

Microbiology PT Evaluation Workshop Training

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My Background

- Governmental Institute for Public Health of the federal state of Lower Saxony /Germany
Niedersächsisches Landesgesundheitsamt = **NLGA**
- Water testing laboratory within NLGA:
 - Drinking water
 - Pool and spa water
 - Bathing waters (EU bathing water directive)
- Provider for drinking water proficiency testing schemes (PTs)

Proficiency testing schemes - NLGA

Drinking water	methods	Rounds/ year	participants
E. coli /Coliform Bacteria	ISO 9308-1:2001 ISO 9308-2:2014 ISO 9308-1:2014	4	600
Colony Counts	ISO 6222:1999	4	600
Intestinal Enterococci	ISO 7899-2	4	600
C. perfringens	ISO 14189	2	600
P. aeruginosa	ISO 16266	2	600
Legionella spec.	ISO 11731	3	600
Other PTs			
E. coli (bathing water)	ISO 9308-3	1	250
Enterococci (bathing water)	ISO 7899-1 ISO 7899-2	1	250
Bacteriophages	ISO	1	30

Training topics



IMPORTANCE OF METHODS
IN MICROBIOLOGY



QUALITY CONTROL
MEASURES



UNCERTAINTY OF
COUNTING



CONTROL CHARTS

Training



Presentations



Discussions



Working
groups



Excercises

Importance of test-methods in microbiology

drinking water

- „clean“ matrix
- Liquid

Safe for
human
consumption

- Legal regulations
- Level of detection e.g. 1/100 ml

indicator
concept

- Correlation with fecal contamination
- Easy to analyse
- Cost effective

Requirements for potable water

Could be

- Safe for human consumption
- Indicator organisms (e.g. E. coli) not detected
- E. coli **not detected**
- No pathogens
- E. coli **absent** in 100 ml 0/100 ml
- Coliform bacteria **absent** 0/100 ml
- Legionella
in warm water <100 CFU/100 ml

Convention method

- Procedure described (e.g. published in a journal or as a standard)
 - “gold standard” in drinking water microbiology usually is culturing
 - Culturing = based on counts
 - ability of microorganism to grow on or in a specific medium
 - Colony forming unit might originate from 1 cell or thousands of cells
 - Method where the analyte is determined by the method
- Any change to the method can/will also change the analyte!

Standardized methods (e.g. ISO)





- what's out there
- how does it get there
- what they can do for you
- how to use them

- what they **cannot** do for you

- ISO International Standardization Organization
- Technical committees
- Member Bodies: national standardization bodies
- written and discussed by experts
- well known and used method
- best available „compromise“

TC 147 - Water quality

Items to be displayed:





- | | |
|--|--|
| <input type="checkbox"/>  Published standards | <input checked="" type="checkbox"/>  Standard |
| <input type="checkbox"/>  Withdrawn standards | <input type="checkbox"/>  Projects |

Subcommittees

 Subcommittee	 Subcommittee Title
TC 147/SC 1	Terminology
TC 147/SC 2	Physical, chemical and biochemical methods
TC 147/SC 3	Radiological methods
TC 147/SC 4	Microbiological methods
TC 147/SC 5	Biological methods
TC 147/SC 6	Sampling (general methods)








TC 147/SC 4 - Microbiological methods

Items to be displayed:

- | | |
|---|---|
| <input checked="" type="checkbox"/>  Published standards | <input checked="" type="checkbox"/>  Standards under development |
| <input type="checkbox"/>  Withdrawn standards | <input type="checkbox"/>  Projects deleted (last 12 months) |

Standards and projects under the direct responsibility of TC 147/SC 4 Secretariat

◆ Standard and/or project

-  [ISO 6222:1999](#)
Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium
-  [ISO 6461-1:1986](#)
Water quality – Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) – Part 1: Method by enrichment in a liquid medium
-  [ISO 6461-2:1986](#)
Water quality – Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) – Part 2: Method by membrane filtration
-  [ISO 7704:1985](#)
Water quality – Evaluation of membrane filters used for microbiological analyses
-  [ISO 7899-1:1998](#)
Water quality – Detection and enumeration of intestinal enterococci – Part 1: Miniaturized method (Most Probable Number) for surface and waste water
-  [ISO 7899-1:1998/Cor 1:2000](#)
-  [ISO 7899-2:2000](#)
Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

ISO Standards contain

- normative information
- informative information
- definitions
- precise technical information
 - instrumentation
 - media composition
 - times
 - temperatures
- format of how to report the results
- most often encountered problems

Most important decisions of a laboratory

- What is the purpose of the analysis?
- Is the standard fit for your purpose?

- Example of influence on how the method influences the result of the analysis: coliform bacteria

ISO 9308-1 (2000)

Detection: **metabolites** and
enzyme activity

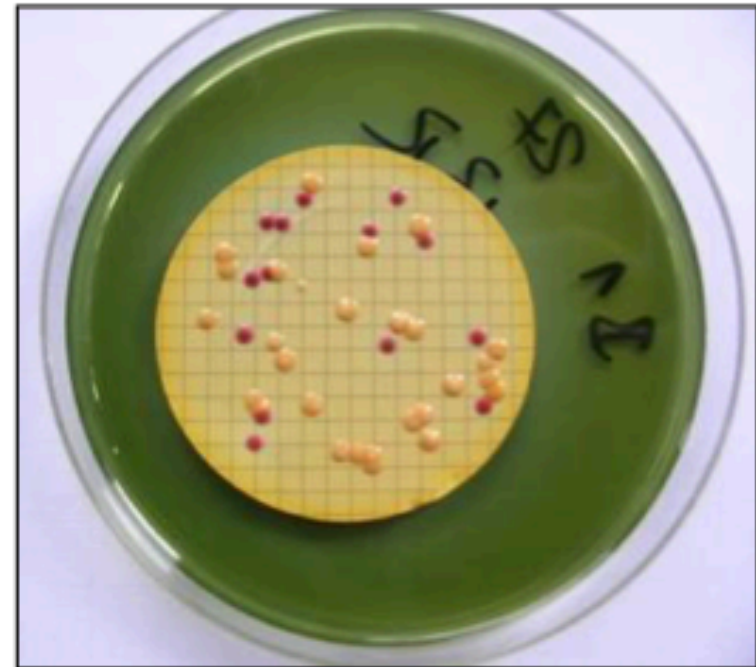
Coliform bacteria:

- acid formation from Lactose
- Oxidase-negative

E. coli:

- Indole production at 44°C

Duration: ca. 48 hours or more for positive results



ISO 9803-1 (2014)

Detection of specific **enzyme activities**

Coliform bacteria:

β -Galactosidase

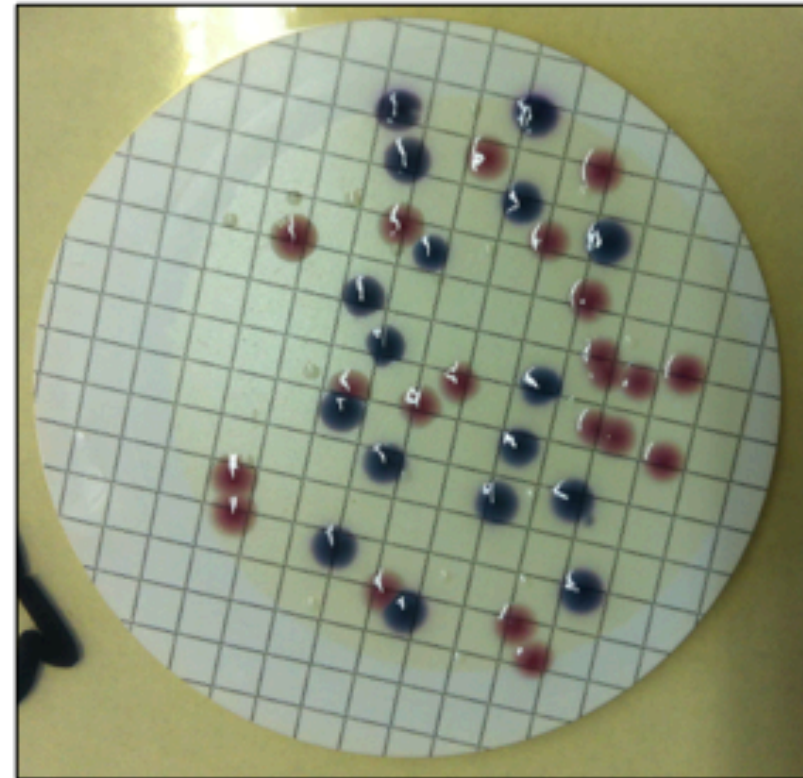
Lactose + H₂O → Glucose +
Galactose (red colonies)

