

## Food Microbiology

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*Proficiency Testing*  
**Microbiology – Food**  
April 2019

**Quantitative analyses**

- Aerobic microorganisms, 30 °C
- Psychrotrophic microorganisms
- Enterobacteriaceae
- *Escherichia coli*
- Presumptive *Bacillus cereus*
- Coagulase-positive staphylococci
- Lactic acid bacteria
- *Clostridium perfringens*
- Anaerobic sulphite-reducing bacteria
- Aerobic microorganisms in fish products, 20-25 °C
- H<sub>2</sub>S-producing bacteria in fish products
- Yeasts
- Moulds

## Abbreviations

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### Media

BA	Blood agar
BcsA	<i>Bacillus cereus</i> selective agar
BP	Baird-Parker agar
CBC	Oxoid Brilliance™ <i>Bacillus cereus</i> agar
DG18	Dichloran glycerol agar
DRBC	Dichloran Rose-Bengal chloramphenicol agar
EC	<i>E. coli</i> broth
EMB	Eosin methylene blue agar
IA	Iron agar
ISA	Iron sulphite agar
LTL5B	Lactose tryptone lauryl sulphate broth
mCP	Membrane <i>Clostridium perfringens</i> agar
MPCA	Milk plate count agar
MRS	de Man, Rogosa and Sharpe-agar
MRS-aB	de Man, Rogosa and Sharpe-agar with amphotericin
MRS-S	de Man, Rogosa and Sharpe-agar with sorbic acid
MSA	Mannitol salt agar
MYP	Mannitol egg yolk polymyxin agar
OGYE	Oxytetracyclin glucose yeast extract agar
PAB	Perfringens agar base
PEMBA	Polymyxin pyruvate egg yolk mannitol bromothymol blue agar
Petrifilm AC	3M™ Petrifilm™ Aerobic Count
Petrifilm Disk	3M™ Petrifilm™ Staph Express Disk
Petrifilm EB	3M™ Petrifilm™ Enterobacteriaceae
Petrifilm EC/CC	3M™ Petrifilm™ <i>E. coli</i> /Coliform Count
Petrifilm SEC	3M™ Petrifilm™ Select <i>E. coli</i>
Petrifilm Staph	3M™ Petrifilm™ Staph Express
PCA	Plate count agar
RPFA	Baird-Parker agar with rabbit plasma fibrinogen
SC	Sulphite cycloserine agar
SFP	Shahidi-Ferguson Perfringens agar
TBX	Trypton bile X-glucuronide agar
TEMPO AC	TEMPO® Aerobic Count
TEMPO BC	TEMPO® <i>Bacillus cereus</i>
TEMPO EB	TEMPO® Enterobacteriaceae
TEMPO YM	TEMPO® Yeast/Mold
TGE	Tryptone glucose extract agar
TS	Tryptose sulphite agar
TSA	Trypton soya agar
TSC	Tryptose sulphite cycloserine agar
VRB	Violet red bile agar
VRBG	Violet red bile glucose agar
YGC	Yeast extract glucose chloramphenicol agar

### Organisations

AFNOR	French National Standardization Association
AOAC	AOAC INTERNATIONAL
ISO	International Organization for Standardization
NMKL	Nordic Committee for Food Analyses
SLV/NFA	Livsmedelsverket/National Food Agency, Sweden

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## General information on results evaluation

### Statistical evaluation of the results

Highly deviating values that did not belong to a strictly normal distribution after  $\log_{10}$  transformation were identified as statistical outliers (Grubbs' test modified by Kelly (1)). In some cases, subjective adjustments were made to set limits based on knowledge of the mixture's contents. Outliers and false results were not included in the calculations of means and standard deviations. Results reported as "> value" were excluded from the evaluation. Results reported as "< value" were interpreted as being zero (negative result). All reported results are presented in Annex 1.

According to EN ISO/IEC 17043, for which the proficiency testing programme is accredited, it is mandatory for the participating laboratories to report method information for all their analyses. Method information is sometimes difficult to interpret, since many laboratories report a medium that is not included in the standard method they refer to. Results from laboratories that report contradictory data on methods/media have either been excluded from the method analysis, or been added to the group of "Others", together with results from methods and media that are only used by 1-2 laboratories.



