonitrosylferrate (III) in 100 ml distilled H_2O (solution can be stored in the fridge for two weeks).

B) Dissolve 2 g NaOH in 80 ml distilled H_2O, allow the solution to cool down and add 0.5 ml NaClO (13%). Fill up with distilled H_2O to a final volume of 100 ml.

SAFETY ADVICES

Phenol (C_6H_6O): toxic, corrosive (R: 23/24/25-34-48/20/21/22-68 S: (1/2)-24/25-26-28-36/37/39-45)
Na-nitroprusside ($Na_2[Fe(CN)_5NO]$): toxic (R: 20/21-25 S: 9-28-36/37/39-45)
Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)
Sodium hypochlorite (NaClO): corrosive, harmful to the environment (R: 31-34-50 S: (1/2)-28-45-50-61)

Practical advices

In the context of analysing nitrogen compounds it is important to prepare blanks for comparison. Calibration curves should be generated with standards lying in the expected concentration range. Keep an eye on clean glass ware and possible precipitates. All used reagents have to be disposed

Procedure

1 ml of solution A is added to 10 ml of sample and mixed. Next, 1 ml of solution B is added and the mixture is again blended. Subsequently, the preparation is incubated for one hour at room temperature, in the dark. The extinction is measured at 635 nm against an ammonium-free blank. Possible precipitates can be removed by centrifugation prior to measuring the extinction. The assay is very sensitive, used glass vessels need to be very clean. Calibration curves are generated based on (NH4)2SO4 using standards with concentrations ranging from 0 to 100 µM.

The principle behind the assay is depicted in Figure 2.

Detection of nitrite

Solutions

A) 1.65 g sulfanilic acid is dissolved in 375 ml of hot water. 125 ml glacial acetic acid is added.

B) 0.5 g a-naphtylamine is suspended in 100 ml of water and 125 ml glacial acetic acid is added. The mixture is stirred until a clear solution is gained. The solution is filled up with water to a final volume of 500 ml. Prevent skin contact and spilling. Use a pipettor and rinse used vessels/pipettes thoroughly.

SAFETY ADVICES

Sulfanilic acid ($C_6H_7NO_3S$): irritant (R: 36/38-43 S: (2)-24-37)
a-naphtylamine ($C_{10}H_9N$): harmful to health and environment (R: 22-51/53 S: (2)-24-61)
Potassium nitrite (KNO_2): toxic, harmful to the environment (R: 8-25-50 S: (1/2)-45-61)

Procedure

0.5 ml of sample is mixed with 0.5 ml of solution A and 2.5 ml of solution B. The extinction is measured after 10 minutes at 530 nm. Calibration curves are generated based on KNO_2, using standards with concentrations ranging from 0 to 100 µM.

The principle behind the assay is depicted in Figure 2.

Detection of nitrate (Goltermann)

SAFETY ADVICES

Hydrazine sulphate ($H_6N_2O_4S$): toxic, harmful to the environment (R: 45-23/24/25-43-50/53 S: 53-45-60-61)
Hydrogen chloride (HCl): toxic, corrosive (R: 23-35 S: (1/2)-9-26-36/37/39-45)

Solutions
A) 1 M HCl

B) 1 M NaOH

C) Reducing agent
C1) 0.039 g $CuSO_4$ * 5 H_2O in 100 ml of distilled H_2O.
C2) 0.12 g hydrazine sulphate N_2H_4 * H_2SO_4 in 25 ml of distilled H_2O.

5 ml of solution C1 and 25 ml of solution C2 are mixed and filled up with distilled H_2O to a final volume of 50 ml. The completed reducing agent and solution C are not maintainable and have to be prepared daily.

Procedure
10 ml of sample (centrifuged, particle- and sulphide-free) (if necessary, remove sulphide using CO_2) are blended with 0.25 ml NaOH and 0.25 ml of solution C. The mixture is incubated for 30 minutes at 26-28 °C. Next, 0.25 ml acetone and after 5 minutes 0.25 ml HCl are added. Nitrite, which is yielded through reduction, is determined as already described (see 2. Analysis of nitrogen compounds, 'Detection of nitrite'). Calibration curves are generated based on KNO_3, using standards with concentrations ranging from 0 to 200 µM.

Principle of the reaction
Nitrate is reduced to nitrite. However, nitrite is not the only product yielded. Out of this reason, it is necessary to generate calibration curves based on nitrate. Calibration curves generated regarding nitrite are not sufficient.

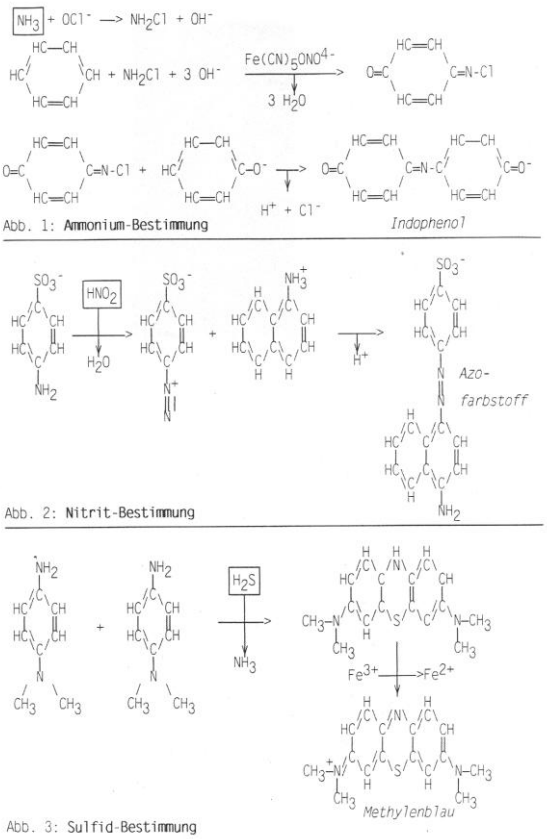


Figure 2: Reaction mechanisms with respect to some photometric assays.

Determination of ammonia/ ammonium (Solorzano, 1969)

There are several colorimetric methods available for determining NH_4^+ concentrations in water samples and soil extract. Here, we use the ‚indole blue method' detecting both NH_4^+ and NH_3. The method is based on the reaction of NH_3 in alkaline solution with phenate to produce a blue colour (indole blue) in the presence of a strong oxidizing agent, such as hypochlorite. The procedure described is in accordance to Solorzano (Limnol Oceanogr. 14, 799-801, 1969) and modified for water samples in the range of 0.01 to 2 mM of ammonia.

Notes:

Glassware should be very clean (rinsed with 0.1 M HCl, followed by rinsing with distilled water). Make sure that water used for preparing for reagents and standards is ammonia free.

Stock reagents

A) Phenol-alcohol reagent: 10 g phenol in 95 % Ethyl alcohol to a final volume of 100 ml (toxic!)

B) Dissolve 1 g Na-nitroprusside in 200 ml distilled H_2O. Store in the dark for not more than 1 month (toxic!)

C) Alkaline complexing agent: Dissolve 100 g trisodium-citrate and 5 g NaOH in distilled H_2O. To a final volume of 500 ml.

D) Oxidizing solution (prepare fresh daily): Mix 25 ml hypochlorite solution (15%) with 100 ml of solution C).

SAFETY ADVICES

Phenol (C_6H_6O): toxic, corrosive (R: 23/24/25-34-48/20/21/22-68 S: (1/2)-24/25-26-28-36/37/39-45)
Na-nitroprusside ($Na_2[Fe(CN)_5NO]$): toxic (R: 20/21-25 S: 9-28-36/37/39-45)
Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)
Sodium hypochlorite (NaClO): corrosive, harmful to the environment (R: 31-34-50 S: (1/2)-28-45-50-61)

Assay

Mix 2 ml sample (Porewater) with 0.08 ml of solution A, 0.08 ml of soultion B and 0.2 ml of solution D in a small, clean test tube. Carefully vortex the mixture. Incubate the mix for 3 hours in the dark, at room temperature. Vortex every hour during incubation. Read the absorbance using a spectrophotometer set to 630 nm.
Standards for calibration: 0-100 µM with $(NH_4)_2SO_4$ (Attention: 1 $(NH_4)_2SO_4$ = 2 x NH_4!)

Determination of nitrite

Reagents (toxic, carcinogenic)

Sulphanilamide solution (store in a dark bottle at 4°C): Add 150 of ortho phosphoric acid carefully to 700 ml of water. Add 10 g sulphanilamide, stir and warm up a little bit. Add 0.5 g naphthyl ethylenediamine dihydrochloride and fill up to 1000 ml.

SAFETY ADVICES

Phosphoric acid (H_3PO_4): toxic (R: 34 S: (1/2)-26-45)
Naphthyl ethylenediamine dihydrochloride ($C_{12}H_{14}N_2.2HCl$): irritant (R: 36/38)

Assay

Put 2400 µl sample into a glass test tube. Add 600 µl sulphanilamide solution and vortex. Measure the extinction at 545 nm against water.

3. Analysis of sulphur and phosphate compounds

Colorimetric determination of sulphide (Cline, 1969)

Reagent
1g N, N-dimethyl-phenyl diammonium dichloride (DMPD) and 1.5 g $FeCl_3$ * 6 H_2O are dissolved in HCl (25 %). The solution is filled up to a final volume of 50 ml using HCl (25 %). **Attention! Reagent is strongly corrosive and carcinogenic.** Prevent skin contact, always use a pipettor and pay attention to adhesive remains. Rinse used vessels and pipettes as soon as possible.

SAFETY ADVICES

Ferric chloride ($FeCl_3$): harmful (R: 22-38-41 S: 26-39)
Sodium sulphide (Na_2S): toxic, corrosive, harmful to the environment (R: 22-24-31-34-50 S: (1/2)-26-36/37/39-45-61)

Procedure
0.4 ml of reagent is added to 15 ml screw-cap tubes. Subsequently, 5 ml of sample are added, the tubes are closed and thoroughly mixed. Wait at least 20 minutes before measuring the extinction at 670 nm. If measured values are higher than one, samples have to be diluted.

Calibration curve
A Na_2S solution is used as standard. In a volurimetric flask, a NaOH pellet is dissolved in 500 ml H_2O and the solution is flushed with N_2 for at least 20 minutes using a cannula. Subsequently, a precisely weighed amount of 0.6 g Na_2S * 9 H_2O, which was washed using distilled water and dried, is added to the volurimetric flask. The flask is closed with a rubber stopper. The prepared stock solution contains approximately 5 mM sulphide (= 5 nmol/µl using 0.6 g Na_2S * 9 H_2O) and is stable for 1 day if kept under nitrogen. For generating calibration curves, 5 ml H_2O are placed in 12 test tubes that are closed using rubber stoppers and flushed with nitrogen for 20 minutes. Using a Hamilton syringe, 0, 2, 5, 10, 20 and 50 µl (0 – 250 nM, each in duplicate) of the stock solution are added. Mix and directly add 0.4 ml of the reagent using a 1 ml syringe. After 20 minutes, the extinction is measured at 670 nm against a sulphide-free blank. In order to calculate the concentration of the Na_2S solution, the molecular weight of Na_2S * 9 H_2O is used. For very precise measurements, it is necessary to add an aliquot of the Na_2S solution to an exactly adjusted acidic J-KJ solution. Next, the excessive iodine is titrated using a $Na_2S_2O_3$ solution.

The principle behind the assay is depicted in Figure 2.

Determination of dissolved sulphide (Ruwisch, 1985)

Sulphide is the final product of dissimilatory sulphate reduction. The presence of dissolved sulphide in cultures can be rapidly proven by its colloidal precipitation as CuS in a copper sulphate reagent, and quantified photometrically.

Copper reagent: HCl (50 mM), $CuSO_4$ (5 mM)
Chemical reaction: $CuSO_4 + H_2S \rightarrow CuS + H_2SO_4$

SAFETY ADVICES

Copper sulphate($CuSO_4$): harmful, harmful to the environment (R: 22-36/38-50/53 S: (2)-22-60-61)
Hydrogen sulphide (H_2S): very toxic, highly flammable, harmful to the environment (R: 12-26-50 S: (1/2)-9-16-36-38-45-61)
Sulphuric acid (H_2SO_4): corrosive (R: 35 S: (1/2)-26-30-45)

Procedure
Remove 0.2 ml of culture from the culture vessel using a syringe. Inject 0.1 ml culture free of gas bubbles into 4 ml of copper reagent (dispensed into glass tubes). Vortex and transfer the solution into a cuvette. The absorbance is immediately measured at 480 nm using a photometer (the colloidal CuS solution remains stable for 20-40s). Copper reagent free of sulphide serves as blank.

Sulphide standard preparation
Washed crystals of $Na_2S \cdot 9H_20$ (~13 g) are dissolved in 50 ml anoxic water to serve as a stock solution (~1M;

the final concentration should be determined via titration). An anoxic dilution series of dissolved sulphide is prepared in the desired range (0-30mM). An aliquot of the stock solution is anaerobically transferred into a Hungate tube containing anoxic water. After shaking, an aliquot of this mixture transferred into a second tube and so on. Calibration curves should be linear up to an absorbance of 0.5.

Turbidometric determination of inorganic sulphate (Tabatabai, 1974)

Reagents

A) Dissolve 10 g citric acid * H_2O in 80 ml distilled water. Mix the solution with 120 ml of glycerine (95 %)

B) Dissolve 0.5 g $BaCl_2$ * 2 H_2O and 5 g citric acid * H_2O in 50 ml distilled water.

SAFETY ADVICES

Citricacid ($C_6H_8O_7$): irritant (R: 36 S: 26)
Barium chloride ($BaCl_2$): toxic (R: 20-25 S: (1/2)-45)

Procedure

2 ml of sample (centrifuged if necessary) are mixed with 2 ml of reagent A (keep an eye on cords). 0.5 ml of solution B is added and the mixture is thoroughly blended to prevent cords. After 30 – 45 minutes, the mixture is blended again and the extinction is measured at 436 nm against a sulphate-free control. To improve the validity of the measurements, always measure in duplicate/triplicate using different diluted samples. Always measure standards covering the expected concentration range along with the test series.

Calibration curve

Using standards ranging from 0.1 - 5.0 µmol sulphate. In order to generate depth profiles, sulphide- and particle-free water samples (1 l) are used.

Principle

Citric acid acidifies the preparation and complexes the Ba^{2+} ions. Crystals offering a high optical density arise based on the complexed ions and sulphate. Glycerine slows down the sedimentation of yielded crystals.

Sulphate – rapid test

Procedure

1 ml of culture is acidified with 2 drops of HCl and shaken. 2 drops of $BaCl_2$ are added. Immediately observed turbidity indicates sulphate, a slowly establishing turbidity suggests thiosulphate (=> sulphur).

SAFETY ADVICES

Barium chloride ($BaCl_2$): toxic (R: 20-25 S: (1/2)-45)
Hydrogen chloride (HCl): toxic, corrosive (R: 23-35 S: (1/2)-9-26-36/37/39-45)

Photometric analysis of thionates (Kelly et. al., 1969; Fitz and Cypionka, 1990)

The photometric analysis of thiosulphate, trithionate and tetrathionate relies on the alkaline cyanolysis of thionates ending up in thiocyanate equivalents. The differentiation between individual thionates is possible by performing the cyanolysis at different temperatures and using CuSO4 as catalyst. Using iron (III), obtained thiocyanates can be quantified photometrically.

(0°C) I $S_4O_6^{2-} + 3\ CN^- + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + 2\ HCN + SCN^-$

(0°C, CuSO4) II $S_2O_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^-$

(100°C, CuSO4) III $S_3O_6^{2-} + 3\ CN^- + H_2O \rightarrow SO_3^{2-} + SO_4^{2-} + 2\ HCN + SCN^-$

Only the concentration of tetrathionate is determined by measuring preparation I. Measuring preparation II allows the determination of tetrathionate, thiosulphate yielded from cyanolysis and thiosulphate already present in the preparation. Preparation III identifies all present thionates.

Solutions

1.) NaH_2PO_4 - NaOH - buffer 1 M, pH 7.4

2.) KCN 1.25 M

3.) $CuSO_4 * 5\ H_2O$ 0.375 M

4.) $Fe(NO_3)_3 * H_2O$ 1.5 M
dissolved in 4 M $HClO_4$, increased volume due to dissolving!
=> 100 ml $HClO_4$ + 92 g $Fe(NO_3)_3$ => 150 ml total volume

SAFETY ADVICES

Potassium cyanide (KCN): very toxic, harmful to the environment (R: 26/27/28-32-50/53 S: (1/2)-7-28-29-45-60-61)
Copper sulphate($CuSO_4$): harmful, harmful to the environment (R: 22-36/38-50/53 S: (2)-22-60-61)
Ferric nitrate ($Fe(NO_3)_3$): oxidising, irritant (R: 8-36/38 S: 26)

As standards, 1 mM solutions of thiosulphate, tetrathionate and trithionate are prepared daily.

Procedure

0.06 ml of solution 1.) are placed in each test tube. Up to 2.25 ml of sample can be added. Distilled water is used to fill up the preparations to a final volume of 2.31 ml. A standard is prepared as mixture of all three thionates. For each preparation, a blank is set up. 3 Preparations (I, II, III).

Preparation I

Preparation I is cooled down to 0 °C for 10 minutes before 0.06 ml solution 2.) and 0.06 distilled water are added (mix!). The preparation is placed in a water bath for 20 minutes (icy water).

Preparation II

Prepartion II is cooled down to = °C for 10 minutes, before 0.06 ml of solution 2.) are added (mix!). After incubating for 10 minutes, 0.06 ml of solution 3.) are added (mix!), the preparation is kept for additonal 10 minutes in the water bath (icy water).

Preparation III

After adding 0.06 ml of solution 2.), the preparation is cooked for 45 minutes (water bath). The test tubes are closed using glass marbles. Subsequently, the test tubes are cooled down to 0 °C (~ 10 minutes) before 0.06 ml of solution 3.) are added (mix!). The preparations are kept in the water bath for additional 10-15 minutes (icy water). Finally, 1 ml of solution 4.) is added (mix!). As soon as the test tubes have reached room temperature, the extinction is measured at 460 nm. According to given reaction equations the concentrations of thionates are determined as follows.

Concentration tetrathionate: preparation I
Concentration of thiosulphate: preparation II - 2 x preparation I
Concentration of trithionate: preparation III – preparation II

Colorimetric determination of sulphite (Pachmayr, 1960)

Solutions
A) decolourised fuchsine solution

400 mg fuchsine is dissolved in distilled water and 125 ml concentrated sulphuric acid. Distilled water is used to fill up the solution to a final volume of 1 l.

B) formaldehyde (32 %)

SAFETY ADVICES

Fuchsine ($C_{20}H_{19}N_3$ x HCl): harmful (R: 40
S: 36/37)
Sulphuric acid (H_2SO_4): corrosive (R: 35 S: (1/2)-26-30-45
Formaldehyde (CH_2O): toxic (R:

Procedure
Using distilled water, the sample is filled up to a volume of 8.9 ml. Next, 1 ml of reagent A and 0.1 ml of reagent B are added (mix!). After 10 minutes, the extinction is measured at 570 nm against a sulphite-free preparation.

Colorimetric determination of sulphur (Chan and Suzuki, 1993)

Solutions
(1) 10 ml distilled water + 190 ml acetone
(2) 0.2 g NaCN + 125 ml of solution (1)
(3) 0.4 g $FeCl_3$ * 6 H_2O + 5 ml distilled water
(4) acetone
(5) petroleum ether
(6) 6.4 mg S° in 10 ml DMSO (final concentration: 20 mM)
(7) 3.2 mg S° in 10 ml petroleum ether

SAFETY ADVICES

Acetone (C_3H_6O): highly flammable, irritant (R: 11-36-66-67 S: (2)-9-16-26-46)
Sodium cyanide (NaCN): very toxic, harmful to the environment (R: 26/27/28-32-50/53 S: (1/2)-7-28-29-45-60-61)
Ferric chloride ($FeCl_3$): harmful (R: 22-38-41 S: 26-39)
Petroleum ether: highly flammable, harmful, harmful to the environment (R: 11-38-65-67-51/53 S: 9-16-29-33-60-61-62)

Procedure
- prepare a S°standard series using solution (6) (white precipitate) and buffer ranging from 5-1000 µM, 0 µM = blank
- extraction: 0.5 ml bacterial suspension/standard + 1.0 ml of solution (5) in Eppendorf tubes
- mix for 30 seconds
- centrifugation: 14 000 rpm, 10 minutes (Eppendorf centrifuge), supernatant becomes clear
- preparation: 0.5 ml supernatant + 1.0 ml of solution (2) in Eppendorf tubes
- mix and allow reaction to occure (2 minutes)
- measurement: 0.95 ml solution (4)
  + 0.05 ml solution (3)
  + 0.50 ml sample in Eppendorf tubes

- mix, a brownish precipitate develops
- centrifugation: 14 000 rpm, 10 minutes (Eppendorf centrifuge)
- measure extinction at 464 nm

Photometric determination of orthophosphate

SAFETY ADVICES

Sulphuric acid (H_2SO_4): corrosive (R: 35 S: (1/2)-26-30-45
Sulfamic acid (H_2NSO_3H): irritant (R: 36/38-52/53 S: (2)-26-28-61)

Reagents
A) molybdenum sulphuric acid reagent:
14.4 ml of concentrated H_2SO_4 (d = 1.84) are dissolved in 30 ml of distilled water. After cooling down, the following ingredients are added:

1 g sulfamic acid in 10 ml distilled H_2O;
1.25 g $(NH_4)_6\ MO_7O_{24}$ * 4 H_2O in 20 ml distilled H_2O;
34.4 mg antimony potassium tartrate in 10 ml distilled H_2O
distilled water is used to fill up the solution to a final volume of 100 ml

B) 1.0 g ascorbic acid in 10 ml distilled H_2O, prepare daily anew

Procedure
10 ml of a filtered water sample are mixed with 0.4 ml of reagent A and 0.25 ml of reagent B. After at least 10 minutes, the extinction is measured at 865 nm in 1cm cuvettes against water.

Calibration curve
Using KH_2PO, standards are prepared ranging from 0.2 - 40 µmol/l. For comparison, also prepare standards with concentrations of 400 and 4000 µmol/l.