Oxidase negative

E. coli:

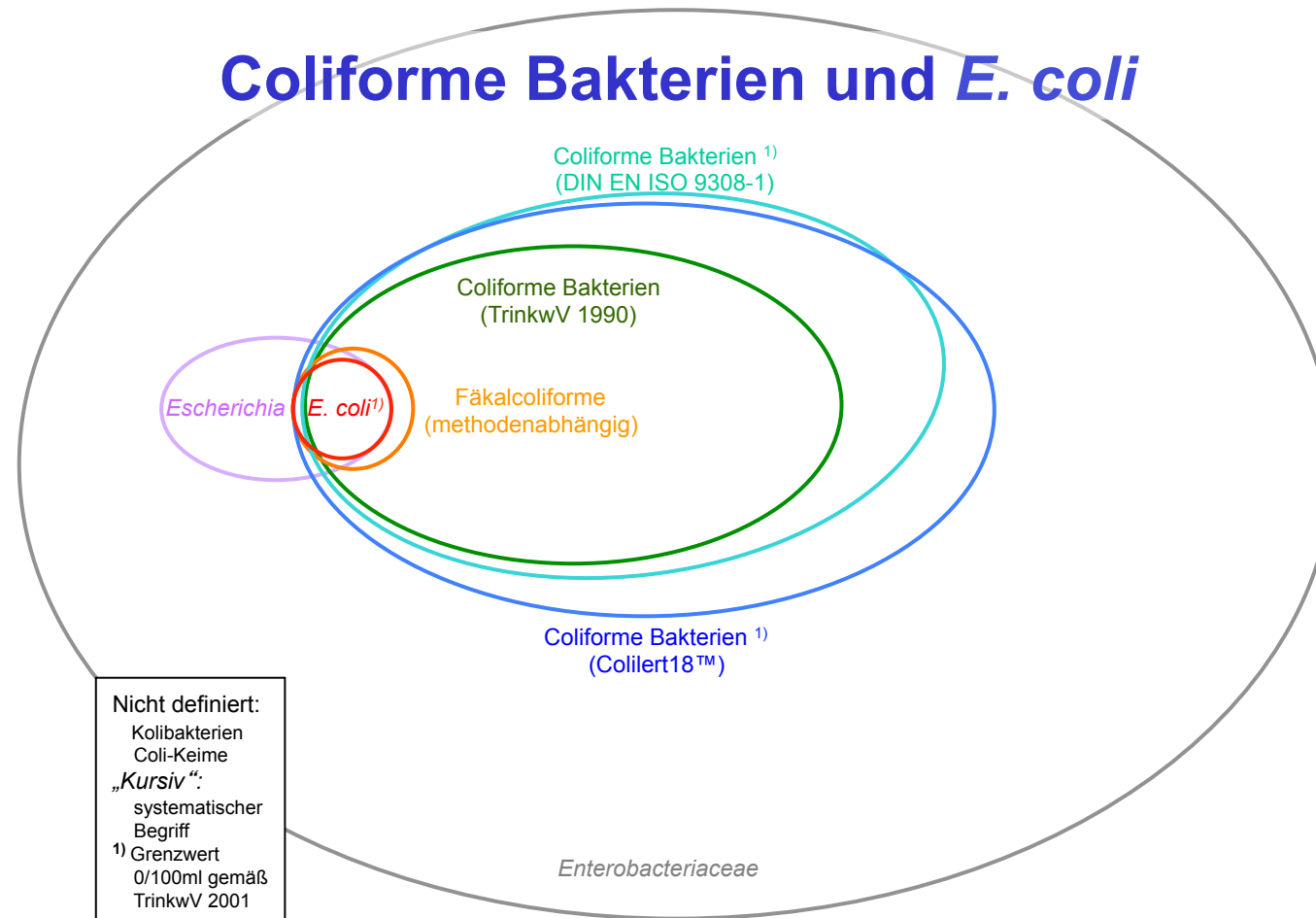
additional β -Glucuronidase (blue colonies)

duration: 21 ± 3 h



Definition of Coliform bacteria

Coliforme Bakterien und *E. coli*



13. Jahrestagung Trinkwasserringversuche Osnabrück, 29. Februar 2012

Coliform bacteria defined by method

“historic”	ISO 9308-1	Colilert®-18
Escherichia Klebsiella Enterobacter Citrobacter	Escherichia Klebsiella Enterobacter Citrobacter Yersinia Serratia Hafnia Pantoea Kluyvera	Escherichia Klebsiella Enterobacter Citrobacter Yersinia Serratia Hafnia <u>Pantoea</u> <u>Kluyvera</u> <u>Cedecea</u> <u>Ewingella</u> <u>Moellerella</u> <u>Leclercia</u> <u>Rahnella</u> <u>Yokenella</u>

Working groups

What factors have an impact on the detection (and quantification) of bacteria

- 1) general aspects (handling, bacterial requirements)
- 2) Membrane filtration methods
- 3) Pour plate methods
- 4) MPN methods

general aspects	Potential quality control measures
Type of container	Sterile, Volume, material
Temperature	Ice in a cooler box
sampling point	Follow the correct procedure: e.g. flaming, spray with disinfectant
sampling procedure	SOP
Environmental conditions	Record
Weather instability	Record
Sampler	Training for competency, qualification records, in house training supervision, demonstration
Technician	Competent
Sample keeping	cool and transport within required time span
Transportation	temperature datalogger, extra sample for temperature reading at start and end of transport
Distance	shortest 6hrs
Sample bench	to be analyzed within 1hr on sterile bench
Method	fit for the purpose by validation data including a decision based on that data and the knowledge of the purpose of the analysis
General lab condition	Temp monitored and controlled, check for airborne contamination by using open non selective plates decide on warning and action limits

Working group 2

Membrane filtration methods	Potential quality control measures
type of membrane filter	pore size, material, test batch to batch compatibility of membrane and medium (see upcoming ISO 7704)
volume of water	graduated funnel, volume measurement
sterility of membranefiltrarion apparatus	flaming, autoclaved, positive and negative control samples
environmental condition	if in the laminar flow bacterial contaminationof air
sterility check of the media	expiry date, pH,
type of water used for media preparation	pH, conductivity 25, heavz metal content
maitanance of the apparatus	no leakage, pump working properlz
verification of the media	pH,
place filter on plate	no air bubbled trapped, visual inspection of the plate during reading
broth and absorbant pad (thickness)	control at reception of the pads with the amount of broth intended for use< pipetting control (weighing)

Working group 3

Pour plate methods	Potential quality control measures
Weighing	Calibration of balnce, mass of media measured
Reconstitution of media	ph, conductivity of water, volume
Media quality	Brand, quality, expiry status, instruction for preparation
Sterilisation of media	Control time, temp, pressure, add spores to autoclave an incubate after run in growth conditions
Glassware	cleaning, sterilisation
Volume of sample measured	Calibration of measuring apparatus
Homogeneity of sample	Mix properly
Serial dilution	Calibration of pipette, technique
Time between serial dilution and pouring	Not more than 15min
Temperature of Agar	Not to exceed 44-47oC
Volume of culture media poured	Not < 10ml, not> 20ml

Working group 3 continued

Pour plate methods	Potential quality control measures
Mixing technique of sample and media	Aseptic technique
Use of singular/duplicate plates	
Stacking of plates	Not more than 6 high
Calibration status of incubators	
Counting of colonies	Identification, accuracy of counting
Calculation of results	Check accuracy of formula
Reporting results	Proper units.
waterbath	temperature control

Working group 4

MPN methods	Potential Quality Control Measures
Pipetting technique	Trained and authorised personnel to use micropipettes
Sample homogeneity (esp. for 1ml aliquotes)	Homogenise samples as prescribed
Volume dispensed	Use pipettes calibrated at points of use
Volume loss during autoclaving	Aseptically transfer dilution water after autoclaving
Cross contamination between dilution steps	Pipette tip sterility tests
incubation temperature & time	Stick to documented limits
Sample colouration (Colilert-18)	Incubate sample without substrate added and compare with one containing substrate
Intensity of colouration (Colilert-18)	Use valid Colilert-18 comparator
Reading MPN (approximate number)	
Foam formation in quantitrays	Use anti-foam
Presence of gas in durham tubes before incubation	Eliminate air from the tubes during inoculation

Factors of impact need to be

- sorted
- weighed

Judgements have to lead to decisions on which ones need to be controlled or tested...

What are acceptable limits for the factors and how can they be controlled?

WHAT IS FIT FOR PURPOSE?

Decisions have to be made.

→ decisions and the reasons should be documented for future use (accreditation?)