Mean values and standard deviations are normally provided for the different analyses. When the total number of reported results for an analysis is fewer than 20, the median is provided instead of the mean value. For method groups with fewer than 5 results, only the number of false results and outliers are provided.

### Uncertainty of measurement for the assigned values

The uncertainty of measurement for an assigned value is calculated as the standard deviation divided by the square root of the number of correct results ("standard error"). The assigned value of evaluated parameters is the mean value of the participants results.




### Table and figure legends

#### Tables

N	number of laboratories that performed the analysis
n	number of laboratories with satisfactory result
m	mean value in $\log_{10}$ cfu ml <sup>-1</sup> (false results and outliers excluded)
s	standard deviation (false results and outliers excluded)
F	number of false positive or false negative results
<	number of low outliers
>	number of high outliers
	global results for the analysis
	values discussed in the text

#### Figures

Histograms of the analytical results for each mixture and parameter are presented. The mean value of the analysis results is indicated in each histogram.

	values within the interval of acceptance (Annex 1)
	outliers
	false negative results
*	values outside of the x-axis scale

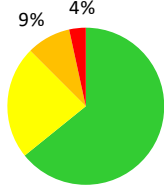
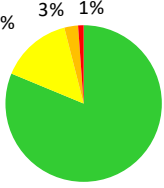
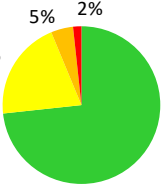
# Results of the PT round April 2019

## General outcome

Samples were sent to 182 laboratories, 42 in Sweden, 124 in other European countries, and 16 outside of Europe. Of the 176 laboratories that reported results, 90 (51 %) provided at least one result that received an annotation. In the previous round with similar analyses (April 2018) the proportion was 62 %.

Individual results for each analysis in the PT round are listed in Annex 1 and are also available on the website after logging in: <https://www2.slv.se/absint>.

**Table 1.** Composition of the test material and proportion of deviating results (N: number of reported results, F%: false positive or false negative, X%: outliers)

	Sample A				Sample B				Sample C			
<b>% participants with</b>												
<b>Microorganisms</b>	<i>Candida glabrata</i> <i>Cladosporium cladosporioides</i> <i>Clostridium perfringens</i> <i>Lactobacillus plantarum</i> <i>Pseudomonas aeruginosa</i> <i>Staphylococcus aureus</i>				<i>Aspergillus flavus</i> <i>Bacillus cereus</i> <i>Brandothrix thermosphacta</i> <i>Clostridium perfringens</i> <i>Hanseniaspora uvarum</i> <i>Shewanella putrefaciens</i>				<i>Aeromonas hydrophila</i> <i>Clostridium bifermentans</i> <i>Escherichia coli</i> <i>Hafnia alvei</i> <i>Lactobacillus plantarum</i> <i>Staphylococcus aureus</i>			
<b>Analysis</b>	<b>Target organism</b>	<b>N</b>	<b>F%</b>	<b>X%</b>	<b>Target organism</b>	<b>N</b>	<b>F%</b>	<b>X%</b>	<b>Target organism</b>	<b>N</b>	<b>F%</b>	<b>X%</b>
Aerobic micro-organisms, 30 °C	All	161	0	4	All	160	0	1	All	161	0	6
Psychrotrophic microorganisms	<i>C. cladosporioides</i>	20	65*	0*	<i>B. thermosphacta</i>	19	0	0	All	20	5	5
Enterobacteriaceae	( <i>P. aeruginosa</i> )	141	6	-	-	139	0	-	<i>E. coli</i> <i>H. alvei</i> ( <i>A. hydrophila</i> )	141	0	1
<i>E. coli</i>	-	115	1	-	-	114	0	-	<i>E. coli</i>	118	0	5
Presump. <i>B. cereus</i>	-	118	7	-	<i>B. cereus</i>	118	1	1	( <i>S. aureus</i> ) ( <i>A. hydrophila</i> )	117	7	-
Coagulase-positive staphylococci	<i>S. aureus</i>	110	2	5	-	108	6	-	<i>S. aureus</i>	110	0	5
Lactic acid bacteria	<i>L. plantarum</i>	61	0	3	-	60	30	-	<i>L. plantarum</i> ( <i>S. aureus</i> )	61	0	3
<i>C. perfringens</i>	<i>C. perfringens</i>	61	7	8	<i>C. perfringens</i>	61	2	3	( <i>C. bifermentans</i> )	60	13	-
Anaerobic sulphite-reducing bacteria	<i>C. perfringens</i>	70	3	6	<i>C. perfringens</i>	70	3	3	<i>C. bifermentans</i>	67	4	7
Aerobic microorg. in fish products	All	30	0	0	All	29	0	3	All	30	0	7
H <sub>2</sub> S-prod. bacteria in fish products	-	28	4	-	<i>S. putrefaciens</i>	27	7	0	<i>H. alvei</i>	28	4	4
Yeasts	<i>C. glabrata</i>	143	1	14	<i>H. uvarum</i>	143	1	1	-	140	2	-
Moulds	<i>C. cladosporioides</i>	141	11	4	<i>A. flavus</i>	141	1	1	-	139	1	-