Principle
Molybdenum within the formed molybdenum posphoric acid is reduced to Mo (+IV) by ascorbinic acid as reducing agent, which which forms together with the remaining Mo (+VI) a blue compound consisting of different valency stages.

Photometric determination of sulphide in cultures of sulphate-reducing bacteria (Cord-Ruwisch, 1985)

This quick method allows the quantitative analysis of dissolved sulphide in just a few minutes. The presence of sulphide can be measured based on the rapid formation of colloidal precipitation as CuS in a copper sulphate reagent. The precipitation can subsequently be determined by measuring the absorbance with a photometer at 480 nm.

SAFETY ADVICES

Copper sulphate($CuSO_4$): harmful, harmful to the environment (R: 22-36/38-50/53 S: (2)-22-60-61)
Hydrogen chloride (HCl): toxic, corrosive (R: 23-35 S: (1/2)-9-26-36/37/39-45)

Reagent
Add 1.25g of $CuSO_4$ and 6.51 ml HCl (25%, 4.4 ml of 37%) to water and fill up to 1000 ml**.**

Assay
Fill 4ml of the $CuSO_4$ reagent into small glass test tubes. For sampling the culture, take 0.3 ml of clear culture liquid (avoid sediment particles) with a nitrogen flushed 1 ml syringe. Release gas bubbles if necessary. Put the tip of the needle into the $CuSO_4$ reagent and inject 0.1 ml of the culture. Vortex shortly and tranfer the soltuion into a plastic cuvette. Measure the absorption at 480nm against the pure $CuSO_4$ solution.

Standards (1, 2, 5, 10, 20 mM) of sulphide solution must be prepared with anoxic water. For storage, the headspace of used vessels is flushed with nitrogen.

Analysis of sulphate by ion chromatography

Basic principle

The column for the anion chromatography consists of polystyrole/divinylbenzol carrier material with attached positive molecules. Anions in the sample bind to these positive molecules and are displaced by carbonate-ions which are provided by the eluent. This is repeated several times when the sample flows through the column. The frequency and the period of the absorption are specific for each anion, which results in different retention times.

To reduce the conductivity of the eluent (which enhances the sensitivity) the column is followed by a suppressor unit. In this unit cations are replaced by H^+ which bind to the strongly conductive carbonate-ions and form the less conductive carbonic acid. The suppressor column must be regenerated with sulphuric acid regularly.

The data is recorded by a thermic plotter, which shows peak height and area. The concentrations are determined by the peak area of several calibration standards.

Conditions

column: LCA A09
eluent: 5 $mmol \cdot l^{-1}$ Na_2CO_3; 5 % (w/v) EtOH
flow rate: 2 $ml \cdot min^{-1}$
temperature: 40°C

Eluent preparation

1.) Degassing of 950 ml dd H_2O
2.) Addition of Sodiumcarbonate and ethanol, swaying cautiously

Operation of the ion chromatograph

1.) Connection of the eluent to the pump. Pressure compensation of the eluent must be assured
2.) Degassing of the eluent inlet (Open the air escape valve of the pump head, purge)
3.) Before switching the eluent flow to the system, the suppressor column must be regenerated first.

Oherwise the valve of the suppressor unit does not enable the eluent to flow.
When the baseline stops drifting, the application of the samples can start.

Three standards (50 µM, 250 µM, 1000 µM) are used for the calibration. The calibration curve is not exactly linear. During the analysis the standards should be measured once in a while. For every charge of eluent the standards must be measured again. Different calibration curves are determined for the different concentration ranges.

Usually the suppressor unit must be exchanged after five or six samples. By switching the suppressor unit on the eluent flows to another suppressor column. When the baseline stops drifting, the application of the samples can start again.

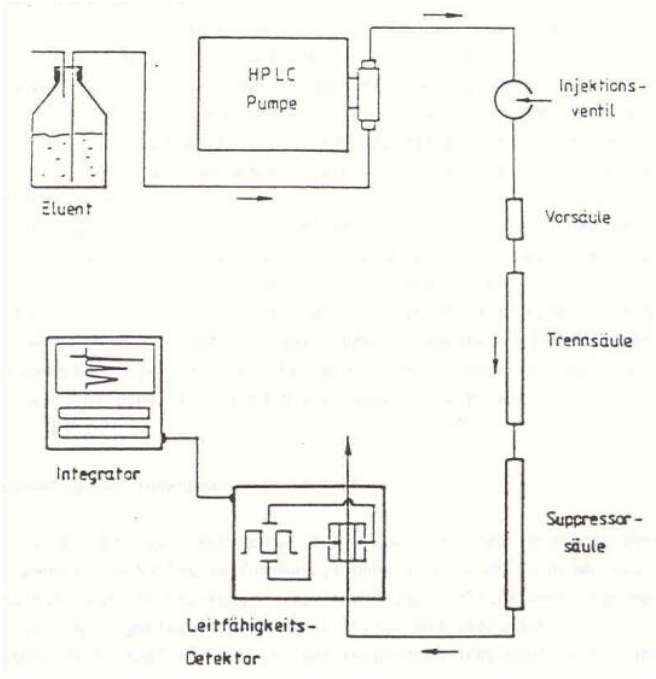


Figure 3: Schematic assembly of the ion chromatograph.

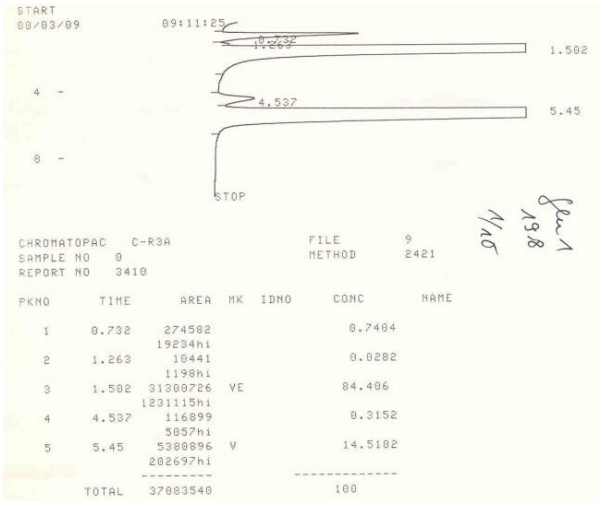


Figure 4: Chromatogram example for a bacterial culture. Peaks: chloride 1.5 min, phosphate 4.5 min, sulphate 5,4 min. The upper value of the data set offers the area and the lower one (hi) the peak height.

4. Determination of proteins and bacterial pigments

Lowry protein assay (1951)

Reagents

• copper reagent: Dissolve 0.1 g $CuSO_4$ * 5 H_2O in 20 ml K-Na-tartrate solution (1 %).
1 ml of the solution is mixed with 50 ml of Na_2CO_3 solution (2%). The solution is prepared daily anew.

• Folin's reagent: 1 moiety of Folin-C.-reagent (Merck) is mixed with 2 moieties of distilled water.

• NaOH: 0.3 M

SAFETY ADVICES

Copper sulphate($CuSO_4$): harmful, harmful to the environment (R: 22-36/38-50/53 S: (2)-22-60-61)
Sodium carbonate (Na_2CO_3): irritant (R: 36 S: (2)-22-26)
Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)

Practical advices

Calibration curves should be generated with standards lying in the expected concentration range. All used reagents have to disposed properly using provided containers. Running samples and standards in multiple parallels increases the validity of yielded data significantly.

Procedure

10 ml cell suspension are centrifuged in the refrigerated centrifuge (6000 g, 10 minutes) and washed with saline solution (0.6 %). The pellet is thoroughly resuspended in saline solution and is filled up to a volume of 10 ml. The protein content is determined in triplicate, using 1 ml of the suspension respectively. In order to digest cells, 0.5 ml of 0.3 M NaOH are added and the samples are incubated at 60 °C for 90 minutes (water bath). After cooling down, 5 ml of copper reagent are added while shaking. The samples are kept in the dark for 10 minutes, 0.5 ml of Folin's reagent are added (mix!) and the samples are kept for further 30 minutes in the dark. Subsequently, the samples are centrifuged (6000g, 10 minutes) and the extinction is measured at 623 nm against a blank. Serum albumine is used for preparing a standard series ranging from 10 – 200 g per preparation.

Determination of proteins according to Schmitt (1969)

(modified Biuret protein assay according to La Riviére, 1958)

Reagents

(A) NaOH	4 M (= 160 g/l)
(B) K-Na-tartrate	5 g
NaOH	4 g
$CuSO_4$ * 5 H_2O	1 g
KJ	2.5 g
in H_2O	400 ml

SAFETY ADVICES

Copper sulphate($CuSO_4$): harmful, harmful to the environment (R: 22-36/38-50/53 S: (2)-22-60-61)
Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)

Procedure

- 10 ml of cell suspension are washed with saline solution (0.9 %) and centrifuged, the supernatant is discarded
- resuspend pellet in 5 ml saline solution (0.9 %)
- add 0.5 ml of reagent A, mix
- cook samples for 10 minutes (water bath, seal test tubes with glass marbles)
- immediately cool down (icy water)
- add 2ml of reagent B, mix
- incubate for 30 minutes at 37 °C (water bath)
- if solutions become turbid, centrifuge down particles
- measure extinction at 546 nm
- generate a calibration curve using BSA standards(stock solution: 50 mg/ml) ranging from 0 bis 10 mg/preparation

Bradford protein assay (1976)

The assay is based on the binding of Coomassie Brilliant Blue G-250 to protein. When binding to protein occurs the absorbance maximum of the dye shifts from 465 nm to 595 nm. Therfore, absorbance can be measured photometrically at 595 nm. The assay is quick and reliable since a visible colour change occurs after 2 min and the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. Furthermore, both hydrophobic and ionic interactions stabilize the anionic form of the dye, i.e. there is no or neglible disturbance by natrium and kalium ions or carbohydrates like sugars. Disturbances are only known from concentrated detergent like Sodiumdodecylsulfate (SDS), Trition X-100 or commercially available solutions. Controls are recommended.

Bradford reagent

Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved. Final concentrations are 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% 8w/v) phosphoric acid. The Bradford reagent should be light brown/reddish in color. In case of blue components filtrate the solution using a round filter.

SAFETY ADVICES

Coomassie brilliant blue G-250 ($C_{47}H_{48}N_3NaO_7S_2$): S: 22-24/25
Phosphoric acid (H_3PO_4): corrosive (R: 34 S: (1/2)-26-45)

Standard procedure

1) Transfer up to 5 ml of homogenised growing culture into 15 ml- centrifuge tubes. Centrifuge for 15 min/4000rpm/4°C. Decant supernatant and freeze the pellet until the Bradford assay is carried out.
2) Preheat a water bath (100°C)
3) Add 500 µl bidestilled water and 500 µl NaOH (0.5 M) to the cell pellet and mix thoroughly.
4) Cook the suspension for 10 min at 100°C
5) Transfer 200 µl in three parallels into 1.5 ml Eppendorf reaction tubes. Mix with 800 µl of Bradford reagent.
6) Incubate for 30 min at room temperature.
7) Transfer the whole volume into a half-micro disposable cuvette.
8) Measure the absorbance at 595 nm using the photometer. Don’t forget blind controls.
9) Note all values and use the mean value for further calculations.

Calibration curve

A calibration curve is made from bovine serum albumin (BSA). A stock solution (1mg/ml) is diluted with bidistilled water in triplicates to prepare standard solutions with concentrations ranging from 0 to 8 µg protein/200µl.

Determination of carotinoides (Eichler and Pfennig, 1986)

a) Harvesting bacteria
Well-grown cultures are centrifuged (9000 rpm, 20 minutes); the supernatant is carefully removed and discarded.

SAFETY ADVICES

Petroleum benzine: highly flammable, harmful, harmful to the environment (R: 11-38-65-67-51/53 S: 9-16-29-33-60-61-62)
Acetone (C_3H_6O): highly flammable, irritant (R: 11-36-66-67 S: (2)-9-16-26-46)

b) Extraction of carotinoides
Carotinoides are unstable if exposed to light and air!

Extraction reagent:ethanol (100 %) : acetone = 1:1

The pellet is resuspended in the remaining supernatant (if necessary add 0.2 ml distilled water) and transferred to a 10 ml centrifuge beaker. Next, 8 ml of extraction reagent are added (mix well), nitrogen is introduced and the vessel is thoroughly sealed using a butyl rubber stopper. Extraction is allowed to take place for approximately 1 hour at room temperature. Mix again and centrifuge down (15 min.) cell remnants, remove the stopper before. Concentrate the coloured supernatant at 25 °C using a rotary evaporator under a black rag. Resuspend the bottom in 0.75 ml extraction reagent. Transfer the solution into a tube, introduce nitrogen and seal the tube with a butyl rubber stopper. Store in the dark.

c) Thin layer chromatography

eluent: petroleum benzine : acetone = 9:1

The chromatography chamber is lined with blotting paper, nitrogen is introduced and the chamber is closed. 100 ml eluent are poured into the chamber and allowed to saturate for 1 hour. The extract is placed to dry on the starting line of a silica gel-thin layer-plate under nitrogen exposure (place ~ 100 µl along 3 cm, besides 50 µl along 3 cm). Treat reference extracts the same. The plate is developed in a shaded chamber. If the eluent has risen roughly 16 cm (mark with a pencil), shortly dry the plate with nitrogen and develop the plate a second time. Dry the plate. Mark bands on the dried plate carefully using a spatula, cover the plate with a glass plate and copy the pattern rapidly to a transparent foil and paper. Note down the colours of the bands and identify reference organisms based on the obtained pattern.

Determination of chlorophylls (Oelze, 1985; Steenbergen and Korthals, 1982)

2 - 5 ml of a bacterial culture are filtered out using glass fibre (f 25 mm) or membrane filters (pore size: 0.2 µm, f 25 mm) if bacterial cells are tiny. After transferring the cells to 3 ml of acetone, chlorophylls are extracted over night in the dark at 4 °C. The extinction is measured against pure extraction reagent at 771 nm (Bchl a), 663 nm (Bchl c), 652 nm (Bchl d) and 647 nm (Bchl e). The pigment concentration is calculated according to the Lambert-Beer law.

$$E = c * d * \varepsilon$$

E = extinction at absorbance maximum
c = pigment concentration
d = width of cuvette (1 cm)
ε = extinction coefficient at absorbance maximum for:

Bchl a = 92.3 ml * mg^{-1} * cm^{-1}
Bchl c = 92.6 ml * mg^{-1} * cm^{-1}
Bchl d = 98.0 ml * mg^{-1} * cm^{-1} (same for Bchl e)

5. Microscopy and cell counts

Cell number determination of (MPN) dilution series

For the quantification of viable cell, a MPN dilution series with appropriate medium is prepared in three parallels. For this purpose, the culture is diluted stepwise 1: 10 and incubated at least for one week. The number of wells or tubes that show microbial growth can be correlated to the MPN-index which refers to guideline values.

Table 8: MPN index.

number of positive tubes with			MPN-Index cells / ml	confidence interval (95%) upper	lower
100 µl	10 µl	1 µl			
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

Quantification of MPN dilution series with SybrGreen I

Practical advices

Generally, prevent fluorescent dyes from being exposed to light. The exposition to light favours fading an decreases the signal intensity.

Preparation of staining-solution

The SybrGreenI stock solution (10.000 x) is diluted in TAE buffer (pH 7.4) containing 1% of a ascorbic acid solution (1 M). The working solution is prepared freshly each day and must offer a five times higher concentration compared to the desired final assay concentration.

Quantification

200 µl sample from each well of the 2 ml 96-well plates and 50 µl SybrGreen I solution are transferred to black microtiter plates and mixed cautiously. Fluorescence is measured after 2 hours of incubation in the dark. Fluorescence intensities are determined in a microplate reader (Fluostar Optima) at 485 nm (excitation) and 520 nm (emission). All measurements are carried out in three reading cycles, with integration of 20 flashes, 0.5 s delay time between reading, and without shaking before each cycle.

Determination of bacterial counts and colony forming units (cfu) from water and sediment samples (Cavalli-Sforza, 1972)

Depth profiles (sediment surface, tidewaywater and other depth layers) of living cell counts (aerobes and anaerobes) are generated. For the determination of living cell counts, dilution series of tidewaywater and sediment samples are generated using artificial seawater (Tidewaywater: 1:10, 1:100, sediment: 1.100, 1:1000, 1:100000) and 100 µl are transferred and spread on agar plates (see figure). Every dilution factor is made up in duplicate. One is incubated under oxic, one under anoxic conditions. The progress of colony formation is recorded daily. Therefore, the colonies are counted, distinguished macroscopically as well as microscopically as colony forming units and specified morphologically.

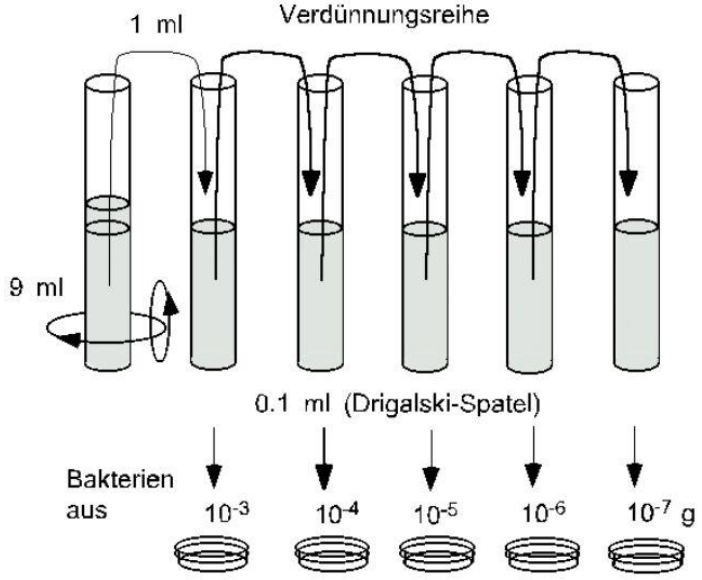


Figure 5: Preparation of dilution series.

The living cell counts will be determined using the following equation :

$$x = \frac{\Sigma_i C_i}{\Sigma_i (n_i * z_i)}$$

x = average count of colony forming units in the volume of the inoculum

C_1, C_2, ... , C_i = number of counted colonies on the plates

n_1, n_2, ... , n_i = plates/dilution series

z_1, z_2, ... , z_i = dilution factors

Example:

Dilution: 10^{-6}: 303, 290, 285 colonies

10^{-7} : 32, 21 colonies

Average cell number $x = 931/(3*10^{-6} + 2*10^{-7}) = 2.91*10^{8}$

Preparation of slides coated with agarose for microscopy (Pfennig and Wagener, 1986; modified after Cypionka)

100ml of an agarose solution (2%, w/v) are heated – prepared in a Schott flask with blue cap – in the microwave. The solution should be free from cords. A preheated water bath (~ 45°C) is used to keep the solution liquid. In order to get a smooth agarose film, clean microscopic slides are preheated (free from fluff) on a clean flat underground by using infrared light. 2 ml of agarose solution are dispensed in zigzag lines onto one slide by using a clean glass pipette. Avoid drainage of the agarose solution.
Before usage, the agarose slides are air dried for a few days. The slides can be kept in closed boxes for some time.

Preparing microsope slides for observation
First of all, cell concentrations in cultures should be high enough for photography. Otherwise, a centrifuge should be used to concentrate the cells. Three drops of ~20, 22 and 25 µl are transferred onto an agarose-coated slide by using a micropipette (0.1 – 0.2 ml). Each drop is covered immediately with a cover slip (18x18 mm). Normally, the liquid part of the cell suspension is soaked into the agarose, while the bacterial cells are arranged on the top.
In some cases it is necessary to prevent the cell suspension from being evaporated. A paraffin solution is used to seal all sides of the cover slip by using a warm spatula.

Don't forget to take some nice pictures of your cells! It is worth it.

See also "How to get the perfect photomicrograph" of Heribert Cypionka
(http://www.icbm.de/pmbio/lehre/ws0708/ringvl/digitalemikrofotos.pdf)

Determination of gram type – Gram differentiation (Bartholomew, 1962; Gregersen, 1978)

Gram-negative bacteria do not retain the initial crystal violet stain. They are decolourised by the organic solvent and hence show a pink/red counterstain. Gram-positive bacteria instead retain the violet dye. This difference basically lies in the cell wall structure of the bacteria.

a) Gram staining
1) Plate freshly grown cells (not older than 24 h) onto a clean object slide or cover slip and allow the film to air dry. Fix the dried film by passing it quickly through the Bunsen flame (‚heat fixation'; do not pyrolyse the cells!). Two reference strains, one gram-negative, and one gram-positive strain are treated the same way.

2) Stain the fixed cells for up to 1 min with Huckers' ammonium oxalate-crystal violet reagent.
3) Drain the staining solution. Wash off briefly with water for 5 sec. Drain.
4) Flood the slide with Gram's Iodine solution for ca. 1 minute. Wash off with water. Drain.
5) Drain staining solution. Wash off briefly with water for 5 sec. Remove excess water.
6) Counterstain: 3 x 30 sec each in 3 drops of n-propanol.
7) Wash off with water for 5 sec.
8) Check quality using a microscope.
9) Flood slide with safranin solution and allow to counterstain for 30 seconds. Drain counterstain solution and wash off with water. Remove excess water. Dry.
10) Find dry and stained bacteria under 10x-40x lenses. Then examine the bacteria using oil immersion but no cover glass.
11) Compare your results with the gram test of gram-negative and gram-positive reference strains.

SAFETY ADVICES

Ammonium oxalate crystal violet: irritant, harmful
Iodine solution: irritant, corrosive
Propanol (C_3H_8O): highly flammable, irritant (R: 11-41-67 S: (2)-7-16-24-26-39)
Safranin ($C_{20}H_{19}ClN_4$): irritant (R: 36/38 S: 24/25)
Potassium hydroxide (KOH): corrosive (R: 22-35 S: (1/2)-26-36/37/39-45)

b) KOH solution test

Put some drops of KOH solution (3%) onto a clean microscopic slide. Add cell material (colonies or cell pellets) by using an inoculating loop and mix for ~5 to 10 seconds. Carefully pull back the platine loop. Slimy filaments indicate gram negative cells. In comparison, treating gram positive cells with KOH does not end up in the formation of slimy threads.