Quality control measures

in a microbiology laboratory analyzing drinking water

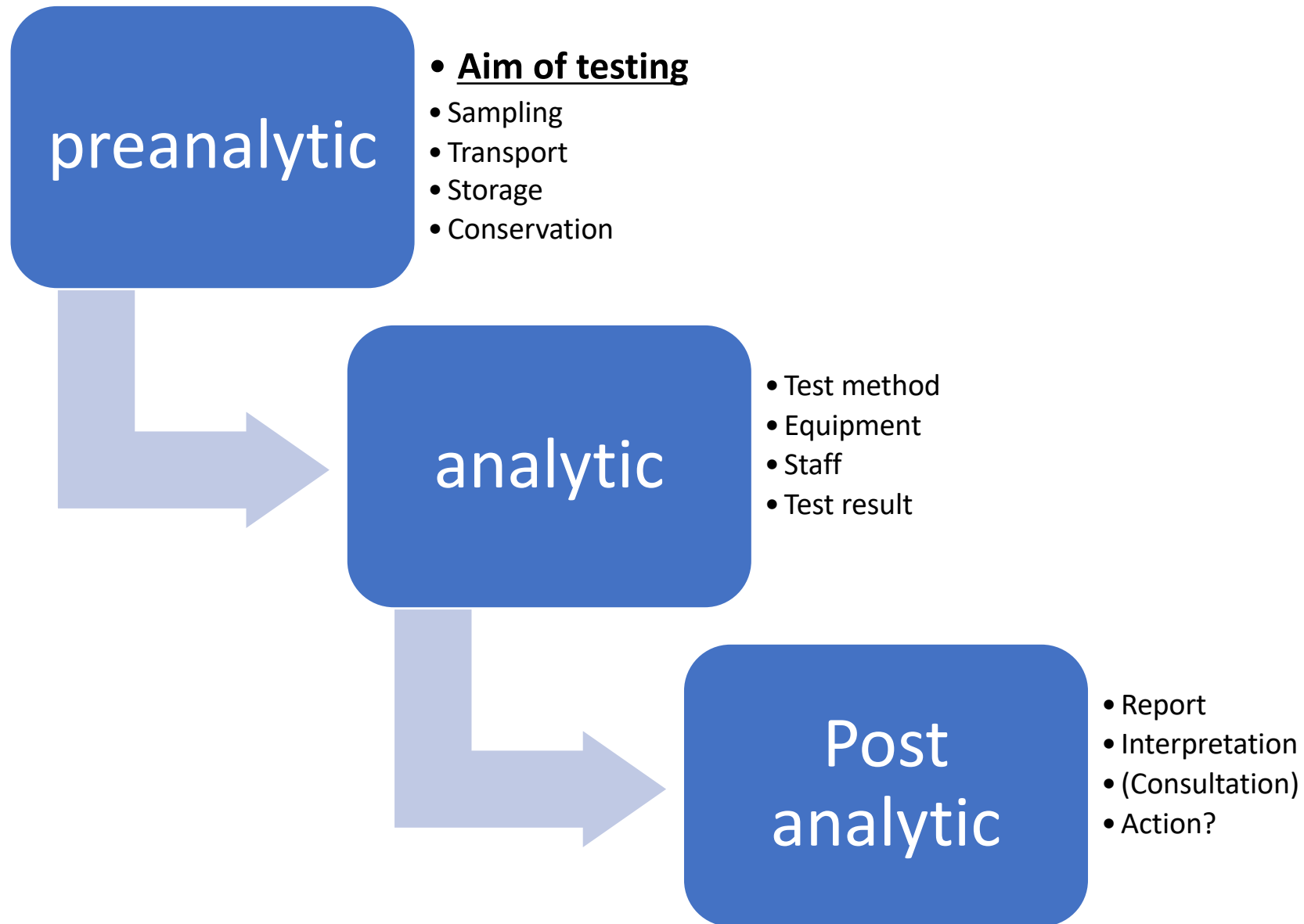
Quality control

WHO:

“The total process whereby the quality of laboratory reports can be guaranteed.”

Quality control

- QC is part of quality assurance that primarily deals with errors in the performance of tests and verification of test results
- QC must cover **all aspects** and **all procedures** contributing to the test report
 - Practicable
 - Affordable
 - Achievable



Test method

- Medium
 - Composition
 - No inhibitory contents like copper or other heavy metals
 - appearance
 - shelf life (storage conditions)
 - Recovery
 - Water used for the preparation (conductivity)
 - Thickness of the plates
- Positive controls
 - Typical appearance
- Negative controls
 - Inhibition of non-target strains (sufficient)
- Combination of each lot of medium with each lot of membrane (ISO 11133)
- Incubation times (documented?)
- Incubation temperatures (see equipment)

Equipment

- Incubators
 - temperature control
 - on different racks
 - over time
 - Different usage (weekend/weekday)
 - Drying of the plates (incubators with fan)
- Membrane filtration apparatus
 - Sterility
 - Pressure (negative or positive)
- Pipettes/Volumetric Devices: check volumes regularly (weighing)
- Glassware
 - discard chipped glassware
 - Sterility
- Autoclaves

staff

- well trained
 - experienced
 - competent
-
- Knowledge on methods
 - Conducting the different steps of the procedure (e.g. filtration, reading, calculation of test result)
 - Reading of plates

Standard Operating Procedures

- All quality control procedures need to be written down as detailed instructions
 - Serves to achieve a consistent quality in the analysis
 - could be part of the instructions for the test method itself in order to minimize the risk of errors
 - helps preventing unwanted “shortcuts” or variations in the procedure

Uncertainty of counting

in a microbiology laboratory analyzing drinking water

ISO 13843:2017

Water quality - Requirements for establishing performance characteristics of quantitative microbiological methods

Contents

1. Scope
2. Normative References
3. Terms and Definitions
4. Basic Concepts
5. Specifications: **some guideline values**
6. Designs for determining performance characteristics of a method
7. Designs for single laboratory verification of a method

Annexes

- A. Mathematical models of variation *(informative)*
- B. Assessment of the lower limits *(normative)*
- C. Assessment of the upper limits *(normative)*
- D. Determination of the operational variability in repeatability and intralaboratory reproducibility conditions *(normative)*
- E. uncertainty in counting** *(normative)*
- F. Determination of the operational variability (interlaboratory reproducibility) in a collaborative performance study *(normative)*
- G. Glossary of principal symbols *(informative)*

Scope

This document deals with the characterization of microbiological methods.

In terms of this document, characterization means the study of parameters that can be measured to describe how the method is likely to perform in a given set of conditions, which can be described as performance characteristics.

The document describes procedures for the determination of performance characteristics which can be used for subsequent validation or verification of methods.

applies to selective quantitative methods (all types of water)

Definitions (41)

robustness insensitivity of an analytical method to small changes in the procedure

sensitivity fraction of total number of positive cultures or colonies correctly assigned in the presumptive inspection

specificity fraction of total number of negative cultures or colonies correctly assigned in the presumptive inspection

verification performance of a second characterization by a different laboratory to confirm the results of the original characterization

Robustness (Uncertainty)

- uncertainty of counting: repeat counting
 - same plate same person (RSD)
 - same plate different person (RSD)
 - beginning and end of designated incubation period (RSD)
- important: calculate **and judge** these data
- this will allow a first assessment of the methods potential problems

Uncertainty in counting

- Read the same plates repeatedly under uniform conditions (short interval of time = max 1 hour) (single person or intralaboratory/more than one)
- Randomly selected plates with more than 20 colonies (no unusual plates)
- count 30 plates

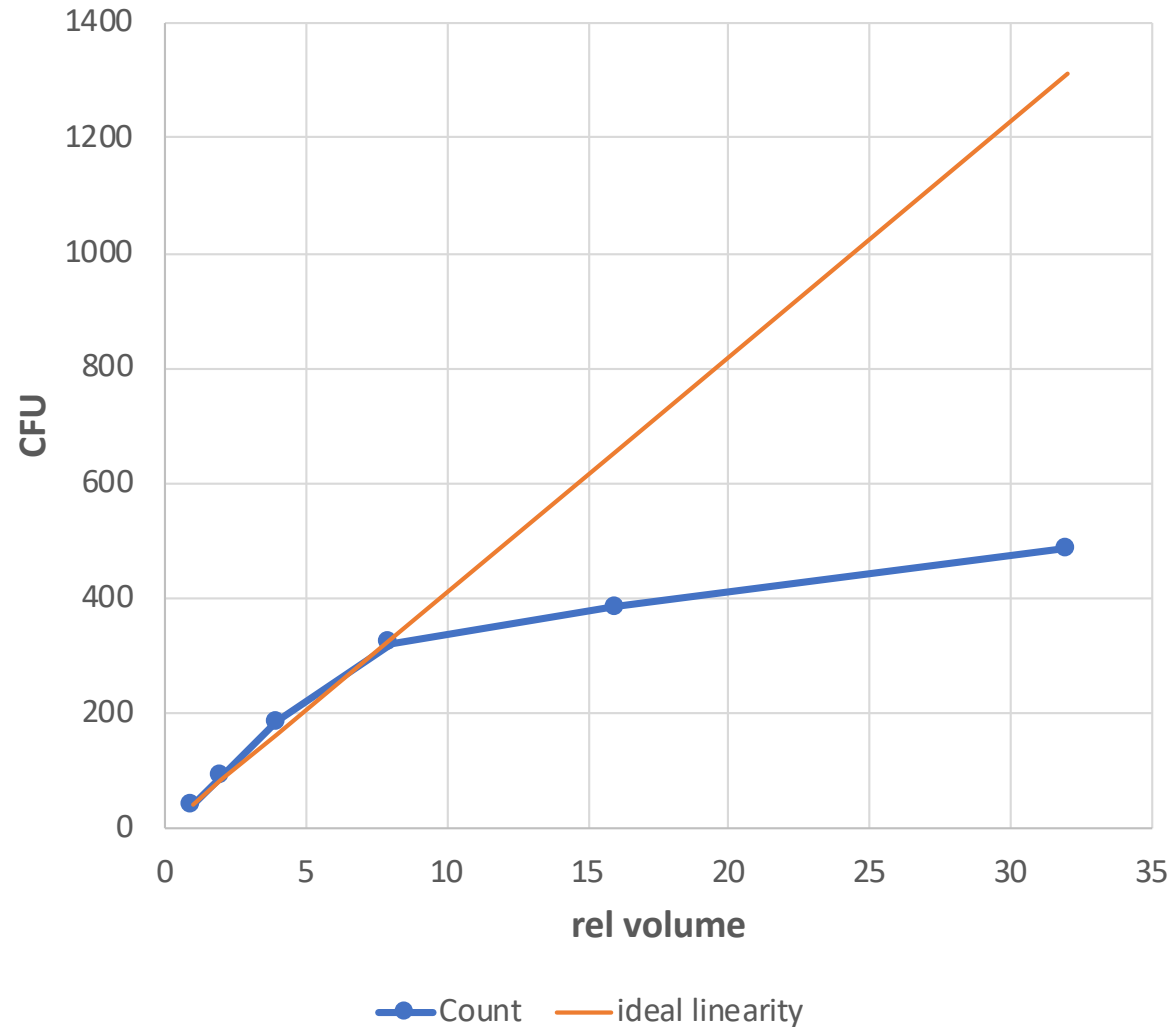
Linearity (Proportionality)