- no target organism or no value  
\* the results are not evaluated

(microorganisms) = false positive before confirmation

## **Aerobic microorganisms 30 °C**

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### **Sample A**

The strains of *P. aeruginosa*, *S. aureus* and *L. plantarum* were present in the highest concentrations and were thus the main target organisms. One low and five high outliers were reported.

### **Sample B**

The strains of *B. thermosphacta*, *B. cereus* and *S. putrefaciens* were present in the highest concentrations and were thus the main target organisms. The results were distributed with a main peak at around  $\log_{10}$  4.0 cfu ml<sup>-1</sup> and a smaller peak at around  $\log_{10}$  4.8 cfu ml<sup>-1</sup>. One high outlier was reported.

The presence of two peaks is likely due to the fact of whether *B. thermosphacta* has been detected or not. *B. thermosphacta* was present in an expected concentration of  $\log_{10}$  4.7 cfu ml<sup>-1</sup>, whereas the remaining microorganisms were present in concentrations lower than  $\log_{10}$  4,0 cfu ml<sup>-1</sup>. The results in the main peak were mainly associated with the use of PCA and MPCA, whereas the results in the higher peak were mainly associated with the use of Petrifilm AC. *B. thermosphacta* is a psychrotrophic microorganism, but can also grow at 30 °C. It is possible that the use of Petrifilm AC is more gentle to *B. thermosphacta* compared to the pour-plate method that is often used with PCA. It can also be noted that *B. thermosphacta* appears to have been detected at a concentration around  $\log_{10}$  4.7 cfu ml<sup>-1</sup> in the analysis of both psychrotrophic microorganisms and the analysis of aerobic microorganisms in fish and fish products. In both these analyses, incubation is however at temperatures lower than 30 °C.

### **Sample C**

The strains of *E. coli*, *L. plantarum* and *A. hydrophila* were present in the highest concentrations and were thus the main target organisms. Three low and six high outliers were reported.