Flagella staining (Ryu, 1937)

Based on a bacterial cell suspension, a microscopic specimen is prepared. As soon as the majourity of cells is fixed, staining solution is added to the preparation. After allowing the staining to take place (5-15 minutes), the flagella should be visible.

SAFETY ADVICES

Phenol (C_6H_6O): toxic, corrosive (R: 23/24/25-34-48/20/21/22-68 S: (1/2)-24/25-26-28-36/37/39-45)
Crystal violet ($C_{25}H_{30}ClN_3$): harmful, harmful to the environment (R: 22-40-41-50/53 S: (2)-26-36/37/39-46-60-61)

Solution I:	phenol solution (5 %)	10 ml
	tannic acid	2 g
	$AlK(SO_4)_2$ * 12 H_2O, saturated solution	10 ml
Solution II:	saturated solution of crystal violet (12 g/100 ml MeOH)	

Staining solution: I : II = 10 : 1

Total cell count

Nowadays, a combination of epifluorescence microscopy and membrane filtration has established as standard method for the direct counting of microorganisms. Before or after filtration, microorganisms are stained using fluorescence dyes like acridine orange or DAPI (4´,6-Diamidino-2-phenyle indole). Acridine orange binds to the phosphate groups of nucleic acids. Stained bacteria fluoresce green, partially orange. Foreign particles appear red, orange or yellow. DAPI binds double-stranded DNA. Stained bacteria fluoresce pale blue. Foreign particles appear yellow. For filtration, membrane filters consisting of polycarbonate or aluminium oxide (Anodisc®) are used. The pore size should not exceed 0.2 µm.

Materials

- filtration top part (Millipore, 20 mm glass filter retainer)
- 0.2 µm Anodisc® filters (Whatman)

Reagents

- 0.5% TWEEN 80 (sterile-filtrated)
- 0.22 µm sterile-filtrated ddH_2O
- 0.22 µm sterile-filtrated PBS buffer (130 mM NaCl, 5 mM NaH_2PO_4; pH 7.2)
- fixative (4% paraformaldehyde + 0.1% Triton X100 in 1 x PBS; pH 7.25)
- DAPI solution (10 µg/ml; steril)
- mounting solution DABCO (25 mg diazabicyclo octane + 1 ml PBS + 9 ml glycerine)

SAFETY ADVICES

Paraformaldehyde ($(CH_2O)_n$): harmful (R: 20/22-36/37/38-40-43 S: 22-26-36/37)
Triton X 100 ($C_{14}H_{22}O(C_2H_4O)_n$): harmful (R: 22-41 S: 24-26-39)
DAPI ($C_{16}H_{15}N_5$): irritant, target organ damage
Diazabicyclo octane ($C_6H_{12}N_2$): harmful (R: 22-36/38 S: 22-26)

- **staining solution**

1ml fixative
+ 930 µl ddH_2O (sterile-filtrated)
+ 70 µl DAPI solution

Procedure

100 µl TWEEN 80 are added to sediment samples fixed with glutardialdehyde (6 ml, 3 %) and the samples are sonicated (5 x 5 seconds). 10 ml PBS buffer are placed in combusted test tubes. Samples are vortexed and 10 µl are transferred to the buffer. Solutions were vortexed immediately before performing the filtration using Anodisc® membrane filters and underlaid glass fibre filters. Test tubes and the filtration tower were rinsed using sterile-filtrated water. The membrane was dry-filtered and carefully removed. Between every filtration, the filtration tower was thoroughly cleaned using ddH_2O.
DAPI solution was made up in a small weighing dish. The filter was applied to the staining solution with the sample featuring side facing upwards and stained for five minutes in the dark. The back of the filter was stained in the dark using kitchen roll paper. The filter was embedded on a microscopic slide using DABCO. A cover slip was applied and the filter was enumerated.

Determination of the total cell count with SybrGreen I (Lunau et. al., 2004)

Dissolution of the cells with methanol and staining with SybrGreen I

SAFETY ADVICES

Glutardialdehyde ($C_5H_8O_2$): toxic, harmful to the environment (R: 23/25-34-42/43-50 S: (1/2)-26-36/37/39-45-61)

To break the particles in the initial sample apart and to remove adherent bacteria, the samples are treated with 100 % methanol in 1.5 ml Eppendorf® reaction tubes (final concentration 10 % [v/v]). Afterwards, samples are sonicated for 15 minutes at 35° C in the ultrasonic bath. To remove detrital and inorganic particles, the samples are centrifuged for 1 minute with 2.000 rpm. 500 – 1.000 µl of the supernatant are filtrated on a black 0.2 µm polycarbonate filter and stained with SybrGreen I. In case of sediment samples, the supernatant is diluted 1:100 with TAE buffer before filtration. The filter is triply flushed in each case with 1 ml TAE buffer and transferred to a microscopic slide. 6 µl staining- and mounting solution are dropped in the centre of a cover slip (18 mm x 18 mm). The cover slip is brought to the object slide with the staining solution towards the filter. To distribute the staining solution equally on the filter, you can carefully press with tweezers onto the cover slip. After an "incubation time" of 15 to 30 minutes at 4° C (refrigerator) the total cell count is determined via epifluorescence microscopy. The storage of specimens at -20° C allows the determination of the cell count also a few months after staining.

Alternativ

Take 1cm^3 sediment by a 2ml syringe and extrude it into a sterile 15ml centrifuge tube (Roth) containing 9 ml Fix Mix (samples can be stored 4°C 2weeks)
Mix it and put 1 ml of this mixture into an 2ml- Eppendorf tube
Treat the samples 15 min 35°C with ultrasonication and mix again.
Dilute 5-100µl of this solution (the supernatant) in 10 ml TAE
Vaccumfilter this solution on 0.2µm polycarbonatefilter,black(Whatman)and rinse 3 times with 3 ml TAE-puffer

alternatively 20 µl of the supernatant is distributed equally on a slide, that is placed on a 20*20 mm square-template
8 µl SybrGreen staining solution is given on a cover slip and the dried filter (alternative: the square-sample) is covered with it

Preparation of the mounting medium
In a 25 ml polypropylene tube ("Falcontube") 2.4 g of polyvinylalcohol 4-88 (moviol 4-88, Fluka, Switzerland) are added to 6 g of glycerol and vigorously mixed at room temperature. Moviol does not dissolve completely. Thereafter, 6 ml double-distilled water is added and the solution is stirred for two more hours (at room temperature). Some of the moviol still remains undissolved. By adding 14 ml of TAE buffer and mixing for another 2h at 50° C moviol is completely dissolved. Finally, the solution is filtered through a 0.2 µm Nalgene® filter and aliquots of 200 – 1.000 µl are stored frozen at -20° C.

Preparation of the staining solution
The mounting medium is added to the SybrGreen I stock solution The dilution depends on the sample fixation procedure. Samples which are fixed with formalin are diluted 1:15, and those that such are fixed with glutardialdehyde are diluted 1:200. In addition, a freshly prepared 1 M ascorbic acid solution, dissolved in TAE buffer, (pH 7.4) is added at a final concentration of 1 % as an antioxidant. The staining solution can be kept at 4° C for several weeks (do not freeze!).

Fix Mix
63ml destilled water
30ml Methanol
2ml GDA(Glutardialdehyde(25%))
5ml Tween 80(0.5%)
The solution is filtered (0.2µm,Nalgene)

Total cell counting

A minimum of 30 squares must be counted, each with 30 – 50 cells

a) Determination of cell number / ml „Prilwasser”

$$\textbf{cell number/ml} = y * (A/a) / V$$

y:= mean cell number per square

A:= filtration area (314,16 mm^2 , d = 20 mm)

a:= square area (0,01 mm^2)

A/a := total cell numbers of the squares; y*(A/a) := number of cells per filter

V:= filtration volume (ml)

b) Determination of cell number / g sediment

$$\textbf{cell number/g} = y * (A/a) / m_{analysed}$$

$m_{analysed}$:= analysed sediment weight (g) = weight (g) / dilution factor

c) Counting tool „Count Them“

Examine specimens (40 x magnification, largest field of vision), document by digital fotography and

quantify using the counting tool „Count them“ (introduction by H.Cypionka)

Total cell counts of water samples (Hobbie et. al., 1977)

2-5 ml of a water sample are mixed with ~ 200 µl of a particle free acridine orange solution (0.1 %) and stained for 2 minutes. Next, the solution is filtered through a Nuclepore® filter (pore size: 0.2 µm) and pre-stained using Irgalan black solution (0.1 %). As supporting filter, a cellulose membrane filter is used. The Nuclepore® filter is dried on a microscopic slide, fluorescence-free immersion oil is added and a cover slip applied. The filter is examined under the microscope. In general, per filter 10 squares are counted. Each square should offer 30-50 bacteria.

cell number/ml = (y • A)/(a • V)

y: mean cell number per square
A: filtration area (μm^2)
a: square area (μm^2)
V: filtration volume (ml)

Counting of growing cells using nalidixic acid (Kogure et. al., 1979)

Principle
Determining the total cell count, does not allow to draw conclusions regarding the physiological state of the cells. Out of this reason, the 'direct viable count' was developed. This method involves the addition of an antibiotic to a bacterial culture, which suppresses the doubling of DNA. Although bacteria can grow, they cannot divide to double. It is assumed, that viable cells are growing. The number of growing cells is taken as 'direct viable count'.

Reagents
Nalidixic acid: Dissolve 1 mg in 10 ml distilled water and filter the solution.

Yeast extract: Dissolve 10 g in 1 l of water and autoclave the solution.

SAFETY ADVICES

Nalidixic acid ($C_{12}H_{12}N_2O_3$): harmful (R: 42/43 S: 22-36/37-45

Procedure
10 ml of a water sample are spiked with sterile yeast extract to get a final concentration of 50 mg/l. Next, nalidixic acid is added to end up in a concentration of 20 µg/l. As controls, reference samples are set up once without yeast extract and nalidixic acid and once without nalidixic acid. Samples are incubated for 8-24 hours. Subsequently, samples are fixed using paraformaldehyde and the total cell counts are determined, as well as the number of non natural elongated cells.

If obtained cell numbers are low, cells should be concentrated through filtering them out using filters with a pore size of 0.2 µm.

Life-Dead-Staining (Boulos et. al., 1999)

Principle
SybrGreen I and propidium iodine are intercalating agents that fluoresce if being exposed to UV light (Figure 7). SybrGreen I enters all cells, while propidium iodine only enters dead cells due to its charge, since dead cells offer no intact cell membrane. Corresponding cells appear orange if being excited at 450-490 nm (Figure 7).

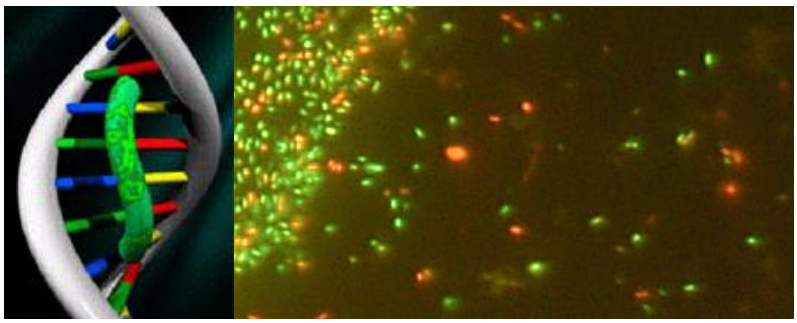


Figure 6: Intercalating substances. Left-hand site: incorporation, Right-hand side: Life-Dead-Staining

SAFETY ADVICES

Phenol diamine ($C_6H_8N_2$): toxic, harmful to the environmentl (R: 23/24/25-36-43-50/53 S: (1/2)-28-36/37-45-60-61

Reagents
all solutions are prepared in combusted Pfennig bottles (12 hours, 180 °C)

- 1 l Tris-HCl buffer 10 mM, pH 8, sterile-filtrated
- Tween 80, 0.5%, sterile-filtrated (only needed for examining sediment samples)

- SybrGreen I, 1:200 dissolved in Tris-HCl buffer

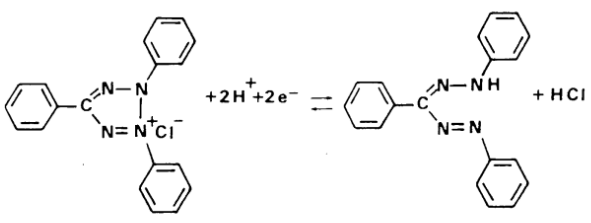
- propidium iodine, 1 mg/ml Tris-HCl buffer

- mounting solution: 1:1 mix of glycerine and Tris-HCl buffer, pH adjusted to ~ 8
  900 µl of this solution is mixed with 100 µl Phenol diamine, keep refrigerated

Procedure
1 g of sediment is suspended in 10 ml Tris-HCl buffer (in combusted gold head tubes) and 100 µl Tween 80 solution are added. The sample is sonicated five times for 30 seconds. 1 ml Tris-HCl buffer is placed in a sterile Eppendorf tube and mixed with 5 µl of sample (maximal 5 µl). Next, 10 µl SybrGreen I solution are added (mix!), before 15 µl propidium iodine solution are added (mix!). The sample is incubated for 10 minutes in the dark. Subsequently, the sample is filtered out on a polycarbonate filter (Anodisc®). Clean the filtration tower and place Tris-HCl buffer before adding the sample. After filtration, the filtration tower is rinsed again with Tris-HCl buffer. The filter is dried in the dark and is embedded on a microscopic slide. Preparations can be stored in the dark at room temperature or in the refrigerator. Filters are counted using the BP 450-490 filter set.

Counting of actively respiring cells

Principle
An artificial electron acceptor is lipophilic and membrane permeable due to hydrophobic side groups. Due to the positive charge of cells offering a membrane potential, the artificial electron acceptor is accumulated. Within the cells, the acceptor is transformed into its crystallised state due to electron transfers. Crystallised states can be coloured (e.g. TTC) or fluorescing (e.g. CTC).



TTC TPF

Abb. 15: Reduktion von Triphenyltetrazoliumchlorid (TTC) zur Triphenylformazan (TPF)

Figure 7: Reduction of triphenyl tetrazolium chloride.

Reagents
CTC: 5-Cyano-2,3-ditolyl tetrazolium chloride (50 mM): dissolve 011 g in 4,6 ml particle free distilled water. Store the solution in the dark at cool conditions. Attention: metabolic poison

SAFETY ADVICES

CTC: irritant, toxic

Procedure
1ml of bacterial cell suspension are mixed with 40 µl of CTC colution (final concentration, 2 mM). As control, a reference sample is killed using formaldehyde (final concentration, 2 %). CTC is added to the reference sample after 5 minutes. Samples are incubated for 4 hours at room temperature. In the following, samples are stained using DAPI, filtered and counted using the DAPI and Rhodamin filter set.

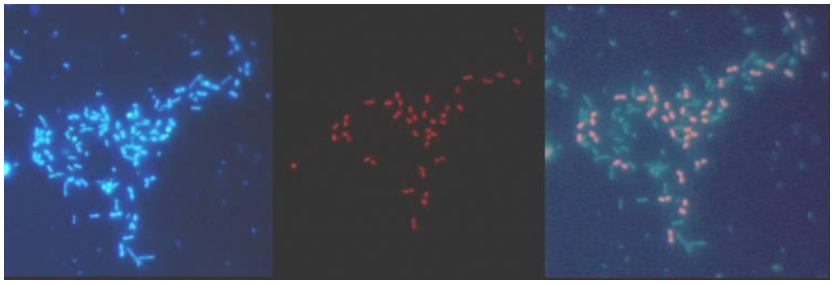


Figure 8: Determining actively respiring cells in a sediment sample taken from Bornhorster lake. Left hand side: DAPI, Middle: CTC, Right hand side: overlay

Fluorescence-in situ-Hybridisation (FISH) using rRNA probes to detect distinct phylogenetic groups

Introduction
Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has developed into an invaluable molecular tool in the late 80`s (Giovannoni *et al*., 1988; DeLong *et al*., 1998; Amann *et al*., 1990) and is now a well-established technique. It is a method for the rapid and specific *in situ* identification of prokaryotes (bacteria and archaea) on different phylogenetic levels independent of whether or not they can be cultured. FISH is a staining technique using fixed cells that are microscopically examined. The selectivity of staining is based on the particular organisation of the ribosomal RNA. Due to comparative sequence analyses, it is possible to identify scopes on the small subunit of the ribosomal RNA (16S or 18S rRNA) which are consistent on distinct phylogenetic levels (class up to domain). Gene sections with a length of 18 base pairs provide the target sequence for the oligonucleotides labelled with a fluorescence dye (e.g. Cy3).
Primarily, the cells of pure cultures or bacterial communities from environmental samples will be fixed on a microscope slide. Afterwards, cells are penetrated and entered by the oligonucleotide probe, which hybridizes with the ribosomal RNA target sequence. In addition, it is necessary to establish particular conditions for every single probe. After the hybridization, non specific probes are washed off and cells are labelled using a conventional dye like DAPI. Finally, samples are analysed by epifluorescence microscopy. The option of different microscopic filters enables the discrimination of non specifically (DAPI) and specifically (FISH) labelled cells. Anyway, some problems could occur. Frequently, cells from natural environmental samples possess a lower amount of ribosomes and therefore feature a lower degree of fluorescence. Also, interfering compounds of the sampling site that are characterized by their own fluorescence (e.g. sediment or precipitations) could have an disturbing effect on the hybridization. Further information can be gained from review articles (e.g. Amman & Ludwig, 2000).

Equipment
Microscopic slides, Epoxy-coated, 8 chambers, ∅ 6mm
Hybridisation chambers (Greiner tubes or falcon tubes)
Hybridisation oven, Water bath, Block thermostat
3 Staining chambers
Fluorescence microscope with DAPI- and Cy3-filter
Washing chambers (Greiner tubes or falcon tubes)

Chemicals and reagents
Oligonucleotide probes, Cy3 labelled
Fixed cells of pure cultures to test the specificity of the probes

SAFETY ADVICES

SDS ($C_{12}H_{25}NaO_4S$) : highly flammable, harmful (R: 11-21/22-36/37/38 S: 26-36/37)
Formamide (CH_3NO): toxic (R: 61 S: 53-45)

Stock solution: NaCl-Stock solution (5 M), autoclaved:
1000 ml = 292.2 g NaCl
100 ml = 29.22 g NaCl

Tris/HCl-Stock solution (1 M, pH 7.2), autoclaved:
1000 ml = 157.6 g Tris/HCl
500 ml = 78.8 g Tris/HCl
250 ml = 39.4 g Tris/HCl
100 ml = 15.76 g Tris/HCl

SDS-Stock solution (1%), sterile filtrated:
100 ml = 1g SDS

Hybridisation buffer with 35% formamide:

Formamide	17.5 ml	3.5 ml	350 µl	175 µl
NaCl (5 M)	9 ml	1.8 ml	180 µl	90 µl
Tris/HCL (1 M)	1 ml	0.2 ml	20 µl	10 µl
SDS (1%)	0.5 ml	0,1 ml	1 0 µl	5 µl
H_2O bidest.	22 ml	4.4 ml	440 µl	220 µl

Washing buffer with 35% formamide (80 mM NaCl):

NaCl (5 M)	16 ml	8 ml
Tris/HCL (1 M)	20 ml	10 ml
SDS (1%)	10 ml	5 ml
H_2O bidest.	ad. 1000 ml	ad. 500 ml

Concentration of NaCl in the washing buffer using different concentrations of formamide in the hybridization buffer:

Formamide in the hybridization buffer [%]	NaCl in the washing buffer [mM]
0	900
5	636
10	450
15	318

20	225
25	159
30	112
35	80
40	56
45	40
50	28
55	20
60	14
65	10
70	7
75	5
80	3.5

Procedure

Prepare microscope slides
Coat the slots of the microscopic slides (8 chambers, ∅ 6mm) with coating solution and air dry them.

Plot the samples
Plot the cell or sediment suspension (3-4 µl) into the slots of the coated microscope slides and air dry them (15 min). If you investigate environmental samples, you can use up to 10 µl of sample.

Lysozyme treatment (use only for gram-positive bacteria)
Pipette 10 µl of lysozyme solution (10 mg/ml PBS-buffer = 1% lysozyme) and incubate at room temperature for 15 min (use a cup as dust protector). Wash off the lysozyme solution using ddH_2O.

Prepare hybridisation chamber (falcon tube)
Put parts of pulp into the tube and pipette hybridization buffer (1.5 ml) in. Afterwards, put the tube into the hybridization oven for 30 min and incubate at 46 °C in order to adjust a continuous vapour pressure.

Ethanol treatment in the staining chamber
Put the microscope slides into the first ethanol bath (50%, 3 min). Afterwards, put the slides into the second ethanol bath (80%, 3 min) and finally, put the slides into the third ethanol bath (96-98%, 3 min). Air dry the slides. The ethanol baths can be used several times.

Hybridisation of the samples
You need 7 µl hybridisation buffer for every single sample (prepare fresh) and 1 µl probe 850 ng/µl). Prepare the master mix for the appropriate amount of sample and preheat them (46°C). Afterwards, pipette the hybridisation solution (8 µl for every sample) into the slots. Put the microscope slides carefully into the hybridisation chambers and incubate them (46°C, 2 h).

Stop the reaction
Put two microscope slides in one Falcon tube (back on back) with cold water, sway it and discard the water.

Remove non specific probes
Put two microscope slides in one Falcon tube (back on back) with preheated washing buffer and sway them (48°C, 20 min). Discard the washing buffer, flush the microscope slides with ddH2O and dry them on air.

Incorporation and counter staining
Pipette 1 µl of Vecta Shield solution into every single slote. Put a cover glas onto the microscope slide and microscope after ca. 5 min or store the slides in the dark.

CARD (Catalyzed Reporter Deposition)-FISH (Sekar et al. 2003; modified by Ishii et. al., 2004)

In environmental samples, single oligonucleotides carrying only one fluorochrome may not provide enough fluorescence signals to detect cells with low ribosome content (Pernthaler *et al*., 2002). The Catalyzed Reporter Deposition (CARD-) FISH uses horseradish-peroxidase (HRP-) labelled oligonucleotides to increase the intensity of fluorescence signals. CARD is based on the deposition of a large number of labelled tyramine molecules by peroxidase activity. HRP reacts with hydrogen peroxide and the phenol part of labelled tyramide to produce a quinine-like structure bearing a radical on the C2 group. This "activated" tyramide then covalently binds to tyrosine residues in the target cell. Each HRP conferred by a probe catalyzes the deposition of many labelled tyramides. FISH signals are up to 20-fold brighter with HRP labelled probes than with conventional single labelled probes (Schönhuber *et al*, 1997).