- Upper limit of the working range of the method can be determined by a linearity test:
 - dilute a sample
1:2:3:4:5:6:7 or 1:2:4:8:16:32:64
 - count three replicates each and analyze the ratio
no of colonies / relative volume

Linearity (Proportionality)

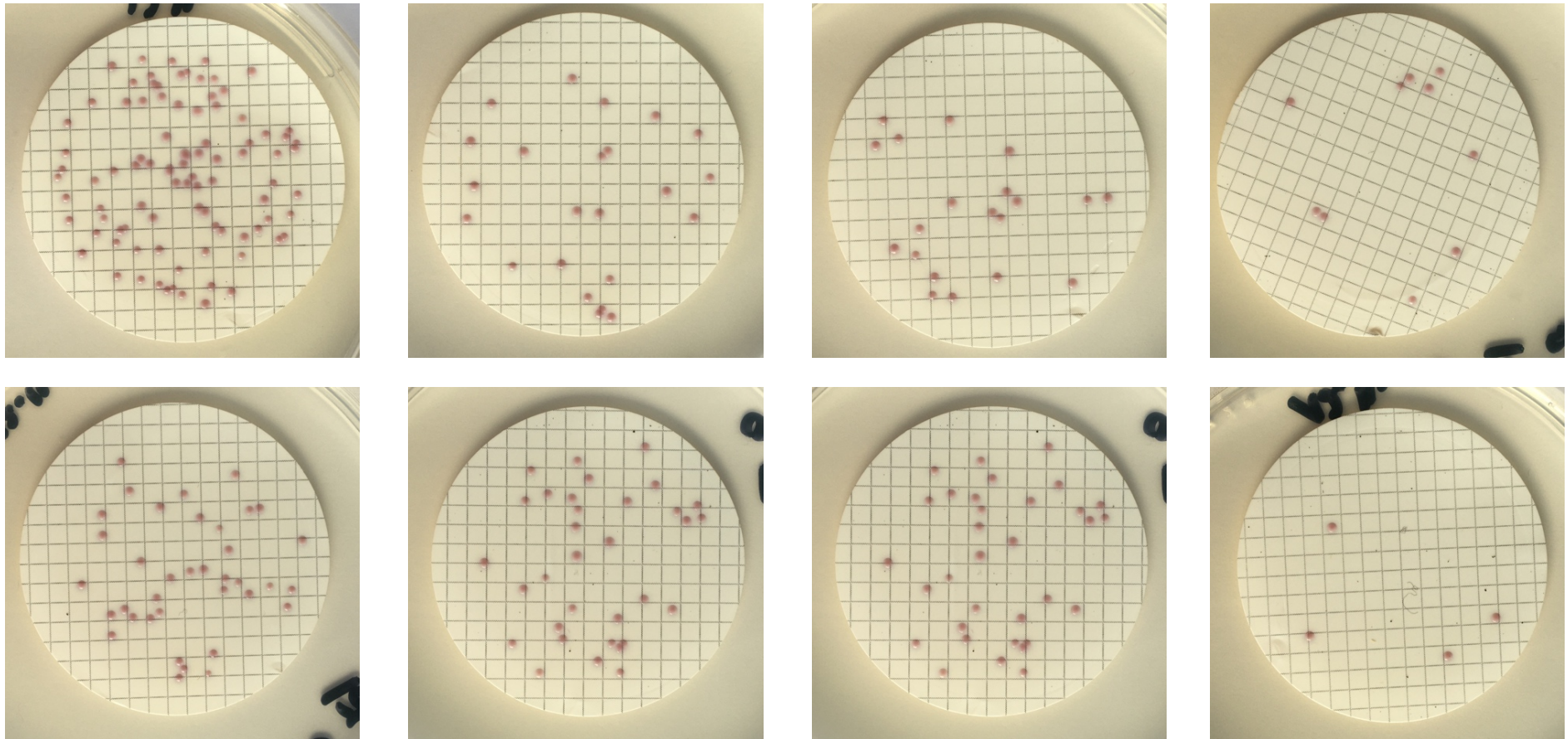
Dilution	replicate counts			sum S_i	relative volume R_i	ratio S_i/R_i
2^{-1}	121	204	162	487	32	15,22
2^{-2}	109	128	148	385	16	24,06
2^{-3}	111	114	97	322	8	40,25
2^{-4}	56	60	68	184	4	46,00
2^{-5}	36	29	24	89	2	44,50
2^{-6}	11	13	17	41	1	41,00

Linearity (example)



S. fonticola (CCA)

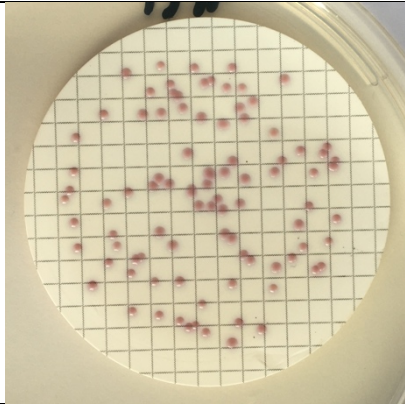
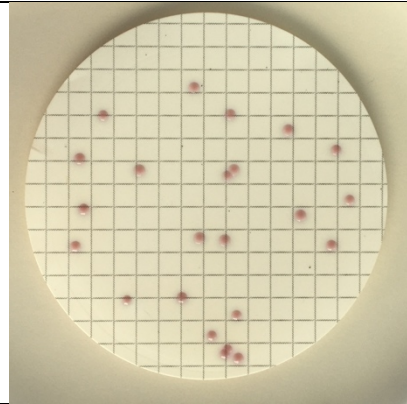
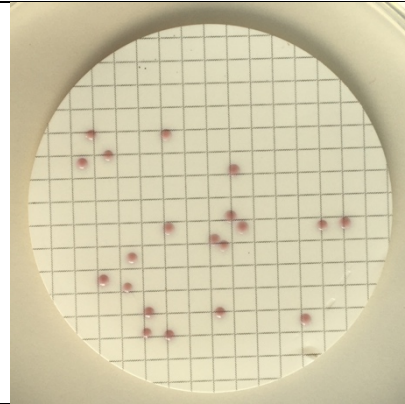
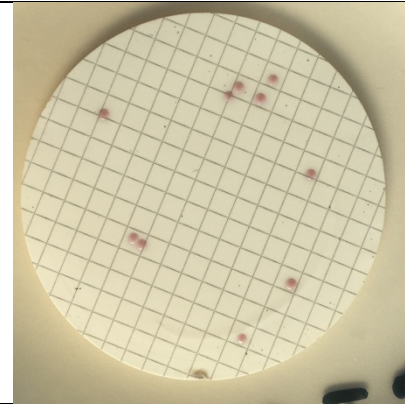
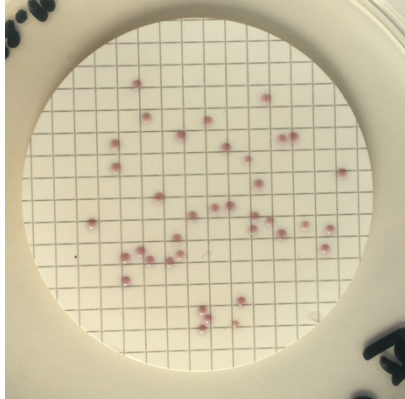
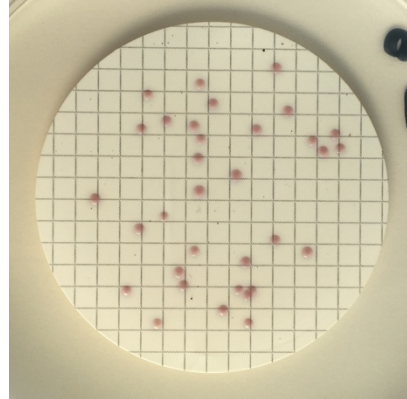
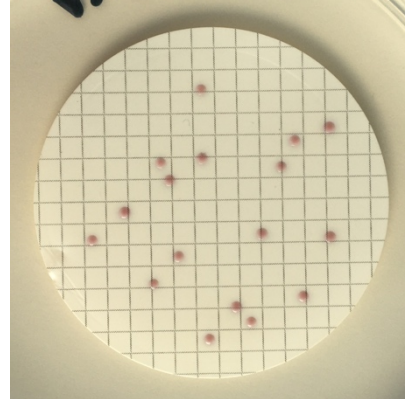
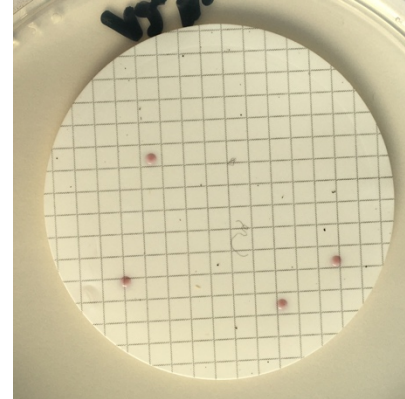
- Count CFU for all membranes (top and bottom membranes are duplicates of the same volume)
- Transfer data to a table and determine if the counts show linearity over the whole range tested
- All data from the top row are collected to calculate the RSD of intralaboratory counting (staff)



The pictures represent a series of plates from different dilutions of a pure culture of *Serratia fonticola* after membrane filtration and incubation on CCA for 22 hours

Exercise:

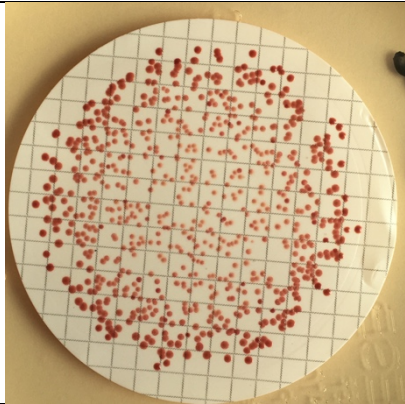
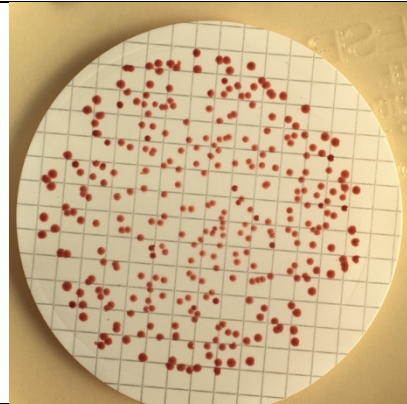
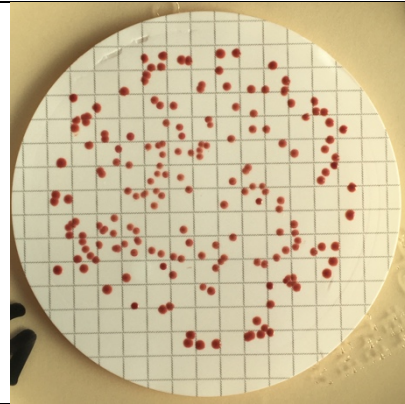
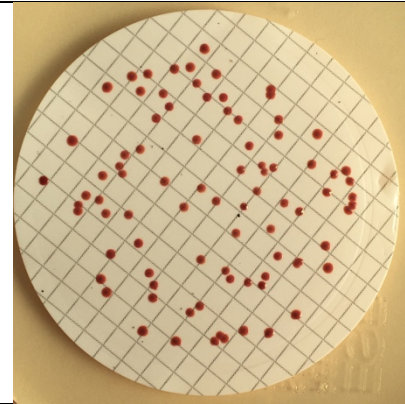
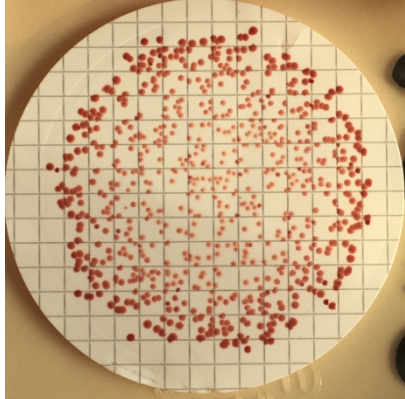
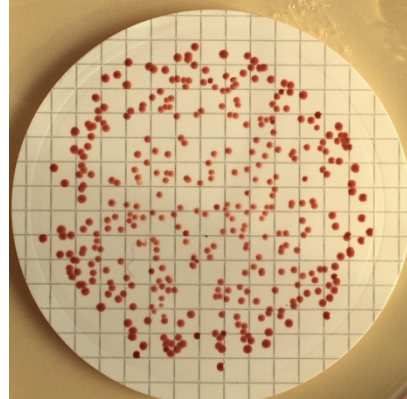
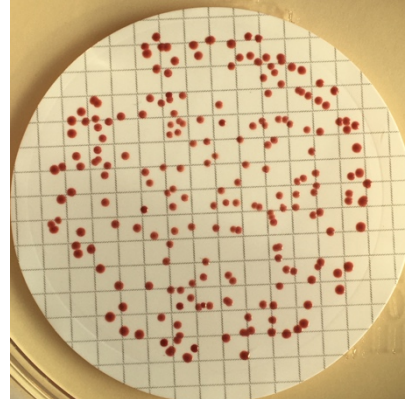
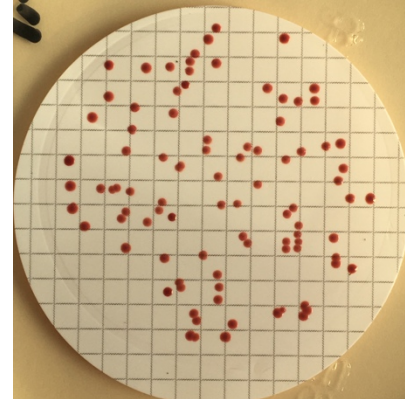
- a) Count all membranes and note the CFU (top and bottom membranes are duplicates of the same volume)
- b) Transfer data to an excel sheet and determine if the counts show linearity over the whole range tested

	I (2^{-1})	II (2^{-2})	III (2^{-3})	IV (2^{-4})
	8 ml	4 ml	2 ml	1 ml
	CFU:	CFU:	CFU:	CFU
A				
	CFU:	CFU:	CFU:	CFU
B				

The pictures represent a series of plates from different dilutions of a pure culture of *Enterococci mundtii* after membrane filtration and incubation on Slanetz-Bartley Agar for 48 hours

Exercise:

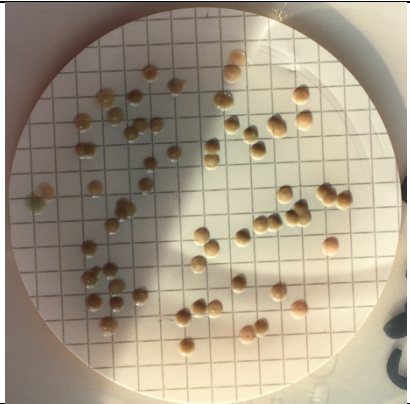
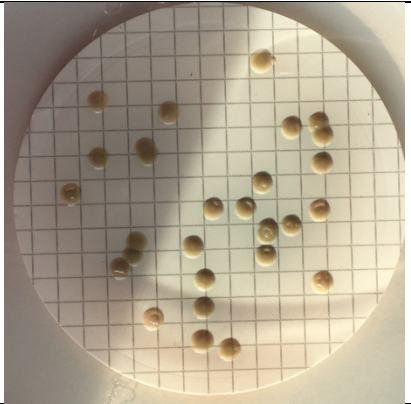
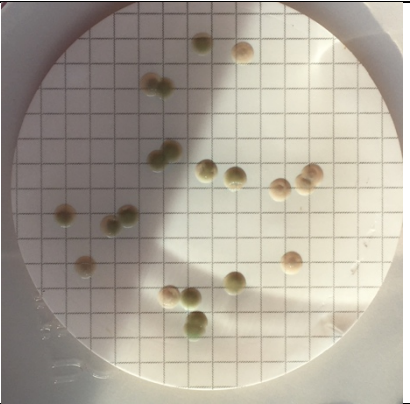
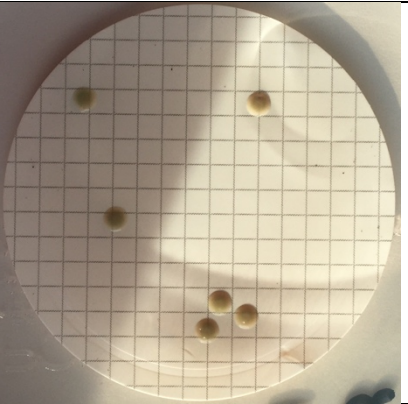
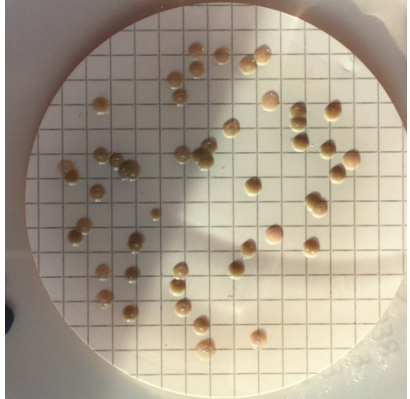
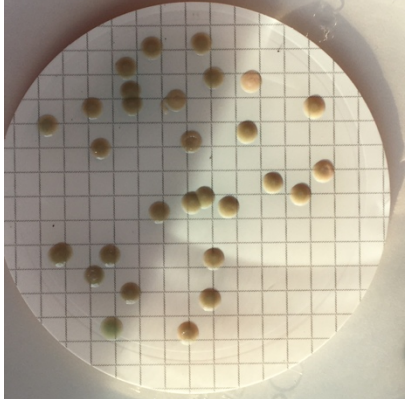
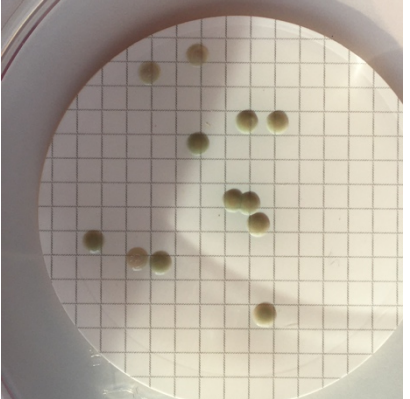
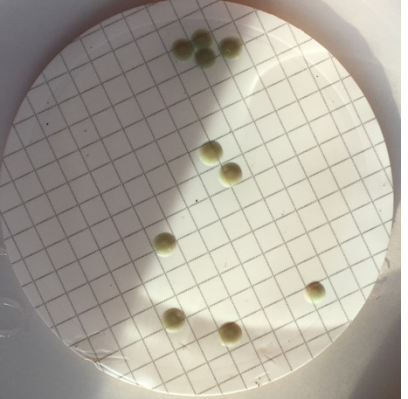
- a) Count all membranes and note the CFU (top and bottom membranes are duplicates of the same volume)
- b) Transfer data to an excel sheet and determine if the counts show linearity over the whole range tested