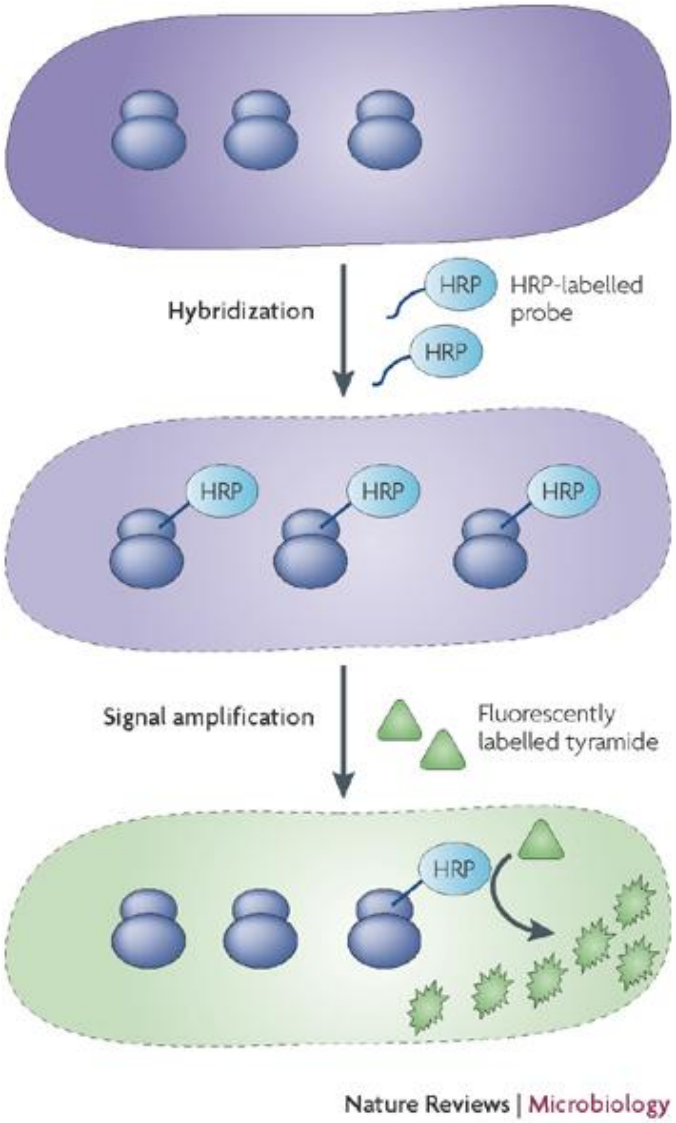


Figure 9: Catalysed reporter deposition (Amman and Fuchs, 2008).

Procedure (according to Ishii et al., 2004)

1. Fixation

Dilute 0.5 ml fresh culture or 0.5 cm^3 sediment in 1ml 3% formaldehyde. Incubate over night at 4 °C. Centrifuge at 16.000g for 5 minutes. Pour off supernatant and resuspend the sample with 1x PBS. Repeat the washing step twice. Store washed sample in a 1:1 mix PBS/ethanol at -20 °C until further processing.

SAFETY ADVICES

Formaldehyde (CH_2O) : toxic (R: 23/24/25-34-40-43 S: (1/2)-26-36/37/39-45-51)
Dimethylformamide (C_3H_7NO): toxic (R: 61-20/21-36 S: 53-45)

2. Sonication and filtration

1. Put fixed sediment sample on ice and sonicate it for 20 sec with lowest intensity.
2. Vortex sample and dilute 20-50 µl of the supernatant in 10 ml 1x PBS buffer
3. Filtrate the diluted sample onto a polycarbonatefilter (0.2µm)
4. Wash with sterile 1x PBS
5. Let filter air dry

3. Embedding

6. Boil Agarose (0.1-0.2%) and let it cool down to 35-40 °C.
7. Dip filters in Agarose and place filters face up onto filter paper and air dry (10 min.).

4. Permeabilization

8. Incubate filter in fresh lysozyme solution (37 °C >60 min)
9. Wash in ddH_2O

5. Inactivation of endogenous peroxidases

10. Incubate in H_2O_2 (0.15% in methanol) for 30 min at RT
11. Wash with ddH_2O (5-10 ml, 1min)
12. Wash with 96% Ethanol (5-10 ml, 1 min) and let filters air dry.

6. Hybridization

13. Cut filter sections
14. Mix hybridization buffer with probe working solution (400µl buffer + 1.3µl probe) in a 0.5 ml vial. Place filter sections in reaction vial (3-4 sections per vial).
15. Incubate at 46 °C for at least 1.5h up to 24h

7. Washing

16. Transfer filter sections to prewarmed washing buffer (48 °C) and wash for 15 min.
17. Remove excess liquid with blotting paper, but don't let filters run dry.

8. CARD

18. Incubate in 1x PBS (10 ml, 1-5 min, RT)
19. Dab filters on blotting paper but do not let filter run dry.
20. Incubate in substrate mix (Mix 200µl PBS with 1µl H_2O_2 (30%). Mix tyramide with 20µl DMF (Dimethylformamide). Mix 1.5 ml Amplification buffer + 15 µl PBS/ H_2O_2+ 3 µl tyramide/DMF) at 46 °C, 20 min, in the dark.
21. Dab filter on blotting paper and wash in 10-50 ml ddH_2O (1-5 min, RT, dark)
22. Wash in 10-50 ml 96% ethanol (1-5 min, RT, dark) To keep the background fluorescence low, it is important to wash the filters in large volumes of water and ethanol.
23. Let air dry (dark).
24. Counter stain with DAPI. Put filter section on glass slide. Stain with 10 µl DAPI solution. Incubate min. 30 min in dark.

Table 9: Frequently used CARD-FISH probes.

Probe	Target	Sequence (5'-3') of probe	FA (%)[a]	Reference
Agro1	*Rhizobium* sp.	GTC TCC AAT GCC CAT ACC	unknown	Süß et al. (2006)
EUB338	Bacteria	GCT GCC TCC CGT AGG AGT	55	Amann et al. (1990)
NON338	None (negative control)	ACT CCT ACG GGA GGC AGC	55	Wallner et al. (1993)
DSB985	Desulfobacter / D'bacula	CAC AGG ATG TCA AAC CCA G	-20	Manz et al. (1998)
Arch915	*Archaea*	GTG CTC CCC CGC CAA TTC CT	55	Stahl & Amann (1991)
Cren537	*Crenarchaeota*	TGA CCA CTT GAG GTG CTG	10	Teira et al. 2004
DSV698	Most *Desulfovibrio* spp.	GTT CCT CCA GAT ATC TAC GG	40	Manz et al. (1998); Mußmann et al. (2005)

[a] formamide concentrations in hybridisation buffer

Solutions

Lysozyme solution (10 mg/ml)

Prepare buffer (1ml 0.5M EDTA, 1ml 1M Tris/HCl and 8 ml ddH_2O). Add 100mg Lysozyme when solution is needed.

Phosphate buffered saline (PBS), pH 7.6

	10 x PBS 1 litre	1 x PBS 1 litre
NaCl	80 g	8 g
KCl	2 g	0.2 g
Na_2HPO_4 * 7 H_2O	26.8 g	2.7 g
KH_2PO_4	2.4 g	0.2

10% Blocking reagent

Blocking reagent is dissolved in Maleic acid buffer to a final concentration of 10% (w/v) with shaking and heating either on a heating block or in a microwave oven. Adjust the Maleic acid buffer to pH 7.5.Then dissolve 10g of Blocking reagent in 100 ml of Maleic acid buffer. This stock solution is autoclaved and stored at -20 °C.

Amplification buffer

2 ml PBS 20x
0.4 ml Blocking Reagent (10%)
16 ml NaCl (5M)
Add sterile H_2O to a final volume of 40 ml
Add 4g dextrane sulphate, heat 40-60°C

1 M Tris/HCl, pH (8.0)

Dissolve 121g Tris base in 800 ml H_2O. Adjust to desired pH with concentrated HCl. Mix and add H_2O to 1 litre.

0.5M EDTA, pH (8.0)

Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 700 ml H_2O.
Adjust pH to 8.0 with 10 M NaOH (ca. 50 ml). Add H_2O to 1 litre.

Maleic acid buffer

100 mM Maleic acid, 150 mM NaCl, pH7.5 (20 °C).

Hybridization buffer

	2 ml	10 ml
NaCl (5 M)	360 µl	1800 µl
Tris/HCl (1 M)	40 µl	200 µl
Dextrane sulphate	0.2 g	1 g
Formamide	x µl	x µl
MQ water	x µl	x µl
Blocking reagent	200 µl	1000 µl
SDS (10 %)	2 µl	10 µl

Keep the mixtures in a water bath (48-60°C) until the dextrane sulphate dissolves.

Washing buffer

	50 ml
NaCl (5 M)	x µl
Tris/HCl (1 M)	1000 µl
EDTA (0.5 M)	500 µl
SDS (10 %)	50 µl
MQ water	to 50 ml

Add SDS finally in order to avoid precipitation.

6. Processing of nucleic acids

Isolation of DNA

Introduction

Nucleic acids can be isolated using multiple techniques. The method of choice depends on the nature of the nucleic acids to be isolated and the intended purpose. Some simple techniques rely on enzymatic digestions (e.g. lysozyme) or mechanical decompositions to break up cells. (e.g. freeze thaw, beadbeater, sonication). Since nucleic acids obtained by these methods are usually strongly contaminated, extraction methods based on organic solvents can be applied to process isolated nucleic acids further.

The origin of taken samples is crucial regarding the cell disruption. Bacterial cells from environmental samples, for example, are partially protected by a sediment matrix from mechanical forces and enzyme activity. The usage of a beadbeater for disrupting bacterial cells cultivated under laboratory conditions would expose them to too much shear forces. Besides, the generation of heat would destabilise/destroy present nucleic acids. In this case, the cell disruption via freeze thawing, which breaks cells physically by sequent cycles of freezing and thawing, would be the method of choice.

In the context of decomposing gram positive cells, it has to be taken into account that the cell wall is significant thicker compared to gram negative bacteria, due to a higher number of peptidoglycane layers. In this case, a pre-treatment by means of lysozyme is necessary to reduce the mechanical stability. Glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid, which form the backbone of murein, are cleaved.

Equipment

- Biocentrifuge
- Freezer (-70°C)
- Heating block

SAFETY ADVICES

SDS ($C_{12}H_{25}NaO_4S$) : highly flammable, harmful (R: 11-21/22-36/37/38 S: 26-36/37)

Reagents

- Lysozyme solutions (0.8 $mg \cdot ml^{-1}$)
- 10 mM Tris-HCl, pH 8
- 10% SDS solution (9.6 ml 20% SDS + 2.4 ml 0.5 M sodium acetate (pH 7,5) + 66,4 ml ddH_2O; autoclaved)
- 3 M sodium acetate solution

Practical advices

When handling DNA it is crucial to work sterile, samples got contaminated very easily.

Procedure

Frozen samples (- 20 °C), stored in Eppendorf tubes, are thawed and centrifuged for 30 minutes (19000 rpm, 4 °C), supernatants are discarded. Per sample, 100 µl lysozyme solution and 100 µl Tris-HCl are added. Samples are reversed ten times and subsequently incubated for 10 minutes stored on ice. Next, 40 µl SDS solution and 60 µl sodium acetate solution are added. The incubation is continued for one hour and samples are still stored on ice. For freeze thawing, samples are snap-frozen (-70 °C) for 3 minutes and subsequently boiled up five times. Afterwards, a phenol/chloroform extraction is performed.

„Freeze & Thaw“ – DNA extraction from growing cultures

Procedure

1) Harvesting cells:
   a) Transfer 2 ml of liquid culture into a sterile 2 ml reaction tube (Eppendorf), centrifuge for 30 min at 4°C and discard the supernatant.
   b) Pick up a colony from agar plates / agar shakes and transfer it into a 1.5 ml reaction tube
2) Add 100 µl of TRIS buffer (50 mM, pH 7.4) or water used for PCR mix until you have a cell suspension
3) Run five „freeze & thaw“ cycles: -80°C ethanol bath, 85°C heater, 3 min each
4) It is recommended to use 2 µl of the fresh DNA extract for PCR (50µl reaction)

Since this is a “quick & dirty” procedure (no DNA stabilisation, no DNA purification etc.) extracts should be used for molecular biological purpose as soon as possible, kept on ice (or in the fridge) and immediately frozen away after use.

DNA/RNA extraction from sediment samples

The extraction of nucleic acids is a necessary prerequisite for using them as template for PCR. Besides the cell disruption, purifying and protecting extracted nucleic acids from degrading enzymes is of key importance. In the context of sediment samples, freeze thawing cannot be applied to decompose bacterial cells, because of the sediment matrix, which lowers the efficiency significantly.
The main problem associated with alternative disruption methods is protecting the nucleic acids. Mechanical and shear forces easily destabilise and destroy nucleic acids so that further investigations are not possible. Moreover, DNases and RNases are set free due to cell disruption.
Purifying extracted nucleic acids is necessary, since sediment samples offer many substances which impair the PCR. A possible way to purify nucleic acids is the phenol/chloroform extraction. Commercially available kits are expensive alternatives, which are not inevitably more efficient. A precipitation is done to absorb the nucleic acids in a certain volume and a proper buffer. After finishing the extraction and if needed quantification, nothing stands in the way anymore to amplify nucleic acids using PCR/RT-PCR.

Equipment

- beadbeater
- rack
- sterile cryo vials + lids
- cold trap
- pump for the Speed Vac
- Speed Vac
- sterile Eppendorf tubes (1.5 ml und 2.0 ml)
- sterile zirkonia pearls (∅ 0.1 mm)
- Biofuge 13 R
- water bath
- Biofuge 15 R

SAFETY ADVICES

Chloroform ($CHCl_3$): harmful (R: 22-38-40-48/20/22 S: (2)-36/37)
Phenol (C_6H_6O): toxic, corrosive (R: 23/24/25-34-48/20/21/22-68 S: (1/2)-24/25-26-28-36/37/39-45)
SDS ($C_{12}H_{25}NaO_4S$) : highly flammable, harmful (R: 11-21/22-36/37/38 S: 26-36/37)

Reagents

- chloroform
- DEPC treated ddH_2O (0.1% diethyl pyrocarbonate (DEPC) Dissolve in ddH_2O and mix well. Incubate over night at 37 °C and autoclave subsequently. DEPC dissolves completely in CO_2 and ethanol).
- DNase (1 U/µl)
- DNase buffer (pH 7.5) (40 mM Tris (Base), 6 mM $MgCl_2$ prepared in ddH_2O, pH adjusted via HCl and autoclaved)

- ethanol (70 %)
- precipitation solution (125 ml ethanol (absolute), 5 ml sodium acetate (3M))
- phenol -saturated with water- (pH 4.0 and 7.5)
- phenol/chloroform -saturated with water- (pH 4.0 and 7.5)
- SDS extraction solution (9.6 ml 20 % sodium dodecyl sulphate (SDS), 2.4 ml 0.5 M sodium acetate (pH 7.5) and 66.4 ml ddH_2O, pipetted into a 100 ml Pfennig bottle and autoclaved)
- TE buffer (pH 8.0) (10 mM Tris (Base), 1 mM EDTA prepared in ddH_2O, pH adjusted using HCl and autoclaved)
- all solutions are set up with DEPC treated ddH_2O to eliminate DNases and RNases
- autoclaving is done for 20 minutes at 121 °C

Cell disrupting using the beadbeater

1 g sediment, 1 g zirkonia pearls and 1 ml SDS extraction solution are transferred into one cryo vial. Using a beadbeater, samples are shaken for 1 minute (~ 5000 rpm). The sediment and the zirkonia pearls are centrifuged down and the supernatant is transferred to a 1.5 ml Eppendorf tube and stored on ice (try to prevent the accidental transfer of sediment). 500 µl phenol are added to the to the cryo vial. After shaking the vial again using the beadbeater, sediment and pearls are again centrifuged down and the supernatant is unified with the previous transferred one. The procedure is repeated again, this time adding 250 µl SDS extraction solution and 250 µl phenol (pH 7.5).
For a combined DNA/RNA extraction, the supernatant is splitted before being subjected to a phenol/chloroform extraction.

Phenol/chloroform extraction

Phenol (pH 4 for RNA, pH 7.5 for DNA) is added in a 1:1 ratio to nucleic acid solution. After reversing the solution ten times and a centrifugation (13000 rpm, 2 minutes), the aqueous phase (usually the supernatant) is transferred to a new 1.5 ml Eppendorf tube. The organic- and interphase contain the proteins and humic acids, which should be removed by the extraction procedure. Phenol/chloroform (pH 4 RNA, pH 7.5 DNA) is added in a 1:1 ratio to the aqueous phase. Again the solution is reversed and centrifuged and the supernatant transferred to a new Eppendorf tube. Finally, chloroform is added in a 1:1 ratio and the solution is treated as before.
The obtained aqueous solution is transferred into a 2 ml Eppendorf tube. Next, an ethanol precipitation is done to remove salts that would impair the PCR and to take up the nucleic acids in a suitable buffer.

Ethanol precipitation

Precipitation solution is added in 2.6 : 1 ratio to the sample (prepare freshly, sodium acetate precipitates over time) and the nucleic acids are allowed to precipitate overnight at -20 °C (alternatively: 4 hours at 4 °C). The nucleic acids are centrifuged down (15000 rpm, 30 minutes). Pay attention to the orientation of the Eppendorf tubes, to ensure the same orientation for the next centrifugation step. After the centrifugation, the supernatant is discarded and 500 µl ethanol (70 %) are added for washing the samples. After a second centrifugation step (15000 rpm, 10 minutes), the supernatant is again discarded and the samples are centrifuged for 5-10 minutes using the Speed Vac at medium temperature. Thereby, remaining ethanol is removed. At least 10 minutes before using the Speed Vac, the cold trap should be turned on to protect the affiliated pump (generates the needed negative pressure) from liquids. The nucleic acids are taken up in 50 µl TE buffer after being dried. After incubating them for 30 minutes at room temperature, samples can be stored ready to be used at 4 °C. For long term storage, nucleic acids can be taken up in precipitation solution (2.6:1) and stored at -70 °C.

RNA extraction and DNase treatment

In order to get pure RNA, it is necessary to prevent/remove DNA contaminations. For extracting RNA, the phenol/chloroform extraction is done at a pH of 4. At this value, DNA partially precipitates in the interphase. The RNA still dissolves completely in the aqueous phase. Since DNA is still present after extraction, purification and precipitation, the sample is treated with DNases. Therefore, the RNA samples are taken up

in 500 µl DNase buffer instead of TE buffer. 5 µl DNase are added and the samples are incubated for 60 minutes at 37 °C using a water bath. Subsequently, the phenol/chloroform extraction is repeated to remove DNases. In order to check the presence of DNA afterwards, a PCR is done.

Summary
- cell disruption using a beadbeater
- splitting of the samples in DNA and RNA subsamples
- Phenol/chloroform extraction (pH!)
- ethanol precipitation
- take up DNA in TE buffer
- DNase treatment of the RNA samples
- Phenol/chloroform extraction (pH 4,0)
- ethanol precipitation
- take up RNA in TE buffer

Rapid test for quantifying DNA

1 µl of sample is pipetted on an EtBr plate (Set up an 1.5 % agarose gel 1 x TAE buffer and boil up the solution using a microwave. After cooling down (~ 60 °C), 15 µl ethidium bromide (10 mg/ml) are added per 100 ml agarose. The agarose is poured into petri dishes). Additionally, 1 µl of herring sperm is added in varying concentrations (e.g. 10, 30, 50, 100, 150 ng/µl). Using a transilluminator, the DNA content can be estimated based on the standard.

SAFETY ADVICES

Ethidium bromide ($C_{21}H_{20}N_3Br$): very toxic (R: 22-26-68 S: (1/2)-28-36/37-45-63)

DNA extraction from sediments with FastDNA®Spin® Kit

Compared to the phenol/chloroform extraction this is a fast but expensive method. A further advantage of a kit is, that filters are used for extraction so that the application of toxic substances such as phenol and chloroform can be minimized. Besides the chemicals, columns and tubes that are included in the kit four sterile 1.5 ml Eppicaps as well as acetate and ethanol are needed. Unless otherwise noted an Eppendorf centrifuge is used by 13.000rpm.

- transfer 0.5 g sediment, 918 µl Sodium Phosphate Puffer, 122 µl MT-Buffer, 40 µl Poly(A)-solution and 20 µl sodiumpyrophosphate-solution to a Lysing Matrix E Tube
- shake the tube slightly for 30 sec and wait two minutes before starting the cell lysis
- the cells are lysed mechanically by a beadbeater at 5000 rpm for 1 min, wait 30 sec before beadbeating a second time
- centrifugation at 15.000 rpm for 15 min at 4°C
- transfer supernatant in a sterile 1.5 ml Eppi and add 250 µl PPS
- invert 10 times and centrifuge for 5 min
- split the supernatant in two sterile 1.5 ml Eppis and add 500 µl Binding Matrix Suspension respectively, vortex the Matrix Suspension before addition
- shake the samples 2 min by hand and wait ten minutes, so that the particles can sediment
- remove 350 µl of the supernatant and transfer 750 µl of the suspended solution to a spin filter, centrifuge 2 min
- remove filtrate, add the remaining supernatant to the supernatant of the second aliquot and transfer it to the filter
- centrifugation for 2 min
- remove supernatant, add 500 µl SEWS-M and resuspend cautiously
- centrifugation for 2 min

– remove filtrate
– centrifugation for 2 min
– place the spin filter into a new catch tube (keep the old tube so that the filter can be placed there later)
– dry the open tube for 5 min under the clean bench
– add 50 µl DES to the filter and centrifuge for 1 min
– transfer the extract to a sterile Eppicap and repeat the DES-step

Usually less than 50ml are needed for amplification. 30µl can be transferred to a sterile Eppendorfcap. After precipitation with 90µl ethanol/ sodium-acetate the DNA can be stored at 70°C.