	I (2^{-1}) 8 ml	II (2^{-2}) 4 ml	III (2^{-3}) 2 ml	IV (2^{-4}) 1 ml
	CFU:	CFU:	CFU:	CFU
A				
	CFU:	CFU:	CFU:	CFU
B				

The pictures represent a series of plates from different dilutions of a pure culture of *Pseudomonas aeruginosa* after membrane filtration and incubation on CN Agar for 48 hours

Exercise:

- a) Count all membranes and note the CFU (top and bottom membranes are duplicates of the same volume)
- b) Transfer data to an excel sheet and determine if the counts show linearity over the whole range tested

	I (2^{-1})	II (2^{-2})	III (2^{-3})	IV (2^{-4})
	8 ml	4 ml	2 ml	1 ml
	CFU:	CFU:	CFU:	CFU:
A				
	CFU:	CFU:	CFU:	CFU:
B				

Results counting exercise

	S. fonticola				E. mundtii	P. aeruginosa			
	8 ml	4 ml	2 ml	1 ml	8 ml	8 ml	4 ml	2 ml	1 ml
mean	86	23	20	11	290	59	27	19	6
SD	5	1	1	1	52	3	1	3	0
RSD	0,06	0,05	0,06	0,06	0,18	0,05	0,03	0,17	0,08
1	84	22	20	11		62	28	20	6
2	87	22	20	10	283	59	27	20	6
3	79	23	20	10	309	60	27	20	6
4	78	22	21	11	303	62	27	20	6
5	88	23	20	11	250	59	27	21	6
6	94	23	20	11	285	60	27	20	6
7	88	22	20	10	305	58	27	20	6
8	82	22	20	11	299		27		
9	88	22	20	10	405	49	24	6	4
10		23			188	59	27	20	6
11	76	21	15	9	296	59	28	20	6
12	89	23	20	11	268	59	27	20	6
13	89	24	20	11		59	27	21	6
14	81	26	19	11		56	27	20	6
15	91	23	20	11		60	27	20	6
16	88	22	20	11		59	27	20	6
17	84	22	20	10		59	27	20	6
18	93	22	20	10		58	27	21	6
19		23	20	11		60	27	21	6

Control charts

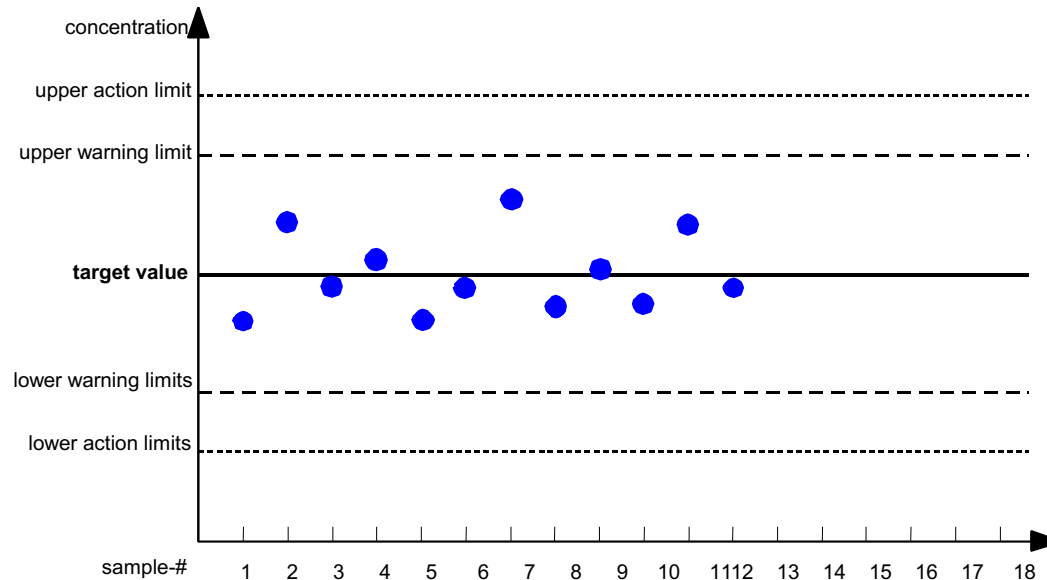
in a microbiology laboratory analyzing drinking water

History

- introduced by Shewhart in 1931
 - originally for industrial manufacturing processes
 - for suddenly occurring changes and for slow but constant worsening of the quality
 - Immediate interventions reduce the risk of production of rejects and complaints from the clients
- From: Quality Assurance in Analytical Chemistry – Training and Teaching (Springer-Verlag Berlin 2003)

Principle

- Take samples during the process
- Measure a quality indicator
- Mark the measurement in a chart with warning and action limits



Control Charts in Analytical Chemistry

- Warning / action limits
 - if data are normal distributed
 - 95.5% of the data are in $\mu \pm 2\sigma$
 - 99.7% are in $\mu \pm 3\sigma$
- $x_{\text{target}} \pm 2s$ is taken as warning limits
- $x_{\text{target}} \pm 3s$ is taken as action limit

Action Limits

- There is probability of only 0.3 % that a (correct) measurement is outside the action limits (3 out of 1000 measurements)
- Therefore the process should be stopped immediately and searched for errors

Warning Limits

- 4.5% of the (correct) values are outside the warning limits.
- This is not very unlikely
- Therefore this is only for warning, no immediate action required

Calculation of Standard Deviation

- measurements marked in the control chart are between-batch
- standard deviation should also be between-batch
- estimation from a pre-period of about 20 working days
- **repeatability STD → too narrow limits**
- **interlaboratory STD → too wide limits**

Control Charts

- Control samples should be stable (monitoring over a long time)
- Should be representative for the matrix
- Concentration within the “normal” range of the analytical method
- Amount sufficient for a long time

- Target value
 - Certified Reference material
 - Often too expensive or
 - Not available
 - Mean of often repeated measurements
 - Values must be different from 0

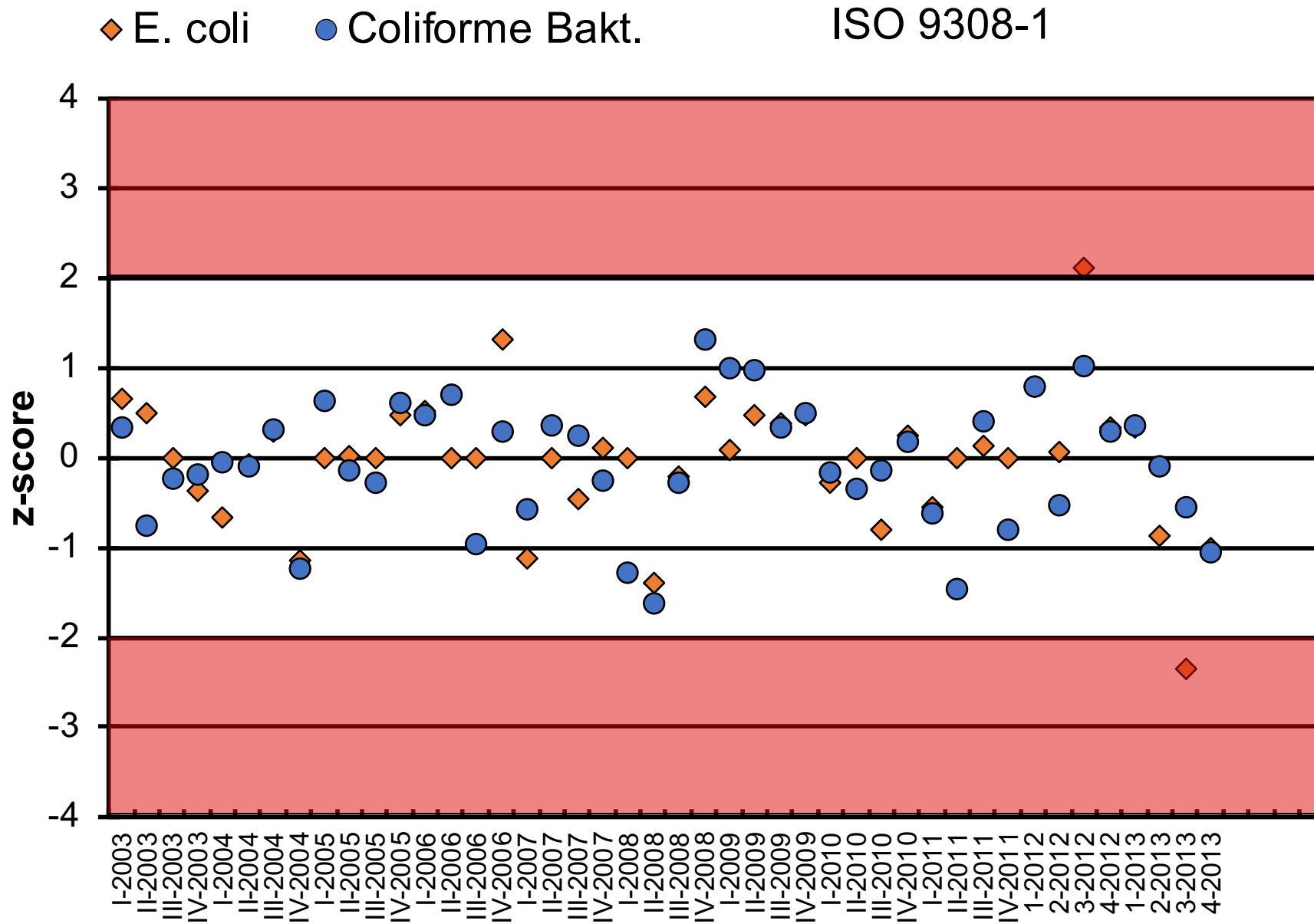
Challenges in Analytical Microbiology

- limited **stability** of samples
- hardly any reference material available
- for drinking water analysis: limit of interest most often 0 CFU (how to cover important range?)
- distribution of the bacteria in the control sample not normal