DNA extraction from liquid samples

SAFETY ADVICES

Chloroform ($CHCl_3$): harmful (R: 22-38-40-48/20/22 S: (2)-36/37)
Phenol (C_6H_6O): toxic, corrosive (R: 23/24/25-34-48/20/21/22-68 S: (1/2)-24/25-26-28-36/37/39-45)
SDS ($C_{12}H_{25}NaO_4S$) : highly flammable, harmful (R: 11-21/22-36/37/38 S: 26-36/37)
Isopropanol (C_3H_8O): highly flammable, irritant (R: 11-36-67 S: (2)-7-16-24/25-26)
Isoamylalcohol ($C_5H_{12}O$): harmful (R: 10-20-37-66 S: (2)-46)

Water samples have been filtered using 0.2 µm polycarbonate filter. In an Eppendorf tube, 0.5 g zirkonia pearls, 20 µl SDS (25 %), 600 µl phosphate buffer and 600 µl phenol chloroform isoamylalcohol have been added to the filter. In order to disrupt the cells and to remove cell remnants and foreign particles from the bacterial DNA, the Eppendorf tube was thoroughly vortexed. Next, the tube was incubated for 10 minutes at 60 °C using a water bath and again vortexed for 3 minutes. The organic phenol phase was separated from the aqueous phase by centrifugation for 10 minutes (10000 rpm) at room temperature. The aqueous phase, containing the DNA, was transferred into a new Eppendorf tube. 300 µl phosphate buffer have been added to the original sample, which was vortexed for 1 minute and again centrifuged for 10 minutes. The aqueous supernatant was unified with the already transferred one. 1 ml phenol chloroform isoamylalcohol was added to remove the remaining impurities and the solution was vortexed and centrifuged as already described. As long as a white precipitate was visible at the interphase, the procedure was repeated by adding 1 ml phenol chloroform isoamylalcohol again.
Subsequently, the aqueous solution was allowed to precipitate over night after adding 30 µl sodium acetate and 2.25 ml isopropanol. By centrifugation (13000 rpm, 30 minutes, 4 °C), present DNA was pelleted. The supernatant was discarded and the pellet washed with 1.5 ml ethanol (70 %). The solution was centrifuged again (13000 rpm, 10 minutes, 4 °C), the supernatant removed and the pellet dried for 3 minutes using the Speed Vac. The precipitated and dried DNA was taken up in 50 µl PCR water, ready for use.

Quantification of DNA using PicoGreen

1µl of sample is mixed with 899 µl TE buffer. For quantification, 100 µl PicoGreen reagent (PicoGreen 1:40 in TE buffer) are added per sample. Using a blank and a standard (blank: 900 µl TE buffer + 100 µl PicoGreen reagent, standard: 899µl TE buffer + 1 µl herring sperm (100 ng/µl) + 100 PicoGreen reagent) the DNA content is determined. By means of a spectrofluorophotometer (Shimadzu), the emission was measured at 540 nm and the extinction at 460 nm. Measured values refer to ng/µl. In this context, the prepared standard should not differ. Before the measurements, samples have to vortexed and incubated in the dark for 5 minutes. During the whole procedure, gloves and lab coats should be worn. Waste is discarded in the 'Färbeabfall'.

DNA-Quantification via a Microtiter plate reader

Since it is necessary to know the accurate amount of DNA for sequencing, *Real-Time* PCR and DGGE, you have to quantify them. The quantification is carried out by using the DNA-fluorescence dye PicoGreen as well as the fluorescence reader. The calculation is carried out via a calibration line resulting from defined concentrations of DNA.

1) Pipette the following DNA-standards

DNA-concentration	TE-buffer pH 7.5	DNA-stock solution	PicoGreen 1:200 diluted in TE-buffer pH 7.5
100 ng/µl	99 µl	1 µl	100 µl
50 ng/µl	99 µl	1 µl	100 µl
25 ng/µl	99 µl	1 µl	100 µl
0 ng/µl	100 µl	0 µl	100 µl

2) Pipette the DNA-samples

TE-buffer pH 7.5	**DNA-sample**	**PicoGreen 1:200 diluted in TE-buffer pH 7.5**
99 µl	1 µl	100 µl

➔ Pipette Pico Green PicoGreen not until measurement
➔ Incubation of 5 min in the reader
1) Turn on the microtiterwellreader
2) Turn on the computer
3) Start program FLUOstar Optima
4) Push the button "Test Protocols"
➔ Test name: „Reinhard Versuch"
➔ Settings:
➔ **Basic parameters**

Test name: „Reinhard Versuch"
Microplate: Nunc F96 Microwell 96
Positioning delay: 0.1
Number of kinetic windows: 1
Number of cycles: 3
Measurement start time: 0.0
Number of flashes per cycle: 20
Cycle time: =variable (depends on the number of samples)
x fluorescence intensity
Number of multichromatics: 1
Excitation filter: 485 nm
Emission filter: 520 nm
Gain: =variable (depends on the highest DNA concentration)
Start: 1
Stop: 1
Pause before cycle: 0

a) Layout

Record the adjustment of your samples- and standards on the plate and push the button „check timing"

5) Push the „traffic light" button -> Push Test name: „Reinhard Versuch"

6) Start the measurement by pushing the button „Start test run"

7) Reporting
Push the „Excel“ button -> choose your test name („Reinhard Versuch“) -> Recording of the raw data

DNA extraction from aquifer sediments (modified from Lueders et. al., 2004 and Gabor et. al., 2003)

Protocol modified from: Lueders, T., Manefield, M., and Friedrich, M.W. (2004) *Environ Microbiol*. and Gabor et al. (2003) *FEMS Microbiol Ecol*

SAFETY ADVICES

Chloroform ($CHCl_3$): harmful (R: 22-38-40-48/20/22 S: (2)-36/37)
Phenol (C_6H_6O): toxic, corrosive (R: 23/24/25-34-48/20/21/22-68 S: (1/2)-24/25-26-28-36/37/39-45)
SDS ($C_{12}H_{25}NaO_4S$) : highly flammable, harmful (R: 11-21/22-36/37/38 S: 26-36/37)
Isoamylalcohol ($C_5H_{12}O$): harmful (R: 10-20-37-66 S: (2)-46)

- Add 0.2 ml (= 1 PCR cup full) of ~1:1 mixed 0.1 mm and 0.7 mm Zirconia/Silica beads (www.biospec.com) to 2 ml bead beating vial with screw cap, autoclave
- Add ~0.8 - 1 g sediment sample (usually ~0.5 – 0.7 ml), make a note of precise weight
  (alternatively, add ~2 mm slices of a 0.2 µm filter membrane cut with a sterile scalpel)
- add 750 µl PTN buffer (pH 8), vortex ~10 sec (also turning over)
- add 40 µl Lysozyme (50mg/ml in TE, stable only for a few weeks at -20°C) and 10 µl Proteinase K (10 mg/ml in TE)
- Mix well manually, incubate 15 min at 37°C in Thermomixer, turn over & shake occasionally
- Add 100 µl SDS (20 %), mix manually and incubate for 15 min at 65°C & 500 rpm in Thermomixer
- Add 100 µl Phenol/Chloroform/Isoamylalcohol (25:24:1) pH8.
- Check that vial is filled maximally to 1.5 ml with minimally 500 µl headspace remaining
- Bead beating 45 sec at 6 m/s
- Spin down 5 min at 7500 rpm (6000 g) & 4°C, take 600 µl supernatant, place in 2 ml vial at 4°C
- Add (up to!) 300 µl PTN buffer to sediment, so that ~400 µl supernatant remain,
- Bead beating 20 sec at 6.5 m/s
- spin down 5 min at 7500 rpm (6000 g) & 4°C, take 300 µl and pool supernatants (= ~ 900 µl), keep at 4°C
- Extract by vigorous shaking (manually) with 1 vol (900 µl) PCI, spin 4 min at 14000 u/min, 4°C
- take ~800 µl supernatant, place in 2 ml “Phase Lock Gel Heavy” tube (Eppendorf)
- Extract with 1 vol (~800µl) Chloroform/Isoamylalcohol (24:1), spin 4 min at 14000 u/min, 4°C
- take 650 µl supernatant, mix thoroughly with 2 volumes (1300 µl) PEG
- Incubate for up to 2 h (min: ~30 min) at 4°C, pellet DNA by spinning over 30 min at 14000 rpm & 20°C
- Remove PEG with pipette, or decant if pellet is clearly visible
- Add 150 µl cold (-20°C) 70 % EtOH, gently wash pellet (should be visible and sticking to the wall)
- spin 5 min at 14000 rpm, 4°C
- Remove EtOH carefully by pipetting, dry DNA briefly at room temperature (<5 min)
- elute each DNA precipitate in 30 µl EB buffer (25 – 100 µl possible, depending on yield)

- mix by flipping, briefly spin down, transfer to 0.5 ml Eppendorf-Cup, store at -20°C

Reagents

PTN Puffer: 120mM $NaPO_4$, 125 mM Tris, 25 mMNaCl, pH8	16,02 g/l Na_2HPO_4, 0,86 g/l NaH_2PO_4, 11,2 g/l Tris-HCl, 6,6 g/l Tris-Base; 1,46 g/l NaCl, adjust to pH 8 with HCl, filter sterilize, autoclave
1 x TE	10 mM Tris-HCl, 1 mM EDTA, pH 8
20% SDS	20 g SDS in 100 ml H2O, prepare with sterilized H2O in baked glassware
30% PEG, 1.6 M NaCl; pecipitation solution	150 g polyethylene glycol 6000 + 46,76 g NaCl in RNAse free water, 500 ml final volume. Prepare in baked glassware, first dissolve PEG in microwave, adjust to final volume, autoclave
EB Buffer	10 mM Tris, pH 8.5, prepare with RNAse free water, filter sterilize, autoclave. Better: take from a fresh QiaQuick Kit.

Agarose gelelectrophoresis

DNA fragments of 200 – 50.000 bp are separated by agarose gelelectrophoresis due to their size. Negatively charged DNA molecules move through an agarose matrix with an electric field. Shorter molecules move faster and migrate further than longer ones. The movement decelerates logarithmically according to the number of base-pairs. After the electrophoresis the separated fragments are stained with **ethidium bromide**. This is an **intercalating fluorescent dye, which is mutagen and carcinogen, so that gloves and a lab coat are required.**

SAFETY ADVICES

Ethidium bromide ($C_{21}H_{20}N_3Br$): very toxic (R: 22-26-68 S: (1/2)-28-36/37-45-63)

Preparation of agarose gels
The gels are prepared in Plexiglas trays which have only two opposite side walls so that the gel is in contact with the electric field. Before preparation the open sides must be sealed with tape. A standard gel contains 1.5 % (w/v) agarose which is dissolved in 40 ml 1xTAE buffer. The agarose is dissolved in a microwave oven until the solution is clear. The solution is then poured into the tray and a comb is inserted so that gaps are developed for the PCR products. After 20 min the gel becomes solid and the comb and tape can be removed cautiously.

1x TAE buffer: 40 mM tris(hydroxymethyl)aminomethane;
1 mM EDTA, pH 7.4 adjusted with acetate

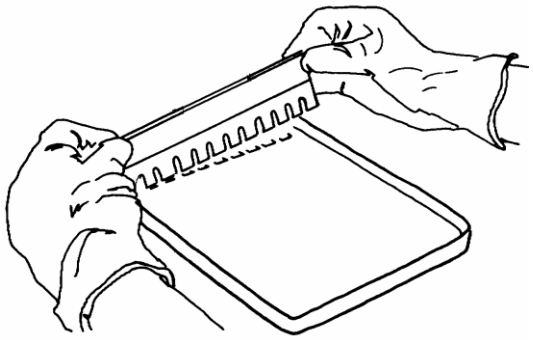


Figure 10: Placing the comb.

Electrophoresis
The tray is transferred to the electrophoresis chamber which contains 1x TAE buffer. Make sure that the gel is covered entirely with buffer.
1 µl 6x loading-buffer is pipetted on a piece of Parafilm and mixed with 5µl PCR product. This mixture is then transferred to the gel. 1.5µl standard solution (concentration 125ng/µl) are applied to the gel. The

electrophoreses is carried out at 100V for 40 min (or 90V for 45min). Pay attention that the correct polarity is chosen.

Loading buffer: 0.25% (w/v) brome phenol blue 40% (v/v) glycerine in 1x TAE

After the electrophoresis the gel is stained for 20 min in an ethidium bromide solution (0.8µg/ml). The documentation is done with a transilluminator.

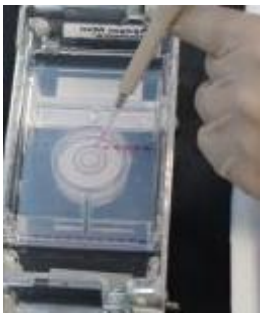


Figure 11: Loading the gel.

Purification of PCR products

The PCR products will be purified using the PCR-Kombi purification kit (Seqlab) and a centrifuge. Before starting with the purification procedure place a Spin filter into a 2 ml Receiver vessel.

1. **Binding of the PCR products**
   Mix 500 ml Binding buffer thoroughly with the PCR sample by pipetting or vortexing. Transfer that solution completely onto a Filter cartridge and centrifuge for 2 minutes at 10,000 x g. Discard the Receiver vessel with the flow-through.
   If the volume of the PCR reaction is higher than 50 µl, split the PCR Mix and add 500 µl Binding buffer to each part. Load both mixes one after the other onto the Filter cartridge. Centrifuge the first part for 1 minute and discard the filtrate. Centrifuge the second part of the mixture for 2 minutes. Then carry on with the elution step.

2. **Elution of the PCR products**
   Place the Filter cartridge into an Elution vessel. Pipett at least 10 µl Elution buffer (or ddH_2O) directly onto the centre of the Filter membrane. (For concentrating the PCR fragments it is possible to perform the elution with a lower volume of Elution buffer than the volume of the initial PCR mixture. The minimum volume is 10 µl. Incubate for 1 minute (best is 5 – to increase the normally already high final DNA yield further, an extended incubation time for up to 5 minutes is recommended) at room temperature, then centrifuge for another minute at 5,000 x g. The Elution vessel now contains the purified PCR product.

DNA-sequencing via Sanger (Chain-abruption method)

using the LiCor DNA Sequencing System 4200 of MWG Biotech

Preliminaries

The sequencing reaction via Sanger is a PCR-like method using just one primer. Since the use of several cycles of temperatures is also necessary, it is spoken of „Cycle Sequencing“. In order to encode one single sequence, four different approaches are necessary, each of them containing another didesoxynucleotide (ddNTP) (= chain abruption nucleotide). The reaction approach contains up to 3% of the „general“ dNTP`s. All ddNTP`s are incorporated just like the „general“ dNTP`s by using the DNA-polymerase. However, the ddNTP`s induce a chain abruption because of the lack of the OH-group on the 3`-end. Depending on a statical distribution of the incorporation of ddNTP`s, you will obtain a mixture of DNA fragments with a

distinct length ending with a particular nucleotide (e.g. the nucleotide of the ddATP approach is an A). (see figure beneath)
The four different approaches will be plotted next to each other on a gel and in the following the mixture of DNA-fragments will be separated due to length by electrophoretic separation (short fragments running faster than the long ones). The detection of the DNA bands is carried out via computer controlled laser and is possible because of the flourescence excitation of the bands. The comparison of the band running length of the four approaches results in the uncovering of the unknown sequence. Finally, the sequence will be transmitted into a internet data bank (e.g. EMBL, RDP) and compared with known sequences.

Material

Equipment:
Thermocycler
LiCor DNA Sequencing System 4200

SAFETY ADVICES

Boric acid (H_3BO_3): toxic (R: 60-61 S: 45-53)

Chemicals and reagents:
DYEnamic Direct Cycle Sequencing Kit (Amersham)
Sequencing primer (IRD-marked)

Stock solutions:
TBE-buffer (10 ×)

Tris Base (890 mM)	108	g
Boric Acid (890 mM)	55	g
$Na_2EDTA \times 2H_20$ (20mM)	7.44	g
H_20 (dest.)	ad. 1	l

Working solution:
Gel solution:

dest H_2O	14.5 ml
Urea NF	12.6 g
10x TBE-buffer	3.6 ml
"Long Ranger 50% gel solution"	4.5 ml

Procedure

Sequencing reaction
The sequencing reaction is carried out via *DYEnamic Direct Cycle Sequencing Kit* (Amersham). For one single sample you will need four 0.2 ml tubes in order to pipette all four nucleotides (G, A, T, C). The DNA-sample (volume = 2 µl) will be transferred in each of the four tubes. Afterward you prepare the master mix (for each sample you need: 1 µl of ddNTP`s + 2 µl of IRD-labelled primer. One aliquot of the master mix contains a volume of 3 µl and will be transferred into your sample tube. Finally, the tubes will be put into the cycler and the you can start the run. (The program depends upon your primer.)

Sequencing-program:

Temp.[C°]	Time [min]	Cycles [n]
95	5	1
95	0.5	
*	0.5	30
72	1	
4	∞	

*The annealing-temperature depends upon the melting temperature (T_M) of the primer you use.

After the completion of the program, you add 5 µl formamide loading buffer to each approach and spin up

the samples by using the centrifuge. Before plotting the samples onto the gel, you have to denature them using a temperature of 70 °C (5 min). Afterwards you put the tubes immediately on ice-water in the dark until you start plotting the gel.

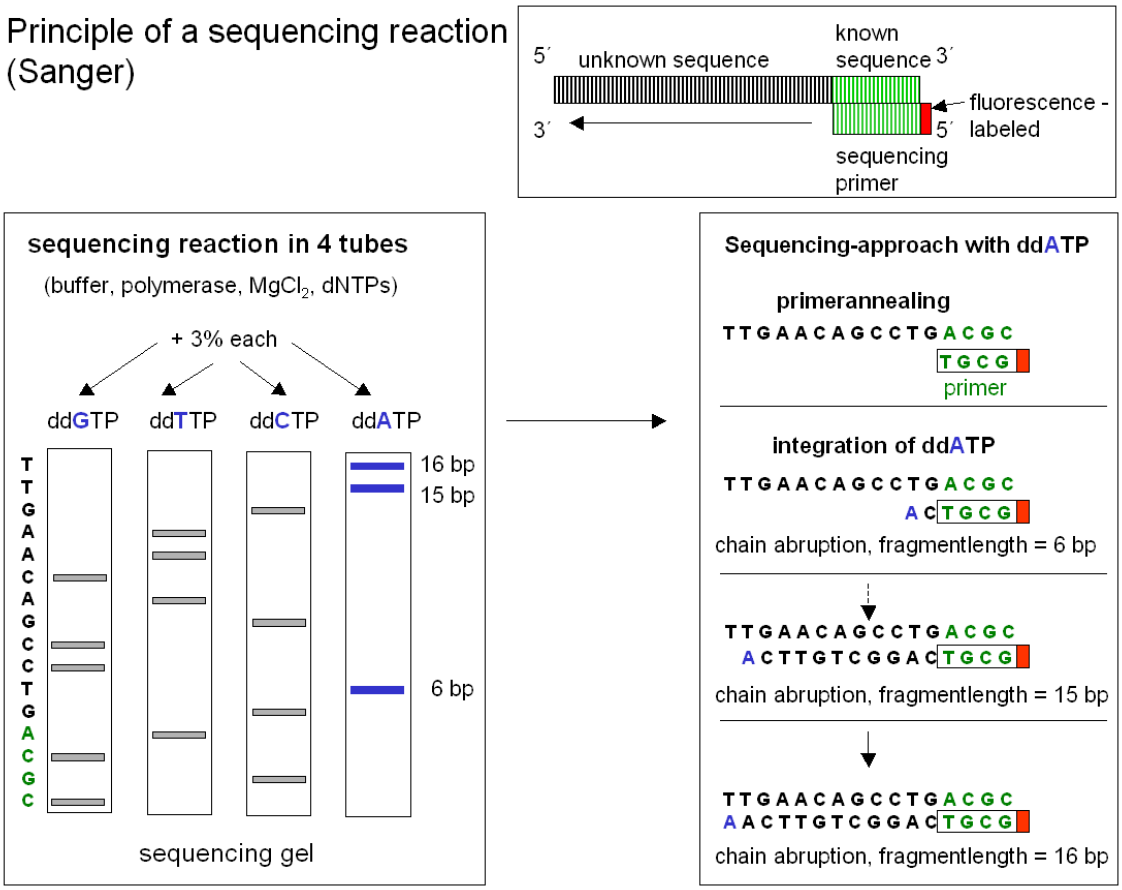


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Figure 12: Scheme of the sequencing principle after Sanger.

Electrophoresis using the LiCor DNA Sequencing System 4200

Prearrange the glass plates

In order to carry out the electrophoresis, you have to put together the „Gel-Sandwich“ (length: 33cm, thickness: 0.25 mm). Wear gloves and a lab coat while working (acrylamide is carcinogenic). The glass plates are marked in order to identify the interior and outerior plates. Spill the plates using SDS (25%), ddH_2O and ethanol (98%). You have to remove ethanol completely because it constricts the polymerization of acrylamide. Afterwards, put the glass plates in the brackets of the equipment.

Casting the gel

Prepare the gel solution not until casting the gel (under the flume hood). Mix the chemicals using the protocol in the beaker on a magnet stirrer. Add TEMED (20 µl) and APS (200 µl) to start the polymerization of the gel and Pipette the gel through a 0.2 µm filter between the horizontal glass plates using a 50 ml syringe. Avoid the formation of air bubbles by knocking gently on the glass plates. Afterwards attach the precomb into the gel. The complete polymerization time lasts 1.5 hours.

Install the „Gel sandwich“

After polymerization is done, you have to pull out the precomb and clean the upper border of the gel. Clean the glass plates in the scope of the detector using ethanol. Install the „gel-Sandwich“ into the sequencer and

mount the upper and lower buffer chamber. Fill both chambers with 1 x TBE-buffer (ca. 1 l) up to the check mark and flush the gel slote with TBE-buffer using a syringe. Finally mount the cover and the electric cable carefully. You can find further information in „DNA Sequencing and Genetic Analysis Manual“, section „Gel preparation and Electrophoresis“.

Electrophoresis settings

Voltage	1500 V
Amperage	35 mA
Power	45 Watt
Temperature	50°C
Motor speed	2
Signal Channel	2
Frames	25

Loading the gel

Stop the electrophoresis after the prerun is done. Take off the cover of the upper buffer chamber and spill the gel slots a second time. Attach the comb (64-sharkstooth-comb) to the gel. Denature the samples (70°C, 3-5 minutes) and store them on ice before plotting them onto the gel using a 8-channel-Hammilton syringe (max. 0,8 µl sample for every single slot). Cover the buffer chamber and start the electrophoresis. The run lasts 5-10 hours. The generated data will be stored automatically.

Denotation of the bases and ambiguities according to the IUPAC glossary:

IUPAC	Meaning	Complement
A	A	T
C	C	G
G	G	C
T/U	T	A
M	A, C	K
R	A, G	Y
W	A, T	W
S	C, G	S
Y	C, T	R
K	G, T	M
V	A, C, G	B
H	A, C, T	D
D	A, G, T	H
B	C, G, T	V
N	G, A, T, C	N

DGGE (Denaturing Gradient Gel Electrophoresis)

The DGGE is a molecular technique used to separate different DNA sequences of the same length from each other. The separation is carried out via a polyacrylamidegel composed of a gradient of denaturing agent (urea and formamide). Diverse DNA sequences run in a different way from each other resulting in a separation due to the amount of GC and generating melting domains (Muyzer et al. 1993).

Figure 13: DGGE apparatus.

I. Materials

DGGE-Equipment (Ingeny)

a) Gel
- Urea
- Formamide
- 50 x TAE buffer pH 7,4 (242 g Trisbase, 57.1 ml conc. Acetic acid, 100 ml 0.5 M EDTA pH 7.4, fill up to 850 ml using ddH_20 (pH 7.4) fill up to 1l using ddH_20.
- Acrylamide/Bisacrylamide 40 % (37.5:1)
- ddH_20
- 10 % Ammoniumpersulfate (APS)
- Tetramethylethylendiamine (TEMED)
- Loading buffer

SAFETY ADVICES

Acrylamide (C_3H_5NO): toxic (R: 45-46-20/21-25-36/38-43-48/23/24/25-62 S: 53-45)
Ammoniumpersulphate (APS) ($H_8N_2O_8S_2$): oxidising, harmful (R: 8-22-36/37/38-42/43
S: (2)-22-24-26-37)
Tetramethyl ethylendiamine (TEMED) ($C_6H_{16}N_2$): corrsosive, highly flammable (R: 11-20/22-34 S: (1/2)-16-26-36/37/39-45)

b)
- Ethanol 70%
- 3 x 50 ml Beaker
- Cannula (yellow) and tubes
- 2 Glasplates (1x block out at the top, 1x block out at the bottom)
- Spacer (U-shaped)
- casting equipment (= electrode chamber)
- Comb (20 - 48 choppers)
- Srew conservation
- Gradient mixer
- DGGE-Equipment
- Magnet stirrer

Figure 14: Gradient mixer.

c) Staining

- 2 black tanks
- 1000 ml 1 x Sybr Gold dye solution (100 µl Sybr Gold (10.000 x concentrated) will be diluted in 1000 ml 1 x TAE-buffer pH 7,4)
- 1000 ml ddH_2O
- Gel beam (UV-permeable)
- Shaker

II. Prearrangement

Work neatly:
- Clean the glas plates, the spacer and the comb using ddH_2O and ethanol.
- Combine the glas plates with the spacer and the screw protection.
- Mount the glass plates into the inductor.
- Put the comb between the glas plates.
- Stick together the tubes of the gradient mixer.
- Put the magnetic stirrer into the first chamber and the contrary volume (tube) into the retral chamber.
- Start the magnetic stirrer.
- Prepare 3 beakers, APS, TEMED as well as the stock solutions (refrigerator).

III. Casting the gel

- Set up the gel solution in the beakers under the fume hood and mix them slightly.
- Add APS and TEMED on the verge of casting the gel (Add APS and TEMED to the 0% solution later on).
- Transfer the lower concentrated solution into the posterior chamber of the gradient mixer.
- Open the plug quickly in order to remove air bubbles.
- Transfer the higher concentrated solution into the anterior chamber of the gradient mixer. Don`t put the cannula on the tube yet, because of the resistance will be too high.
- Afterwards, put the cannula onto the tube.
- Put the gradient mixer onto the magnet stirrer. Hold the tube as high as possible (at least higher than the gradient mixer is situated).
- Connect both chambers by turning over the plug.
- Clamp he cannula between the both glas plates and let the gel run between them. Thereby you should notice the mixture of both solutions in the anterior chamber of the gradient mixer (Formation of striae).
- Let the complete solution drop out of the tube. Thereby, you have to take care that the cannula doesn`t dip into the gel and that the gel solution doesn`t reach the comb during the casting. Preliminarily, you have to remove the cannula and let the residual solution drop out into a beaker.
- Add APS and TEMED to the 0% solution.
- Absorb the solution using a 10 ml syringe and put a cannula onto it.
- Pipette the solution carefully on one side onto the gel. Change sides. Take care that the gradient doesn`t reach the comb.
- Cast the gel up to the border of the plates, so that the gel will almost overflow.
- Wait at leat 2-4 hours until the gel is polymerized.
- Spill all beakers, tubes and equipment you have used with ddH_2O.

Stock solutions 0% and 80%:

Substance	Stock solution 0 % denaturing	Stock solution 80 % denaturing
Urea	-	50.4 g
Formamide	-	48 ml
50 x TAE, pH 7,4	3.0 ml	3.0 ml
Acrylamide/Bisacrylamide	22.5 ml	22.5 ml
Fill up with ddH_2O	150 ml	150 ml

Gel solutions: e.g. gradient of 50-70%

Substance	Gel	Low concentrated (50 %)	High concentrated (70 %)
Stock solution 0 %	9 ml	9 ml	3 ml
Stock solution 80 %	-	15 ml	21 ml
End volume	9 ml	24 ml	24 ml
Add on the verge of use:			
TEMED	8 µl	17 µl	17 µl
APS (10 %)	100 µl	86 µl	86 µl

If you use another gradient you have to mix up the stock solutions in a different proportions to each other. The end volume of the gel solutions need to be a volume of 24 ml. The amounts of TEMED and APS stay the same no matter which gradient you use.

IV. Loading the gel and electrophoresis

- Produce 17 l loading buffer directly in the electrophoresis chamber using the stock solution (50xTAE). You just have to exchange 5 l loading buffer for further runs. In order, you have to remove 5 l used buffer and produce 5 l new buffer using the stock solution (50 x TAE) and fill up the DGGE chamber.
- Attach the chamber cover.
- Put the casting equipment with the gel into the electrophoresis chamber at least 1.5 hours before loading the gel.
- Connect the plugs of the electrophoresis and the pump to the casting equipment and turn on the Low Voltage (LV) supply. Let the buffer heat up to 60°C.
- While heating up the buffer, you have to prepare the PCR samples to load the gel later on. Possibly you have to purify your samples (Purification of PCR-products). Final volume max. 40 µl.
- Load the gel using a „Hamilton“ syringe with max. 40 µl for every single gel slot. Flush the syringe after the loading of each sample.
- Pipette 5 µl loading buffer into the empty slots.
- Pipette 10 µl DGGE-standard (mixture of 16S rRNA products of pure cultures).
- Turn on High Voltage (HV) up to 100 V.
- The running time is 20 hours.

V. Analysing the DGGE gel

- Turn off the voltage.
- Take the gel out of the DGGE brackets and release the screws.
- Take off the upper glass plate carefully.
- Take off the DGGE spacer and mark one edge of the gel by cutting them in order to remember the loading direction later on.
- Put the gel bracket into the staining tank and fill in the staining solution.
- Transfer the gel into the staining tank by inverting the glas plate with the gel and holding it into the staining solution.
- Use 1 x Sybr Gold staining solution in order to stain the DNA (light sensitive!)
- Cover the staining tank with the another black tank.
- Shake slightly 1-2 hours.
- Transfer the staining solution back into the bottle (the solution can be used several times).
- Cover the gel with ddH_2O and incubate it 15 minutes.
- Document and save the gel parameters on the UV/blue light table using a documentation program.

Wear cloves and a lab coat during staining procedures!

Figure 15: Staining tank.

Quantitative PCR

The quantitative PCR, also called *Real-Time*-PCR, is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity (Higuchi et al. 1993). The time point where the target amplification is first detected depends on the amount of targets in the template. This value is referred to as cycle threshold (C_t), the time at which fluorescence intensity is greater than the background signal (Wong et al. 2005). Therefore the quantitative PCR is one potential method to quantify specific targets and thus the number of organisms in the sample.
There are several *Real-time* PCR detection chemistries:

- Application of specific primers in combination with DNA binding dyes (SYBRGreen I), which emit fluorescence when bound to dsDNA. As the double-stranded PCR product accumulates during cycling, more dye can bind and emit fluorescence.

- Application of fluorescence labelled specific probes (For instance: FRET- and TaqMan-probes, Molecular Beacons etc.)

We are using SYBRGreen for detection.

Procedure
Q-PCR amplification is performed in a volume of 25 µL containing 12.5 µL of the DyNAmo HS SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland) and 10 µL of the 1:10 diluted DNA templates. Thermal cycling is performed using a Rotor-Gene, RG-3000 four –channel multiplexing system (Corbett Research, Sydney, Australia) with the following parameters: 95°C initial hold for 15 min to activate the *Taq* polymerase, followed by 50 cycles of amplification, with each cycle consisiting of denaturation at 94°C for 10s, followed by 20 s of annealing at primer specific temperatures and an extension step of 30 s at 72°C. Fluorescence was measured at the end of each amplification cycle for 20 s at primer specific temperatures (Wilms et al. 2006).
To verify the results, every quantification was repeated three times at the same concentrations of all chemicals and templates.

Solution	Volume[µl]
10 x buffer (2mM $MgCl_2$)	2.5
dNTPs (2.5mM each)	2
BSA(20mg/mL)	0.25
$MgCl_2$ (10mM)	0.75
PCR H_2O dest.	7.4
357f (10pmol/µL)	0.5
907R (10pmol/µL)	0.5
SYBRGreen I (1:400)	1
NEB Taq-Polymerase (5U/µL)	0.1
DNA-Template	10
Final volume	25

Temp. [°C]	Time [min]	Cycles
96	4	1
94	1	
55	1	40
72	2	Fl.-detection
72	10	1
25	1	1

The thermo cycler

Thermal cycling is performed using a Rotor-Gene, RG-3000 four–channel multiplexing system (Corbett Research, Sydney, Australia). The cycler operates with a computer using the software Rotor-Gene. For detail see „Rotor-Gene User Manual“. The cycler provides a 37/72 well dual-channel-system. We will use the 36 well rotor using 36 flat-cap tubes (0.2 mL). The rotor has to be completely loaded (no empty positions). The Rotor-Gene-cycler has 2 detection units. Channel 1 detects at 510 nm and channel 2 at 555 nm. Therefore you can run a multiplex detection using 2 dye solutions at the same time. Once the tube passes the detection unit, it will be excited caused by high energy flashes. The resulting emission light will be recorded by a photomultiplier. The data will be transferred to the PC and represent graphically.

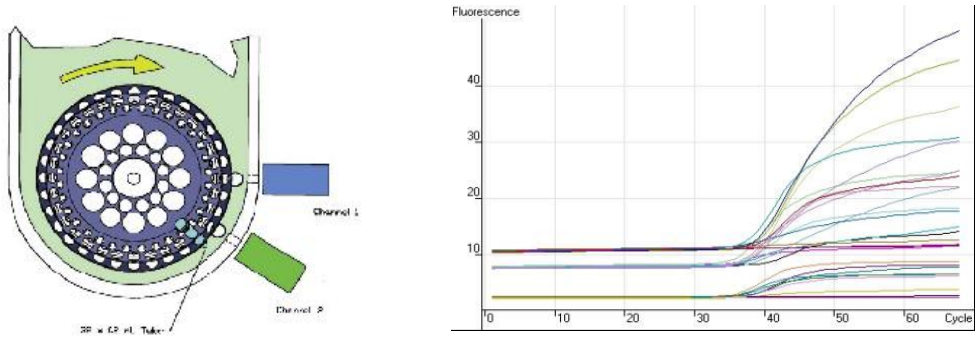


Figure 16: Left-hand side: Rotor-Gene, RG-3000 four-channel multiplexing system Right-hand side: Graphical presentation of recorded data.

The Rotor-Gene Software

Before starting the Rotor-Gene Software you have to switch-on the cycler.
After starting the Rotor-Gene program the main window „new experiment“ will appear.

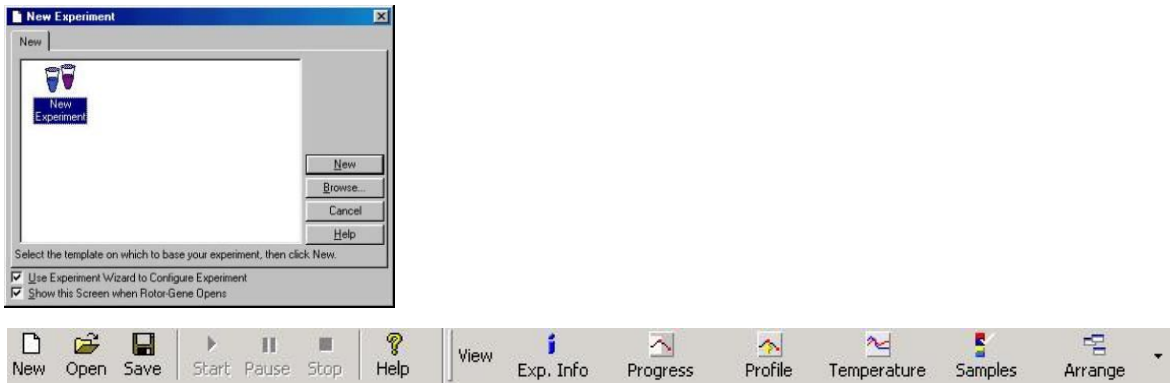


Figure 17: Rotor-Gene Software toolbar.

Before starting the run, set up your experimental parameters using the toolbar.

Exp. Info (settings)
The name of the operator and the name of the experiment has to be inserted. Also the reaction volume, the detection channel and the kind of rotor has to be chosen.

Profile
The temperature program for the run has to be set up. You can choose among the options HOLD, CYCYLING and MELT.
HOLD: Choose a temperature, which will be hold over a certain time period (DENATURE: = special option for the first denaturing step).

Cycling
Choose max. 5 following temperatures and time periods (Denaturing, Annealing, Elongation). Set the time point of fluorescence detection. The fluorescence detection will take place directly after the elongation step (72°C), because the DNA binding dyes can only bind into double stranded DNA. Because of the application of binding dsDNA dyes, unspecific products (for instance: primer dimers) can be detected. To eliminate primer dimers, a temperature of 80°C will be set up at the end of the run and melt on the primer dimers. Furthermore, the presentation of the raw data can be chosen (normal/High Sensitive/Low Sensitive) – you can also choose all of them.

Melt
At the end of the PCR-program a melting curve will be recorded. Therefore, the initial temperature (often 50°C) will be raised to 99°C using 1°C steps. At the same time the fluorescence of all samples will be detected. Before running the melting program the initial temperature of the melting curve has to be hold constantly for at least 1 min.

Samples
Choose the names of the samples. You can choose among SAMPLE, NTC (non template control), STANDARD and NONE. Set up the concentration or the copy numbers of your standards.

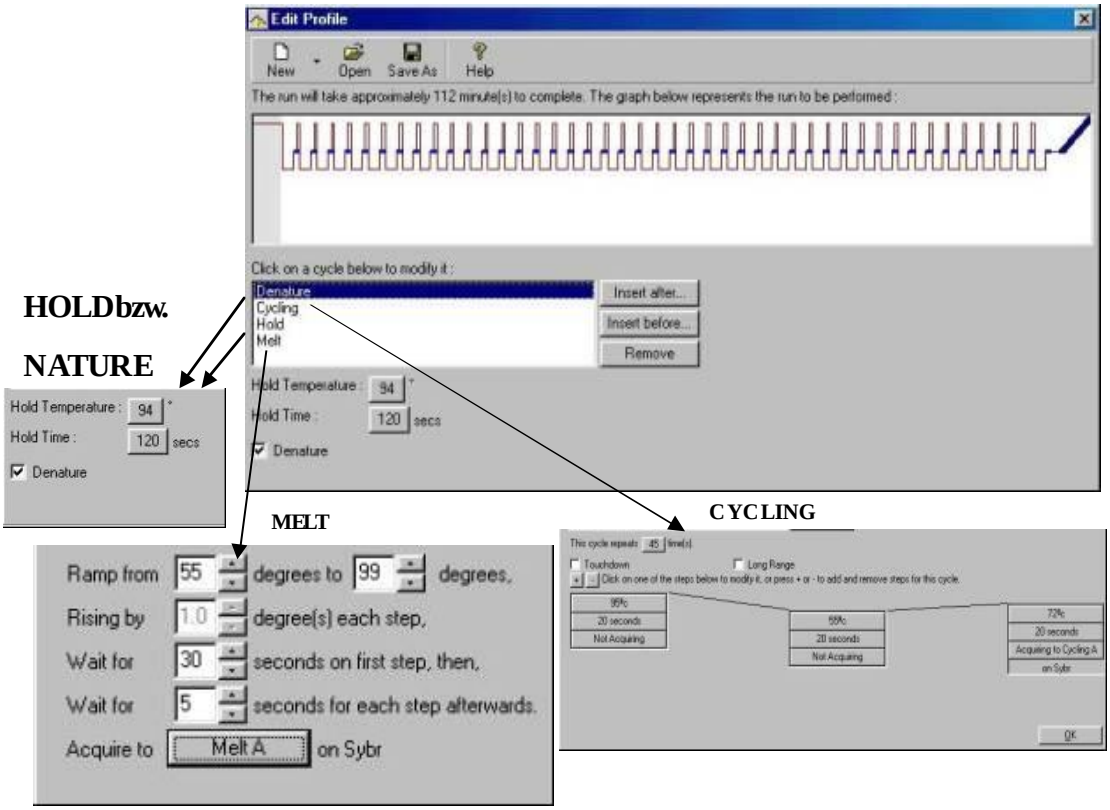
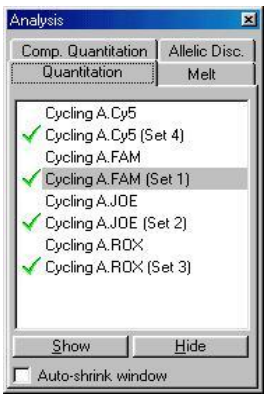


Figure 18: Adjusting the settings.

Start
You can see the PCR profile again before starting the run. Furthermore, the experiment will be saved automatically. You can have a view on the raw data of the PCR run (fluorescence detection, temperature profile) during the entire run.

After the run completed: Choose the window „analysis“.
The window will be opened by pressing the button „Analysis“ in the toolbar.



You can perform new analysis or accomplish older ones. You can choose among: COMP. QUANTITATION, ALLELIC- DISC. ,MELT and QUANTITATION. If you choose one of them, the program will bring up a list of possible channels used for your analysis. Double click on the channel name and the analyses will be shown up.

Figure 19: Analysis software.

Quantitation
Double click on the function „Quantitation“ and 3 windows will be opened: the main window showing the curves of the raw data, the standard curve window, and the results window.

Main window
The raw data will be recorded in a linear and logarithmical scale.

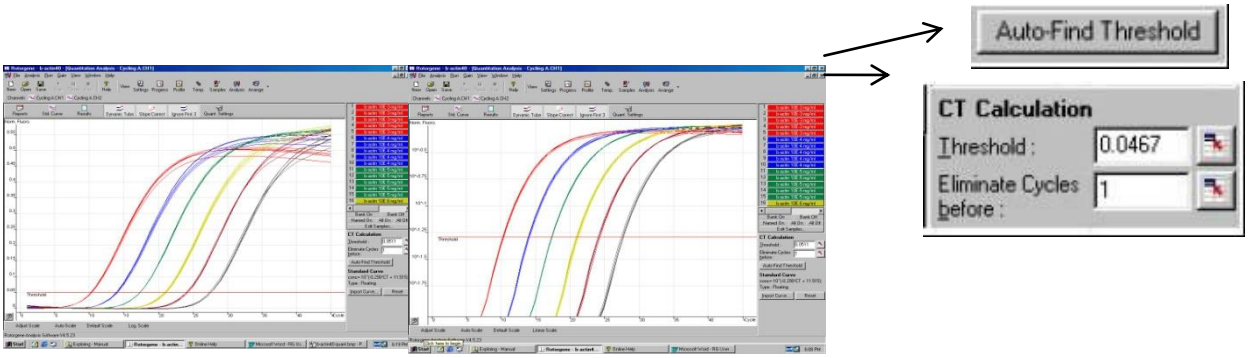
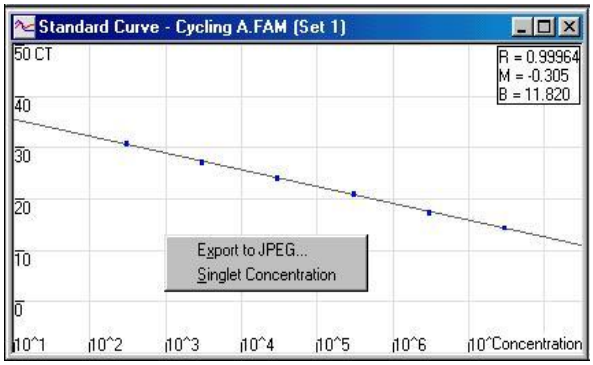


Figure 20: Graphical presentation of recorded in a linear and logarithmic manner.

Ct-Calculation
The Ct-value (cycle-time value) is the point where the threshold passes the amplification curve and indicates the starting point of the exponential amplification The CT-value can be correlated to the initial copy numbers but can also be set up automatically using the AUTO-FIND-THRESHOLD button.

Standard curve
This standard curve shows the Ct-values plotted against chosen units (concentration, copy numbers …). You can chance the standard curve by adding or deleting standard values. The blue points indicate the standard and the red points the unknown samples.