ISO 11133

Microbiology of food, animal feed and water - Preparation, production, storage and performance testing of culture media

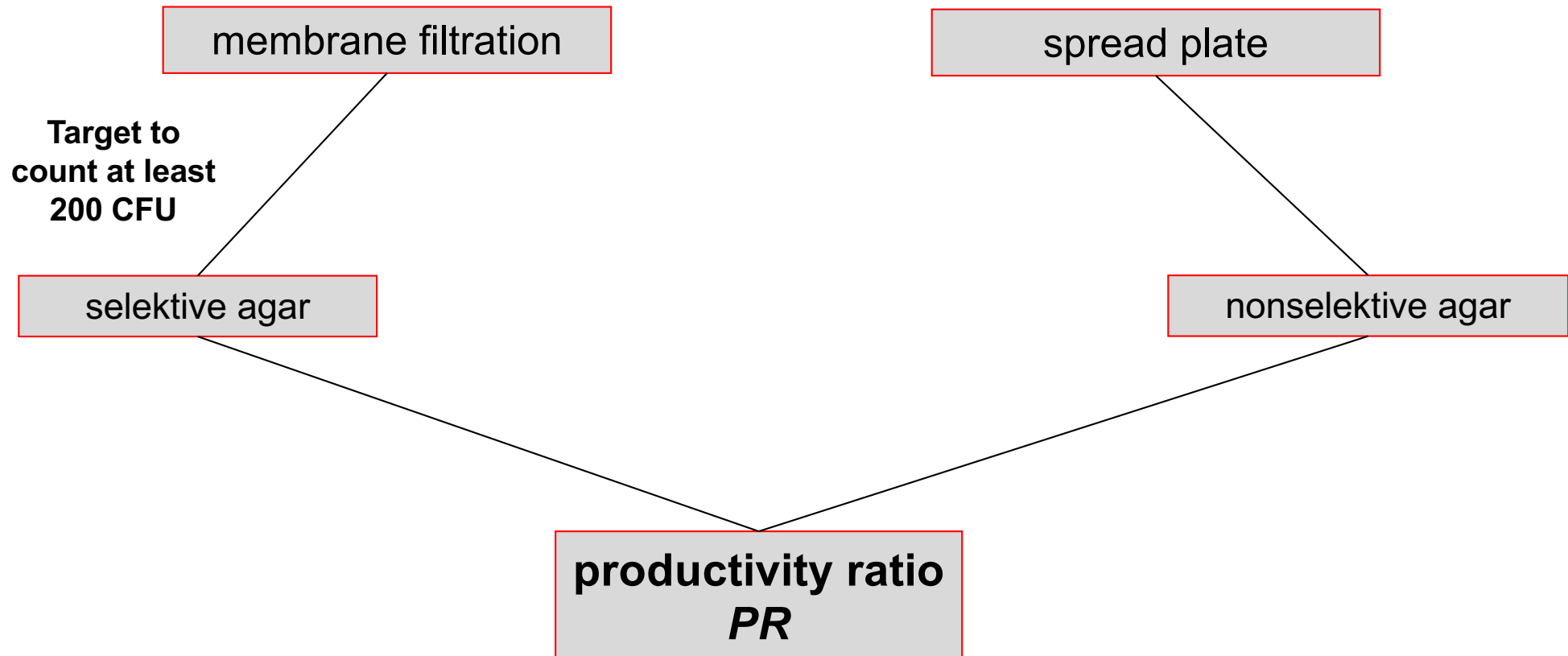
- quantitative methods → quantitative quality control
- method specific control strains
- test method
- test criteria
- example Lactose TTC medium (membrane filtration ISO 9803-1)



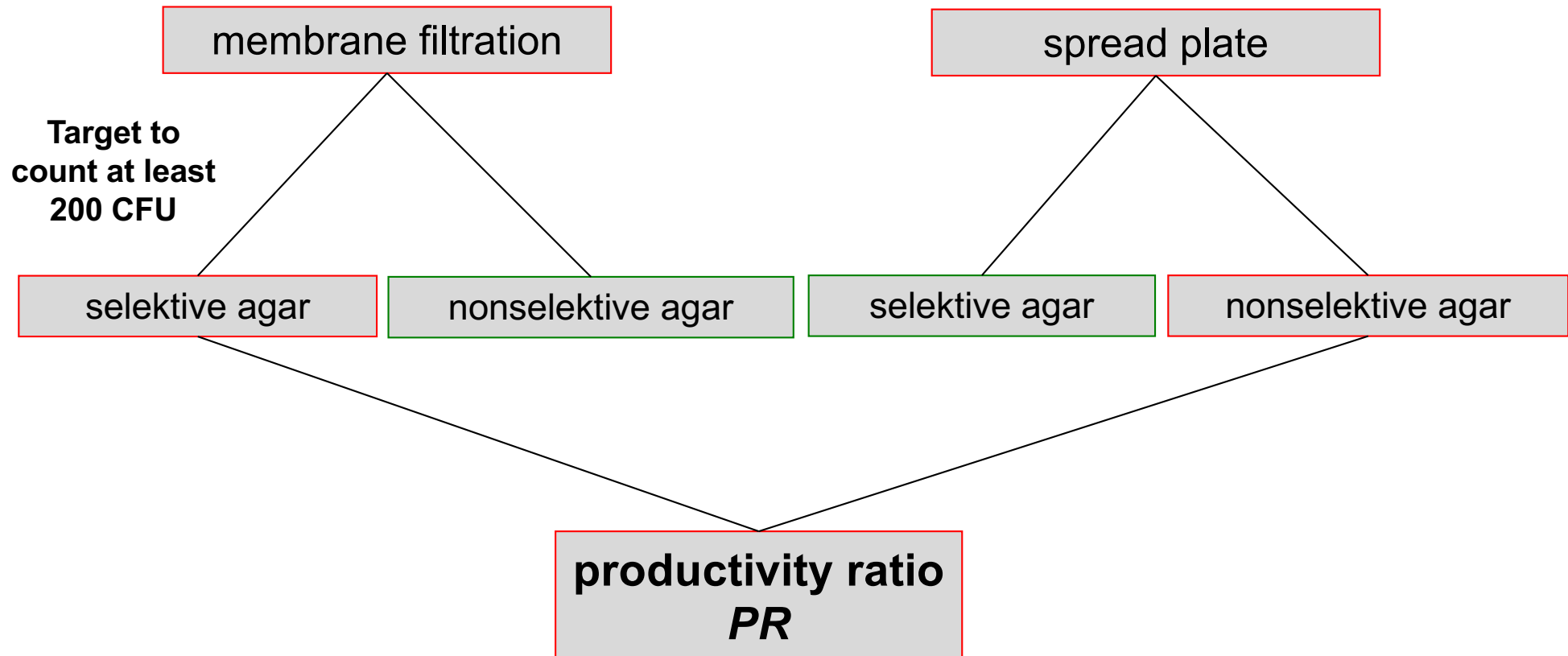
ISO 11133 Annex F (for 9803-1)

Productivity	(21 ± 3) h / (36 ± 2) °C	<i>Escherichia coli</i> 00012 ^o <i>E. coli</i> WDCM 00013 <i>E. coli</i> WDCM 00179 <i>Enterobacter aerogenes</i> WDCM 00175 <i>Klebsiella pneumoniae</i> WDCM 00097	TSA	Quantitative	$PR \geq 0,7$	Yellow colour in the medium under the membrane
Selectivity	(21 ± 3) h / (36 ± 2) °C	<i>Enterococcus faecalis</i> WDCM 00009 ^d <i>E. faecalis</i> WDCM 00087 ^d <i>E. faecalis</i> WDCM 00176 ^d <i>Staphylococcus aureus</i> WDCM 00034 ^d <i>S. aureus</i> WDCM 00032 ^d <i>S. aureus</i> WDCM 00035 ^d	—	Qualitative	Total inhibition	—
Specificity	(21 ± 3) h / (36 ± 2) °C	<i>Pseudomonas aeruginosa</i> WDCM 00024 ^d <i>P. aeruginosa</i> WDCM 00026 ^d <i>P. aeruginosa</i> WDCM 00025 ^d	—	Qualitative	—	Red colonies, blue colour in the medium