R-value: Correlation coefficient: Number between 1 and -1, defines the relation of 2 variables (y = mx+b) in the curve (value between 0.99 and -0.99).

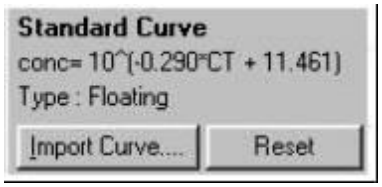


Figure 21: Standard curve.

CONC: Defines the correlation between Ct-value and target concentration of the template.

TYPE FLOATING: The standard curve will be calculated again by changing the threshold.

TYPE FIXED: The standard curve stays unchanged by changing the threshold (For instance, by using the standard curve also for other PCR runs).

IMPORT CURVE: You can choose and import a standard curve from another PCR run and use it for your current calculations.

Quant. Results - Cycling A.FAM

No.	Name	Type	Ct	Given Conc.	Calc. Conc.	CV	Ct Std. Dev.
1	BCL-2 gen. DNA (1:1)	Standard	19.40	100,000	100,000	0.00%	0.05
2	BCL-2 gen. DNA (1:1)	Standard	19.44	100,000	100,000	0.00%	0.05
3	BCL-2 gen. DNA (1:1)	Standard	19.55		100,000	0.00%	0.05
4	BCL-2 gen. DNA (1:1)	Standard	19.43		100,000	0.00%	0.05
5	BCL-2 gen. DNA (1:1)	Standard	19.50	100,000	100,000	0.00%	0.05
6	BCL-2 gen. DNA (1:1)	Standard	19.44	100,000	100,000	0.00%	0.05
7	BCL-2 gen. DNA (1:1)	Standard	19.39	100,000	100,000	0.00%	0.05
8	BCL-2 gen. DNA (1:1)	Standard	19.41	100,000	100,000	0.00%	0.05
9	BCL-2 gen. DNA (1:1)	Standard	19.39	100,000	100,000	0.00%	0.05
10	BCL-2 gen. DNA (1:1)	Standard	19.40	100,000	100,000	0.00%	0.05
11	BCL-2 gen. DNA (1:1)	Standard	19.41	100,000	100,000	0.00%	0.05
12	BCL-2 gen. DNA (1:1)	Standard	19.40	100,000	100,000	0.00%	0.05
13	BCL-2 gen. DNA (1:1)	Standard	19.46	100,000	100,000	0.00%	0.05
14	BCL-2 gen. DNA (1:1)	Standard	19.39	100,000	100,000	0.00%	0.05

Export to Excel

Figure 22: Result window.

Result window
The result window shows the Ct-values and there standard deviation (CT STD. DEV.), the standard defined concentrations (GIVEN CONC.) and the calculated concentrations (CALC.CONC. = m*Ct + b). In optimal case, the GIVEN CONC. And the CALC. CONC. should have the same value, so the CV-value (Variation coefficient) will be 0. The result file can be exported to Excel using the right mouse button.

Melt
The melting curve shows the melting characterization of the PCR samples after finishing the PCR run. So you can distinguish between specific and unspecific products (primer dimer).

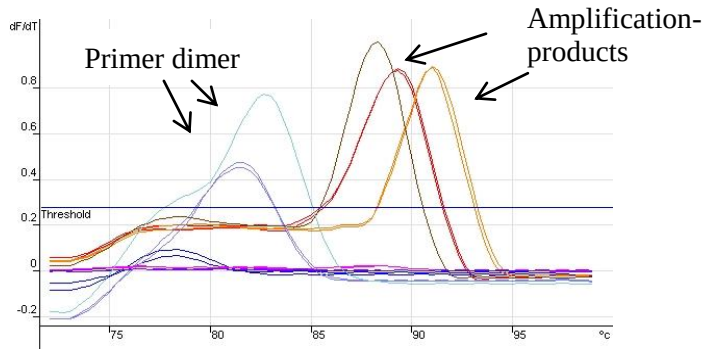


Figure 23: Melting curve

Agarose gel electrophoresis
After finishing the pCR run, 5 µl of PCR products (+ 3 µl loading dye) will be plotted on a 1.5% agarose gel (see agarose gel electrophoresis).

7. Isolation of anaerobic and sediment bacteria

Enrichment and isolation of abundant heterotrophic sediment bacteria

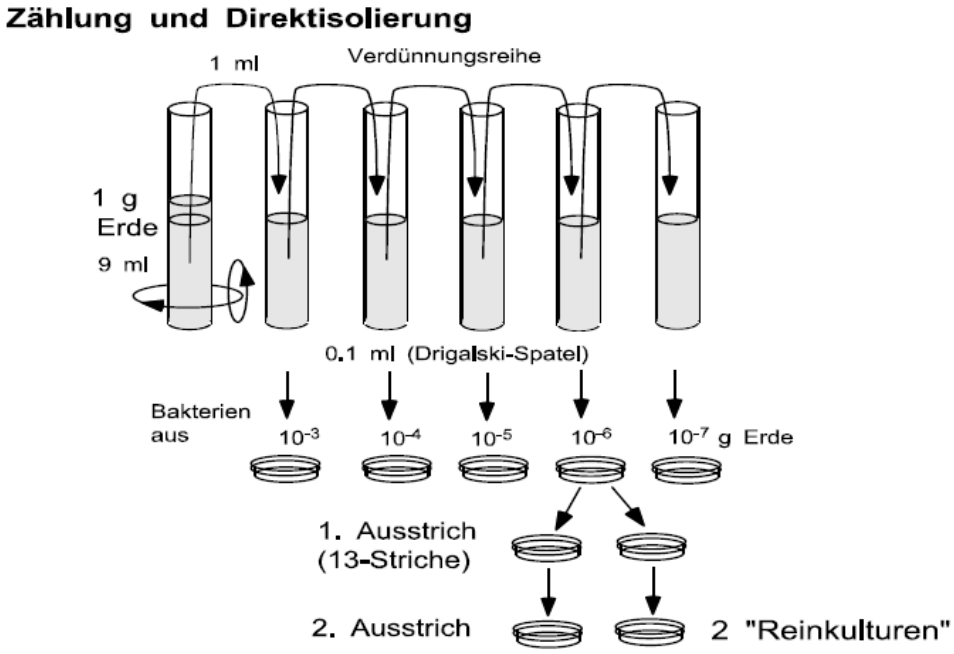


Figure 24: Counting and direct isolation.

Procedure
Liquid dilutions and agar plates are checked for growth (OD measurements, microscopy of colony cells: suspend the colony in 50 µl Tris-buffer, transfer 5 µl of them onto a microscope slide and put a cover slip on it, use the 40x lens). The liquid cultures and agar plates having the highest growth are used to set up subscultures via the 13-streak-method. If subcultures are grown, the form and colour of the colony as well as the cell types will be compared to the initial culture microscopically. New subcultures are set up to yield single and pure cultures. The growth is checked daily, the presence of colonies is recorded and colony types are specified. Pure cultures will be harvested and used for physiological characterization.

OD-measurement
The OD-measurements are done using the same photometer for all measurements. Aerobically grown cultures are examined using the one-cuvette-method. The photometer is adjusted to zero by using water. After drying the cuvette using a filter paper, samples are measured in the same cuvette. The OD should not exceed 0.3, otherwise 70 mM phosphate buffer will be used for diluting samples properly. The screw covered tubes containing the anaerobe growing cells will be kept locked until the end of the experiments (even if the OD > 0.3). They will be measured against a tube containing water. Measurements are performed using a photometer, which can handle tubes. Tubes are adjusted in a way that the measured OD is as low as possible. The precise position is marked. For further measurements, tubes are always set in the same position.

Isolation of anaerobes from agar deeps

Preparation of so-called 'shaking-tubes'
300 ml double-distilled water and 12 g grinded agar are added to a 500 ml Schott bottle (determine the empty weight including lid and magnetic stir bar). The suspension is stirred for 10 minutes. Afterwards the agar is allowed to settle down (~ 20 minutes). The water is carefully decanted. The performed washing step is necessary to remove growth-inhibiting ingredients and hydrolysis products. The procedure is repeated five times. Subsequently, the agar is filled up on a balance (300 g + empty weight of the bottle) and autoclaved (20 minutes, 120 °C).

Place used pipettes in a Erlenmeyer flask filled hot water and boil them out.

Agar dilution series

Needed material/equipment
- per series, 7 properly labelled, sterile test tubes and suitable rubber stoppers (keep some backup stoppers ready)
- 5-times washed, sterile agar (4%)
- water bath preheated to 42 °C
- water bath preheated to 65 °C
- water bath (icy water)
- 2 Bunsen burner
- 1 50 ml bottle (completed medium)
- sterile 1ml, 10ml pipettes

Procedure
The agar is liquidated and kept at 65 °C, 3 ml of agar are added to the single test tubes, which are kept at 42 °C. Next, 6 ml completed medium are added to each test tube. One series of test tubes is removed from the water bath and placed in a rack at room temperature. From the culture of interest, one drop (~ 0.5 ml) is added to the first test tube (slew the tube slightly). Transfer one drop (~ 0.5 ml) from the first tube to the second one. Place the first tube in the cold water bath (icy water) and so on. Before each transfer, wipe the corresponding tube to prevent water drops from being transferred (unwanted dilution). Before incubating the tubes at designated temperatures, N_2/CO_2 (80/20, v/v) is introduced.

Obtained single colonies can be collected for microscopic investigation and for getting pure cultures using a 1ml syringe and a proper cannula.

8. Physiological characterisation

Catalase test

Reagent
5% H_2O_2 solution, produced by diluting a 30% stock solution with distilled water. Store in a cool and dark place. Do not touch with naked hands! Contaminations (e.g. dust) provoke corrosion.

Procedure
Place some bacterial cell material from the middle of a fresh colony on a clean slide. Add one drop of the diluted H_2O_2 solution using a clean Pasteur-pipette. Formation of gas (O_2) shows catalase activity.

Oxidase test

Reagents

H_2O	100 ml
Ascorbic acid	0.1 g
Tetramethyl-p-phenylendiamin-HCl	1.0 g

SAFETY ADVICES

Tetramethyl-p-phenyldiamine:harmful
(R: 20/21/22 S: (2)-28)

Attention, carcinogenic! Reagents (will be provided, freshly prepared every day!). Do not touch with naked hands and prevent spilling! After using, vessels and pipettes are immediately cleaned!

Procedure
Soak a strip of filter paper (cellulose) with some drops of the reagent (not too wet!). Add some bacterial material taken from a colony with an inoculation loop on the strip and rub it using a rounded glass rod. Blue colouration indicates oxidase activity. As control, results are compared with reference strains of known activity (e.g. E.coli).

Determining the substrate spectrum of aerobic isolates

The substrate test is the centre of the physiological characterisation of bacterial isolates. Bacterial growth is tested in respect of 59 carbon compounds. () = final concentration

Complex substrates:	Peptone (0.05 %), Casamino Acids (0.05 %), Yeast extract (0.005 %)
Polymer substrates:	Cellulose (0.05%), Starch (0.1 %), Chitin (0.05 %), Xylane (0.05 %), Laminarin (0.05 %)
Disaccharides:	Saccharose (5), Cellobiose (5), Maltose (5), Trehalose (5)
Monosaccharides:	Arabinose (5), Rhamnose (5), Xylose (5), Fructose (5), Glucose (5), Mannose (5),
Sugar derivatives:	Mannitol (5), Gluconate (5), Glucosamine (5)
Carboxylic acids:	Formiate (5), Acetate (5), Propionate (1), Butyrate (2.5), Valerate (0.5), Capronate (0.5), Caprylate (0.5), Crotonate (0.2)
Dicarboxylic acids:	Malonate (5), Succinate (10), Fumarate (5), Malate (5), Tartrate (2)
Other organic acids:	Glycolate (5), Pyruvate (5), Lactate(10), 2-Ketoglutarate (5), Citrate (2)
Alcohols:	Methanol (2), Ethanol (5), Propanol (5), Butanol (5), Glycol (5), Glycerine (5), Tween 80 (0.001 %)
Amino acids:	Alanine (2), Arginine (2), Asparagine (2), Cysteine (2), Glutamine (2), Isoleucine (2), Phenylalanine (2), Tryptophane (2), Proline (2)
Amines:	Betaine (2)
Aromatic compounds:	Benzoate (2), Salicyclic acid (2)
Heterocyclic compounds:	Niacin (2)

The assay is set up in 200 µl microplates (see below) aseptically. 140 µl medium, 40 µl substrate (from a master plate) and 20 µl of the bacterial cell suspension are pipetted into one well. The plates are incubated at 20 °C. As control, one well is set up without cells and one without substrate.
Important: cells are washed before the inoculation to prevent the entry of foreign substrates. 1.5 ml liquid culture are centrifuged down, the supernatant is carefully removed ,discarded and the pellet is resuspended in 1.5 ml fresh medium. All steps are carried out under sterile conditions (clean bench).
The evaluation is done based on the turbidity due to bacterial growth in the inoculated wells. Spot tests are done regarding single wells as control. It has to be considered that used substrates partially cause a turbidity independent from bacterial growth.

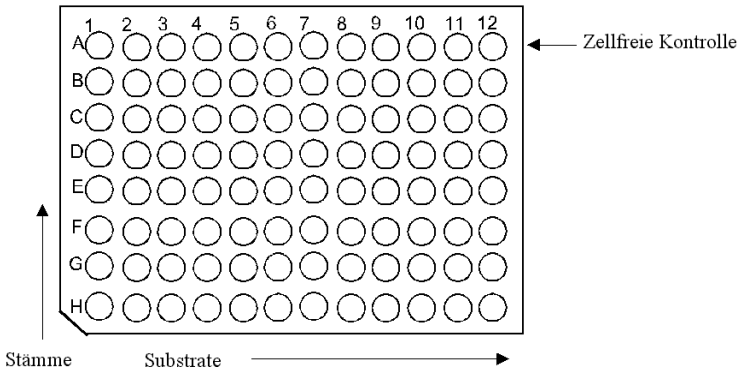


Figure 25: Scheme for the inoculation of microplates with respect to testing various substrates.

Recording of growth curves

The most important parameter in the context of growth experiments is the growth yield regarding specific substrates. This circumstance is reflected by individual dry weights and cell counts. Growth rates of bacteria cultures are determined by turbidity measurements (optical density, OD) using the "Ratio/XR Turbidimeter" (HACH, Germany). To convert the OD to cell counts, a conversion factor has to be determined. Therefore, a culture exhibiting a strong turbidity is analysed in respect to its cell concentration and then serially diluted. The OD of the dilutions is noted and the respective cell counts calculated (cells/OD).

Equation to calculate growth rates:

$$\mu = \frac{ln\,(x) - ln\,(x_0)}{(t - t_0)}$$

µ = growth rates (h^{-1})

x = OD (current date)

x_0 = OD (at the beginning of exponential growth)

$t - t_0$ = period examined (h)

Turbidity measurements of microbial growth
The turbidity ($OD_{436\ nm}$ against water) is measured to estimate the growth yield. The photometer is set to zero using water (3 ml-cuvette, glas cuvettes are preferred) and the OD of the bacterial suspension is measured. Values should not exceed 0.3, otherwise aerobic cultures should be diluted prior to the measurement (using 70 mM phosphate buffer). Cultures grown under anaerobic conditions are not diluted. They are grown in tubes and measured against a tube filled with water. When the tubes are measured for the first time, the minimum absorption for each tube is found by turning the tube around in the photometer. The corresponding positions are marked on the tubes and the same position is used (after adjusting) to measure the OD throughout the experiment.

Taking samples
Immediately after inoculation, and at least every 60 minutes thereafter, 1 ml samples are taken from aerobic cultures and investigated regarding the OD and cell counts (max. once every hour). From anaerobic samples, only the OD is measured. The OD (and maybe other parameters) of slowly growing strains should be checked once a day or even a larger time interval during lag phase. Temperature is an important parameter for biological turnover rates and should therefore remain constant. In no case leave cultures standing at room temperature.
During the exponential growth phase, decrease the intervals at which samples are taken. OD data sets are plotted semi logarithmically.

Determination of β-glucosidase activity

β-glucosidase (=cellulase) hydrolysis the β(1,4)-glycosidic bonds between the glucose units of cellulose.

Principle
The β-glucosidases within a sample hydrolyse the non-fluorescent substrate analogue 4-methylumbelliferyl-β-D-glucoside. The cleaved 4-methylumbelliferone is detected fluorometrically.

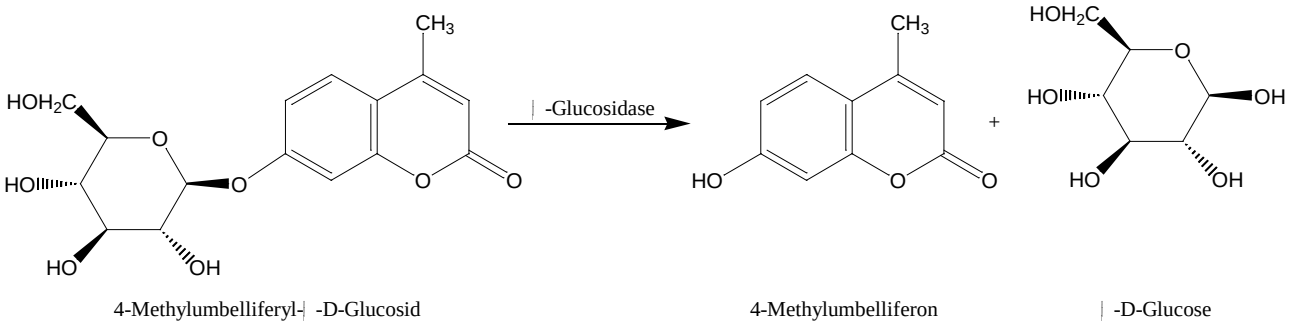


Figure 26: Principle of the assay for determining β-glucosidase activity.

Equipment	Reagents
spectrofluorophotometer	4-methylumbelliferyl-β-D-glucoside
test tubes including a rack	4-methylumbelliferone (MUF)
Vortexer	glycine
magnetic stirrer	sodium chloride
pH-electrode	sodium hydroxide
automatic pipettes	ethylene glycol monomethyl ether
25°C water bath	
centrifuge	

SAFETY ADVICES

4-methylumbelliferone: irritant (R: 36/38 S: 2 – 46)
Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)
Ethylene glycol monomethyl ether ($C_4H_{10}O_2$): toxic (R: 60-61-E20/21/22-10 S: 53-45)

Substrate analogue stock solution
3 mg 4-methylumbelliferyl-β-D-glucoside are completely dissolved in 0.5 ml ethylene glycol monomethyl ether and 0.5 ml sterile, double distilled water are added (in a glown out vessel). The substrate analogue solution has to be prepared freshly each day.

Fluorophore stock solution
10 mg 4-methylumbelliferone are dissolved in 5 ml ethylene glycol monomethyl ether and 5 ml double distilled water are added (glown out vessel). 100 µl of this solution are filled up to a final volume of 100 ml (final concentration: 1 µg/ml) using sterile double distilled water (in a glown out vessel). The solution is stored in the dark at 4 °C.

Glycine buffer
0.75 g glycine and 0.58 g sodium chloride are dissolved in 100 ml of sterile double distilled water (glown out vessel). The pH is adjusted to 10 using 1 M sodium hydroxide.

Procedure
Per sample three parallels are set up. 100 µl of substrate analogue solution are added to 1 ml of sample. Blank: 1 ml of sterile double distilled water (in a glown out vessel) + 100 µl of substrate analogue solution. Samples are incubated at 25 °C for two hours using a water bath. In order to stop the reaction, 75 µl of 0.1 M glycine buffer (pH 10) are added. The samples are well mixed and measured at an excitation wavelength of 360 nm and an emission wavelength of 440 nm against a blank using a spectrofluorophotometer.
If a high background fluorescence is detected, the blank is prepared by mixing 1 ml of sample with 100 µl of sterile double distilled water and treated the same way as the samples. The blank is measured against a fluorometric blank (3.3 ml of sterile double distilled water). The fluorescence of the blank is subtracted from the fluorescence of examined samples.

Calibration curve
For preparing a standard series, between 10 and 70 µl MUF stock solution are added to 3.3 ml of sterile double distilled water (in a glown out vessel). 750 µl glycine buffer are added, the solutions are well mixed and measured against a blank (3.3 ml sterile double distilled water + 750 µl glycine buffer). The standard series has to be measured along with each test series.
The determination of β-glucosaminidase, leucine-aminopeptidase and β-glucosidase activity using microtiterplates is done with slight modifications to the procedure described.

Determination of β-glucosaminidase activity

β-glucosaminidase (=chitinase) hydrolyses the β(1,4)-glycosidic bond between the N-acetyl-glucosamine units of chitin.

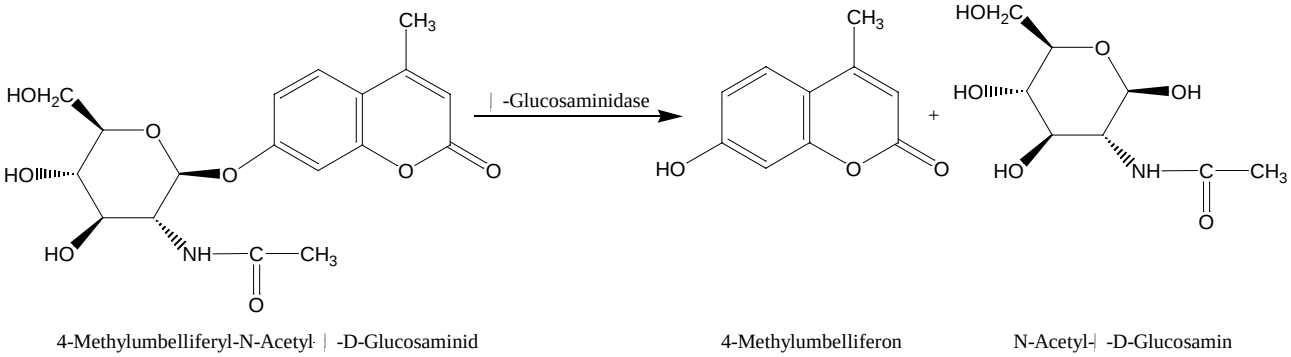


Figure 27: Substrate analogue: 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide.

Determination of leucine aminopeptidase activity

Leucine aminopeptidase cleaves peptone, which is made up by a mixture of peptides and amino acids from animal or plant proteins.