ISO 7704 (revision) draft



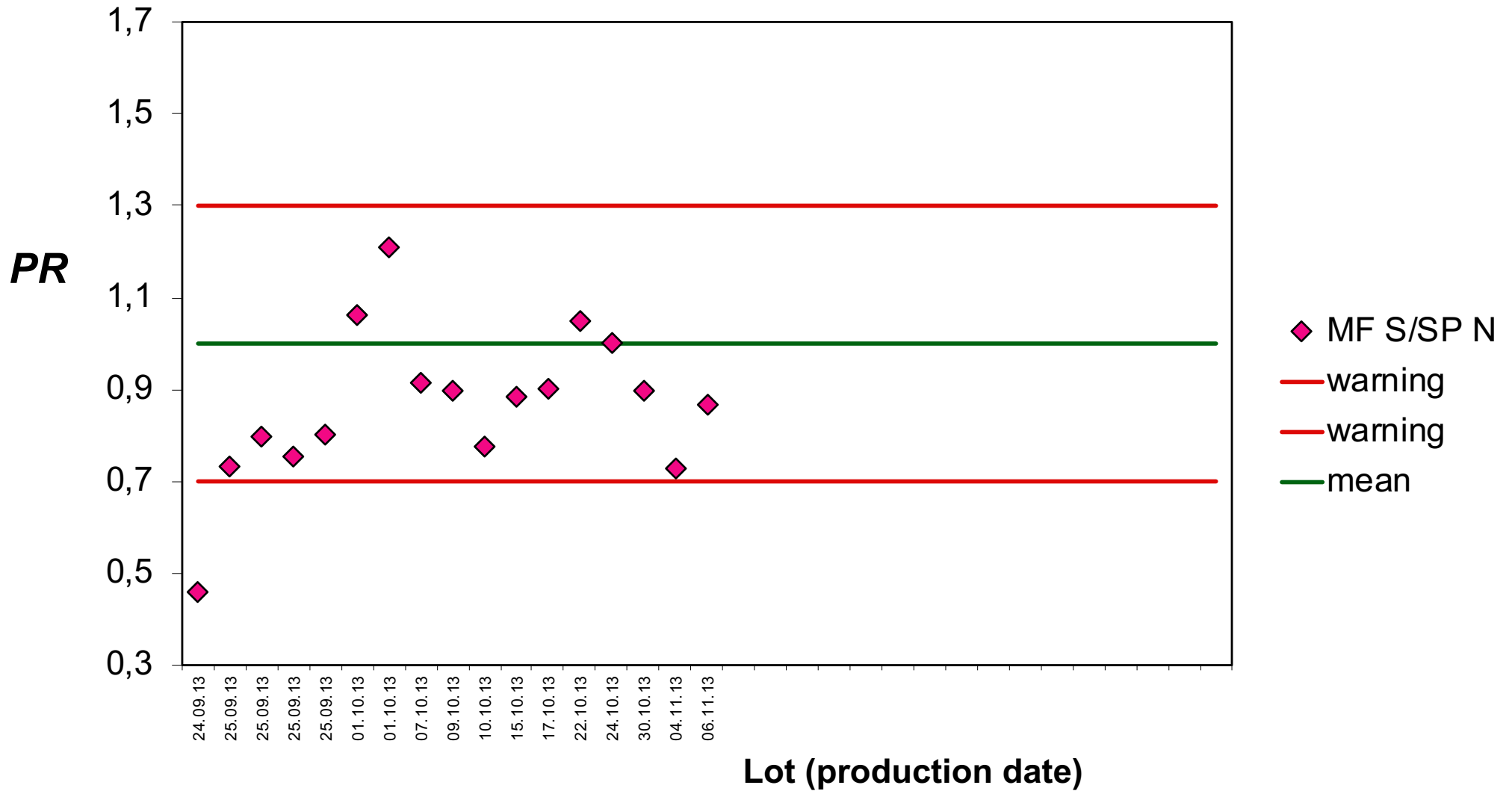
ISO 7704 (revision) draft



A MF S		B MF N		C SP S		D SP N		MF S/SP N	MF S/MF N	SP S/SP N	MF N/SP N
9	13	16	23	5	16	23	25	0,46	0,56	0,44	0,81
64	82	74	73	115	89	107	92	0,73	0,99	1,03	0,74
185	184	218	216	224	241	228	236	0,80	0,85	1,00	0,94
182	179	219	210	218	220	236	244	0,75	0,84	0,91	0,89
183	196	205	217	238	192	228	245	0,80	0,90	0,91	0,89
186	175	198	200	183	150	160	180	1,06	0,91	0,98	1,17
87	75	87	93	93	77	75	59	1,21	0,90	1,27	1,34
52	56	54	58	60	64	60	58	0,92	0,96	1,05	0,95
133	127	140	137	123	132	141	149	0,90	0,94	0,88	0,96
48	46	51	52	42	45	62	59	0,78	0,91	0,72	0,85
39	30					38	40	0,88			
31	33					35	36	0,90			
162	164	148	162	154	82	174	137	1,05	1,05	0,76	1,00
1	1					1	1	1,00			
89	105					106	110	0,90			
54	43	53	67	54	41	61	72	0,73	0,81	0,71	0,90
104	106					118	124	0,87			

MF S		SP N		MF S/SP N
9	13	23	25	0,46
64	82	107	92	0,73
185	184	228	236	0,80
182	179	236	244	0,75
183	196	228	245	0,80
186	175	160	180	1,06
87	75	75	59	1,21
52	56	60	58	0,92
133	127	141	149	0,90
48	46	62	59	0,78
39	30	38	40	0,88
31	33	35	36	0,90
162	164	174	137	1,05
1	1	1	1	1,00
89	105	106	110	0,90
54	43	61	72	0,73
104	106	118	124	0,87

Control chart TTC-Agar with Filter - K. pneumoniae





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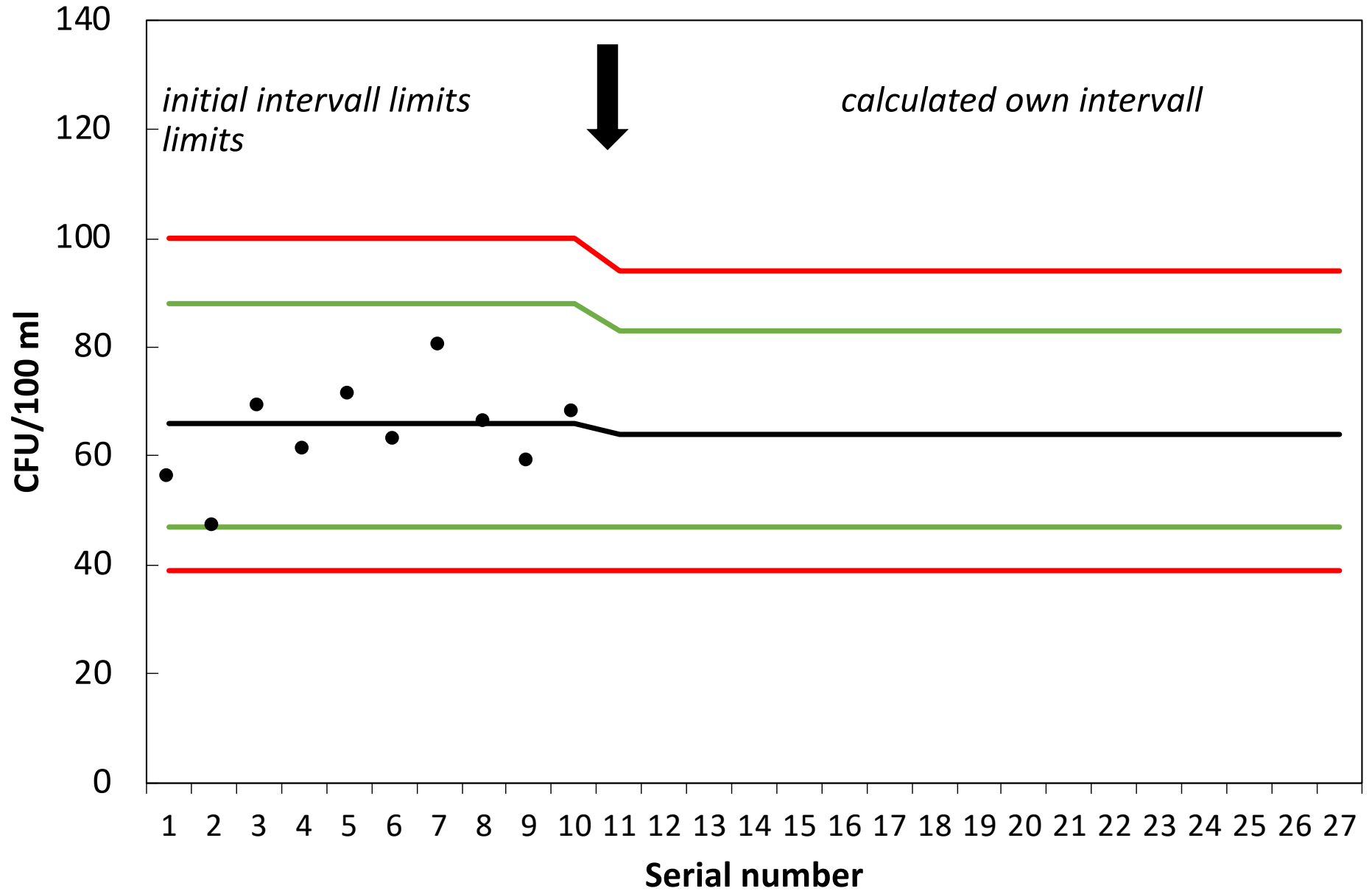
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Microbiology Division
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CONTROL CHART – WATER 1 (4)

2 April, 2013

Construction of control charts for RM, Dw sample types

- stable reference material that can be tested over extended period of time (x-charts)
- for quantitative testing of drinking water methods
- main benefits: good stability, mixed cultures



Benefits of Control Charts

- Graphical display is fast and illustrative
- Changes in quality of analyses can be detected rapidly
- Can serve to demonstrate competence to clients and auditors