Substrate analogue stock solution

- prepare 100 ml of a 2 mM L-leucine-7-amino-4-methylcoumarine solution in sterile double distilled water (in a glown out vessel, 12 hours at 180 °C)
- storage at - 20 °C in the dark
- necessary concentrations are prepared after thawing

SAFETY ADVICES

Ammonium hydroxyide(NH_4OH): corrosive, harmful to the environment (R: 34-50 S: (1/2)-26-36/37/39-45-61)
Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)

Fluorophore stock solution

- prepare 1 l of a0.1 mM 7-amino-4-methylcoumarine solution in 10 ml ethylene glycol monomethyl ether and 990 ml sterile double distilled water (in a glown out vessel)
- aliquotted and stored at - 20 °C in the dark
- necessary concentrations are prepared after thawing

Ammonium glycine buffer

- prepare 1 l of a 0.2 M ammonium hydroxide / 0.05 M glycine solution using sterile double distilled water (in a glown out vessel), adjust the pH to 10.5 using 5 M sodium hydroxide

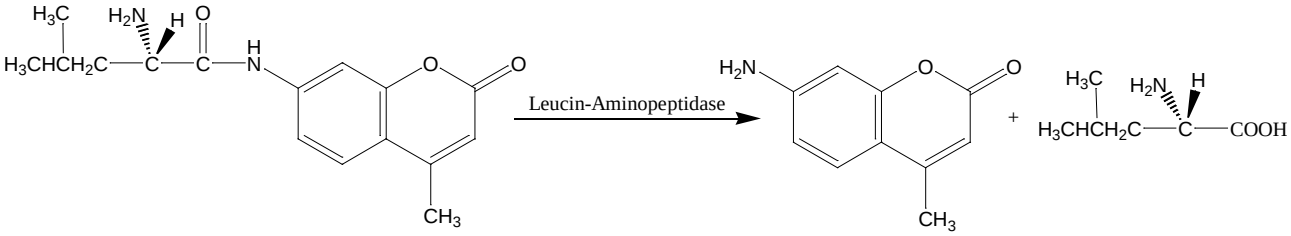


Figure 28: Principle of the assay for determining leucine aminopeptidase activity.

Procedure

1 ml of sample + 1 ml of substrate analogue solution (for calibration, 1 ml of sample + 1 ml fluorophore solution). Blank: 1 ml of sterile double distilled water (in a glown out vessel) + 1 ml of substrate analogue solution (for calibration, 1 ml of sample + 1 ml sterile double distilled water).
In order to stop the reaction, samples are cooked for 3 minutes and cooled down to 25 °C. Next, 1 ml of ammonium glycine buffer is added, particles are allowed to settle down and the supernatant is transferred and centrifuged for 15 minutes at 13000 rpm.

Determination of β-glucosaminidase activity in microplates

Necessary equipment: 96-well plates
automatic multichannel pipettes
microplate reader

Prepare a substrate analogue solution (10-fold amount).
Prepare 1l of a 0.14 M sodium chloride solution.

Procedure
200 µl from each sample are added in triplicate to a microplate and mixed with 20 µl of a substrate analogue solution. As sample blank, 20 µl of sodium chloride solution are added to 200 µl of sample. In order to set up a photometric blank, 200 µl of sodium chloride solution are mixed with 20 µl of substrate analogue solution. The microplates are covered and incubated at 30 °C for 24 hours. After the incubation, the pH of the single wells is adjusted to 10 using 50 µl glycine buffer. The plates are analysed using a microplate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm.

Calibration curve
For preparing standards, between 10 and 70 µl MUF stock solution (at least three standards) are filled up with sterile double distilled water (in a glown out vessel) to 200 µland 20 µl of substrate analogue solution are added. A standard series is prepared along with each test series and treated the same way as the samples. After incubating them at 30 °C for 24 hours, the pH of each well is adjusted to 10. The plates are analysed at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Determination of ATP (Bergmeyer, 1983)

The determination of ATP is based on the reaction of ATP with luciferin and oxygen in presence of the enzyme luciferase.

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow{\textit{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{Light}$$

If the reagents besides from ATP are present in excess, the light emission is proportional to the amount of ATP present. Using a calibrated measuring system, the measured light intensity can be related to the ATP content of the sample. The light emission is determined using a luminometer (LKB Wallac). The intended intensity is adjusted by an internal ^{14}C emitter. A recorder at the outlet of the luminometer collects measured values in mV.

In order to enable the determination of the ATP content of cells, it is necessary to disrupt them first.

Extraction of ATP (Blaut and Gottschalk, 1984)
100 µl of 3 M perchloric acid stored on ice are pipetted into an Eppendorf tube, 100 µl of sample are added and the solution is shaken shortly. The shaking is repeated every half an hour (samples stored on ice). After 1 1/2 , 50 µl of 1 M Tres buffer (pH 7.4) and 112 µl of 3 M KOH are added to adjust the pH to a value between 7 and 8. The potassium concentration in the neutralised extract is sufficient to precipitate the perchlorate, which would interfere with the test reaction. The precipitate is centrifuged down shortly.

Luciferase assay
For the assay, the following reagents are needed:

Tris buffer (EDTA-tris-acetate, pH 7.75)
12.12 g of Tris-(hydroxy-methyl)aminomethane and 0.74 g Na2-EDTA are weighed in and filled up with distilled water to a volume of 900 ml. The pH is adjusted to 7.75 using 2 M acetic acid and the solution is filled up to a final volume of 1 l using distilled water.

Test reagent
The reagent (1243-102 Monitoring Kit, LKB) contains:

I.) firefly luciferase
II.) D-luciferin
III.) 50 mg bovine serum albumine
IV). 0.5 mM magnesium acetate
V) 0.1 µM inorganic pyrophosphate

The freeze-dried test reagent ready to use mixture is dissolved in 10 ml ultrapure water. 1 ml aliquots are distributed to cryo vials and are stored in liquid nitrogen until usage.

P standard (0.5 µM):
30.26 mg ATP (ATP Na_2 H_2 * 3 H_2O, Boehringer, Mannheim) are dissolved in 100 ml Tris buffer (see above) and 1 ml aliquots are prepared and stored in liquid nitrogen. For each test series, P standard is thawed and diluted in three steps 1:1000 using Tris buffer. This ATP standard offers a concentration of 0.5 µM (= 0.5 pmol/µl).

Procedure
160 µl Tris buffer are mixed with 40 µl test reagent in a polystyrene cuvette by rotating the cuvette slowly. The emission of this blank is measured using a luminometer. The cuvette is removed, 10 µl cell extract are added (new volume: 210 µl), and the cuvette is mixed and the light emission measured again. Next, the measuring is calibrated by adding 5 µl of ATP standard two times (2.5 pmol) to the same cuvette (new volumes: 215 and 220 µl). By this internal standardisation, mistakes, which can be caused by the different components of the single samples are excluded.

9. Unrelated experimental procedures

Determination of methane concentrations via Gas Chromatography

Material
- overheated (glown out) 50 ml bottles or Bellco-tubes with defined volume
- sterile rubber stoppers
- 1 M NaOH
- gastight syringes
- methane standard 100 ppmv
- gaschromatograph Varian 3400

SAFETY ADVICES

Sodium hydroxide (NaOH): corrosive (R: 35 S: (1/2)-26-37/39-45)

Preparation
To stabilize the system the standby-modus of the GC should run 24h-48h before the first measurement. The nitrogen valve on the floor must be opened first. The nitrogen pressure within the GC should be adjusted to 0.8 bar. Now the GC can be turned on and method 4 must be started. By pushing "reset" the error message disappears.
The content of the nitrogen bottle must be controlled regularly.

About 2h before measurement the synth. air and hydrogen bottles must be opened. The nitrogen pressure within the GC must be corrected upwards to 5.7 bar. Now open the outlets for synth. air and hydrogen. The pre-adjustments for synth. air and hydrogen are:

synthetic air	hydrogen
4.2bar	2.8bar
300 ml/min	30 ml/min

These two gases are the fuel-gases for the Flame Ionisation Detector (FID). Pay attention that the hydrogen valve is opened **after** the valve for synth. air and that afterwards method 2 is started **immediately**. After additional two hours the base line should be near zero and the measurements can be started.

Calibration
For calibration use method 2 and a methane standard of 100 ppmv. The standard injection volume is 20µl, that means 20µl of the standard is equal to 100ppmv. The volumes listed in the table below are injected into Injector A. Three parallels are measured for calculating the calibration line and the correlated standard deviation.

Before measuring a standard or a sample the syringe must be purged at least thrice with the substance that should be measured.

Volume	ppmv
1	5
2	10
5	25
7,5	37,5
10	50
15	75
20	100
25	125
30	150
40	200
50	250

Methane measurement
After calibration the syringe must be purged thrice with air and then air can be used as blank-value. Homogenize the headspace of the sample and inject 20µl headspace immediately after sampling into the injector – repeat the last step twice.

Determination of methane concentrations

essential parameters for calculation:
- sample vessel volume
- head space volume
- GC area
- corrected GC area
- ppmv methane
- sediment volume

Phage dislodgment and extraction from sediments samples (Danovaro et. al., 2001)

1. 0.5 ml sediment + 4 ml ddH_2O + 1 ml sodium pyrophosphate (final concentration 10mM)
2. incubation for 15 min. with a shaking speed of 100 rpm
3. sonication for 3 min., interrupted for 30 sec. every minute
4. samples were shaken manually for 1 min. and centrifuged for 1 min. at 1000 rpm (800xg)
5. supernatants are filtered 0.2 µm (0.45 µm + 0.2 µm)
6. storage at 4°C

Sodium pyrophosphate working solution:

- 100 ml ddH_2O + 1.463 g sodium pyrophosphate (x10H_2O), pH 8.0
- **Sterilizing at 121°C, 20 min.**

Microcalorimetry

Introduction

Calorimetry is the science of measuring the heat production of physical, chemical or biological reactions.

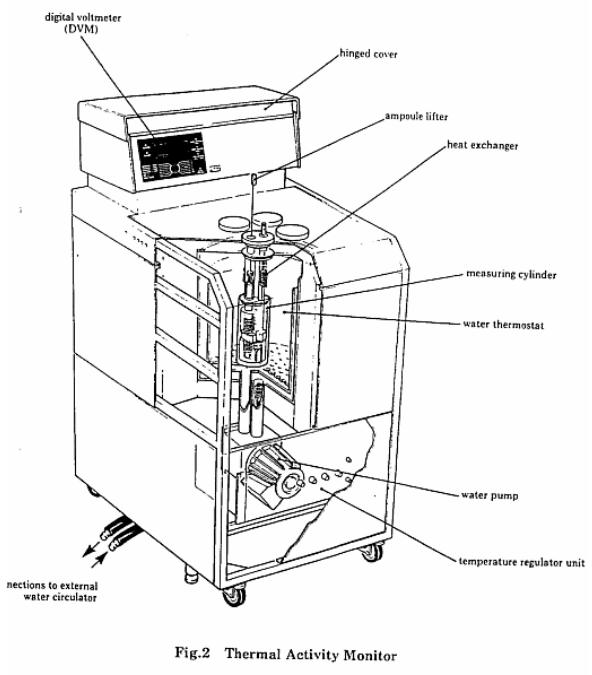


Fig.2 Thermal Activity Monitor

Figure 29: Thermal acitivity monitor.

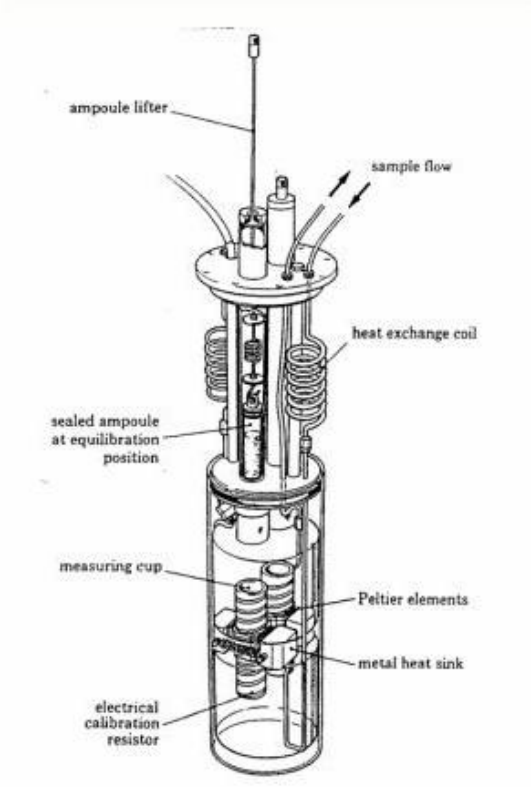


Figure 30: Measuring cylinder.

The microcalorimeter "Thermal Activity Monitor 2277" is an ultrasensitive instrument that measures the heat flow in µW. It consists of four measuring cylinders (Fig.1) that are surrounded by a water bath with a constant temperature (isotherm). Each cylinder is divided into two channels - the left one for the sample and the right one for the reference (Fig.2). When heat is produced in the sample the temperature difference is conversed into an electricity flow by the surrounding Peltier elements (Fig.2). The temperature increase of the internal water bath is compensated by an external thermostate next to the microcalorimeter.

In this case the microcalorimeter is used to monitor growth experiments. Heat is produced when a substrate is degraded due to microbial activity. This is plotted in a heat-production versus time diagram on a computer. With the data set the energy yield can be calculated:

1Joule = 1 Watt*Second

Vice versa the energy yield can be predicted if the reactions in the degradation processes of the substrate are known.

Running the microcalorimeter

- First of all, the thermostate on the left side of the microcalorimeter must be turned on, otherwise the microcalorimeter will start to heat. The tempertature in the internal waterbath of the microcalorimeter keeps it temperature, because it is connected to the thermostate next to the microcalorimeter which can cool down as well as heat. Always pay attention that the external waterbath contains enough water. Because the microcalorimeter itself can only heat, the temperature of the external water bath must be

lower than in the internal one. To adjust the appropriate temperature of the external as well as the internal water bath, have a look at the temperature regulator unit (Fig. 1) in front of the microcalorimeter.

- Now the microcalorimeter and then the computer can be turned on. The password is agcyp.

Recording data
By choosing the calorimetry software "Digitam for windows" four red lights will appear on the front panel of the microcalorimeter.
Choose "Control" and "Initiate experiment"
Fill in the parameters for each channel:

Channel: 1, 2, 3 or 4 (for each measuring cylinder)

Amplifier setting: 300µW, 1000µW or 3000µW (usually 1000µW is enough).

Operator: enter your name

Results name: Use the right mouse button and choose "Browse file" and enter a name for the data file. For example jutta001-3: the first number is the number of the run and the second one the number of the channel. Sometimes it is impossible to enter a new name. The easiest way is to change the name of an already existing file.

Method name: Use the right mouse button and choose "Browse file" and enter a name for your method.

When you have filled in the parameters of the first channel do not press enter, just change in the field "Channel " 1 to 2 and the parameters for channel 1 will disappear.
When you have defined the parameters of all four channels, press "Close".
To close a window, choose the upper left corner not the right one as usual.

- Go to menu "Control" and the submenu "Experiment control" to start the monitoring. Mark "Start Experiment" for all four channels and then press "OK". If you want to monitor an experiment for a longer period than a week, end the running experiment and start a new one with the same parameters (except "results name") and combine the two data sets in "Data plot". This is necessary because the virtual memory is too little.

- Go to menu "Plot" and submenu "Define Screen Plot" and insert the names of the Results name (see above) of the four running channels. Update the plot by pressing "Scale to show all".

End the experiment in menu "Control" and submenu "Experimental control".

Saving the data on a disc
- Menu "File", "Open", "Results file", click on the file to be saved. Ignore the now opening window.
- Go to menu "File", "Export" and mark "Generate numeric data report" and press "OK"
- Mark only "P" in the next window.
- Choose device "A" and the files will be saved as text files.

Analysing the data
Using "Data Plot" is the easiest way to plot and integrate the data.

Preparing the samples
Material:
- 20 ml autoclaved glass bottles 23mm diameter N20 opening
- rubber stoppers and hooks
- medium (page …) or sterilized sea water

- inoculum (e.g. sediment)
- substrate
- optionally inhibitor

For anaerobic growth experiments use the anaerobic chamber to fill the bottles with medium and sediment.

Put the filled and closed sample and reference ampoules (attached to a hook respectively) slowly! into the equilibration position of the microcalorimeter. Make sure that the ampoules are clean and dry. After at least half an hour the ampoules can be lowered to the measuring position (again slowly). The microcalorimeter is so sensitive that it can even measure frictional heat caused by vibrations.

When the heat flow is constant (approximately after two days) the substrate can be added.

Determination of physicochemical gradients

Various physical and chemical gradients of the water column and the upper 50 cm of the sediment are determined. The resolution should be 1 cm.

The oxygen profile is determined by an oxygen electrode, therefore an undisturbed sediment core is needed. The profiles of ammonia, nitrate and nitrite are analysed photometrically. Sulphate is determined via ion chromatography/HPLC and methane via gas chromatography (GC).

Determination of the oxygen profile by a needle electrode

The measurement of the oxygen profile is done by using a steel needle-electrode of the cathode type, which needs an external reference electrode. This kind of oxygen electrode consists of a platinum wire (which is isolated by glas) with a gold tip (Fig.). The electrode is surrounded by a steel capillary. The sensible tip is located in the capillary tip (Ø ca. 500 µm). The oxygen electrode is polarised by a voltage source to a level of 750mV. The power flow that results from the reduction of oxygen is measured by a picoamperemeter.

$$O_2 + 2e^- + 2H^+ \rightarrow 2OH^-$$

The relation between oxygen concentration and power is linear at a voltage of 750 mV. Thus a calibration with only two standards is useful.
→ 100% O_2 = air saturated water = tap water
→ 0% O_2 = oxygen free water = oxygen free sediment layer

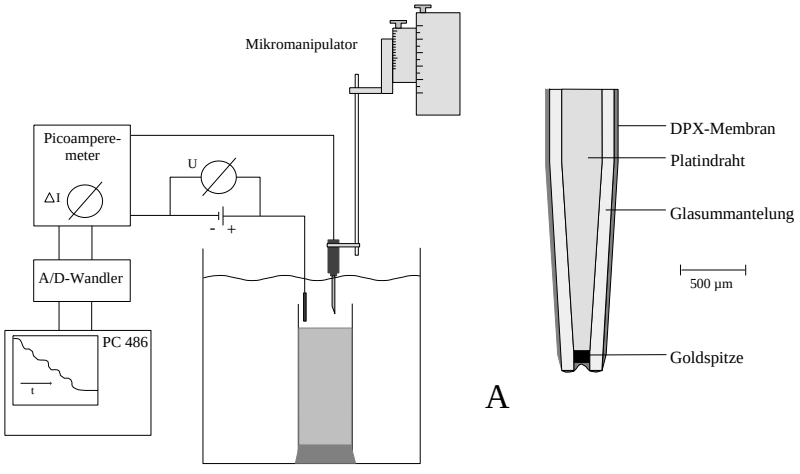


Figure 31: left-hand side: schematic set-up, right-hand side: electrode tip

Molecular diffusion coefficients for various ions and gases in aqueous solutions (CRC, Handbook of Chemistry and Physics, 73rd Ed.)

The values refer to water at 25°C (except oxygen and sulphide)

solute		D [$m^2 \cdot s^{-1}$]
ammonia	NH_4^+	$1{,}96 \cdot 10^{-9}$
methane	CH_4	$1{,}49 \cdot 10^{-9}$
nitrite	NO_2^-	$1{,}91 \cdot 10^{-9}$
nitrate	NO_3^-	$1{,}90 \cdot 10^{-9}$
oxygen[1)]	O_2	$2{,}13 \cdot 10^{-9}$
sulfide[1)]	HS^-	$1{,}61 \cdot 10^{-9}$
	H_2S	$1{,}96 \cdot 10^{-9}$
sulphate	SO_4^{2-}	$1{,}07 \cdot 10^{-9}$

[1)] sea water values at 25°C.

Fick´s law of diffusion for sediments

$$J = -\phi \cdot D_{Sed} \cdot \frac{\delta C}{\delta x}$$

Φ: porosity
D_{sed}: apparent diffusion coefficient for sediment
δC/δx: vertical concentration
J: diffusion flux

The apparent diffusion coefficient for sediments differs from the coefficient for aqueous solutions:

$$D_{Sed} = D \cdot \theta^{-2} \qquad \text{with} \qquad \theta^{-2} = 1 - \ln(\phi^2)$$

Extraction of pore water
The extraction of pore water is done by centrifugation of the sediment with a filter (Fig.). The vessels for the filters are provided by the AG "Organische Geochemie". The centrifuge tubes and the appropriate teflon vessels are washed with 2% HCl and thereafter with ethanol. The Teflon vessels are provided with membrane filters (0.4 µm pore diameter) and filled with ca. 10 g sediment. The centrifuge tubes are tarred by weighing. Centrifugation at 3500 rpm for four minutes. Then the centrifugate is decanted into a syringe and filtrated (0.2 µm pore diameter) into an Eppi cap.

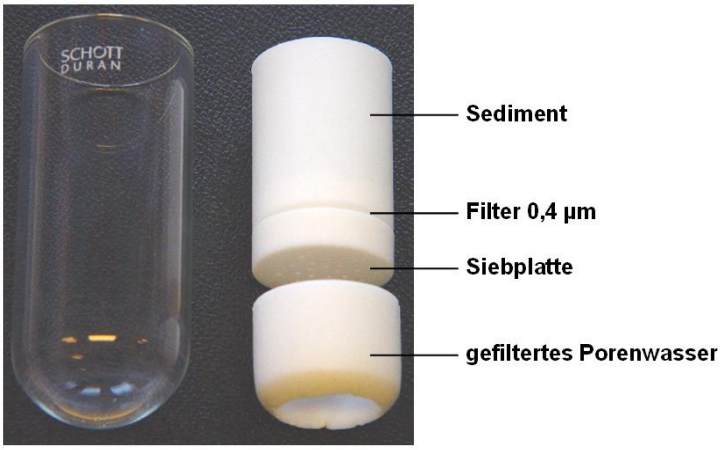


Figure 32: Filter used for the extraction of pore water.

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