### **General remarks**

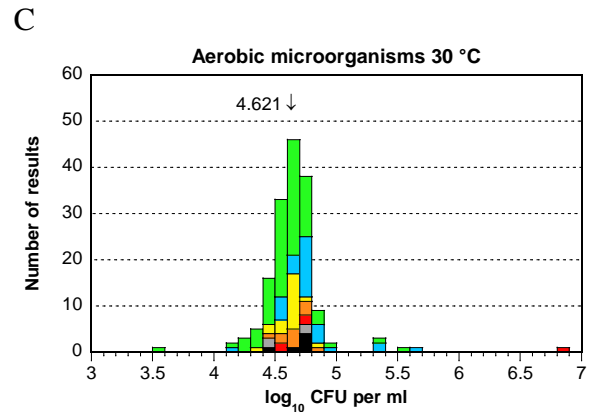
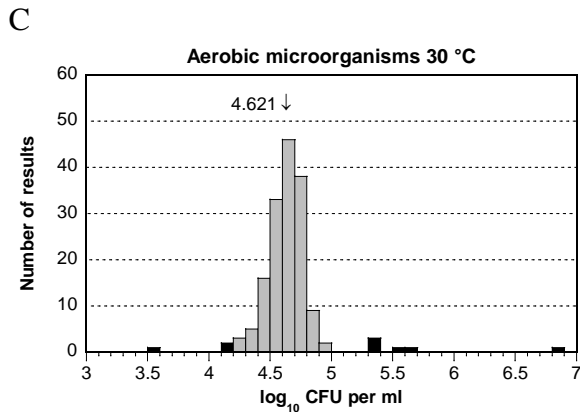
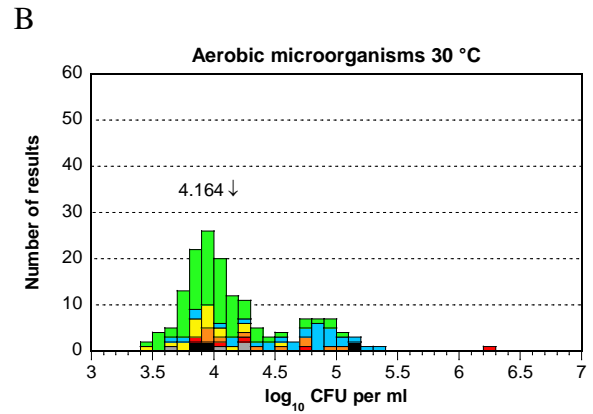
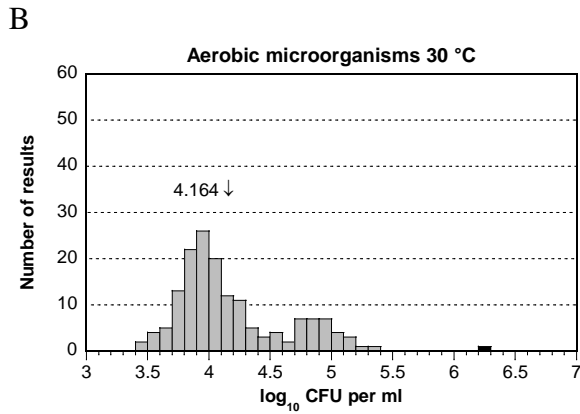
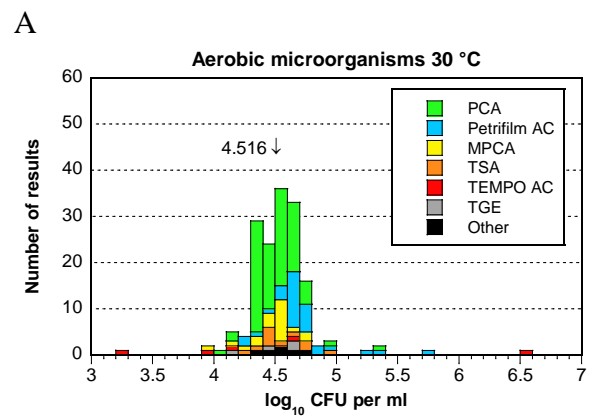
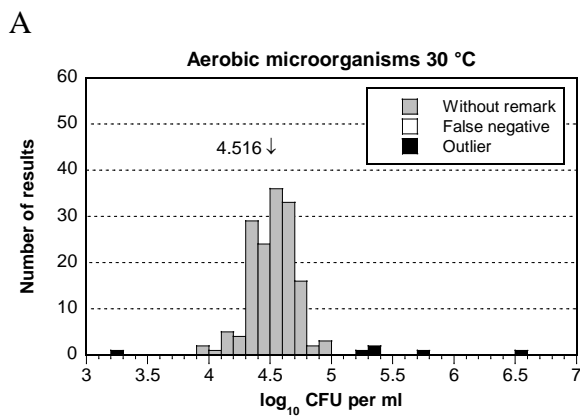
The majority of the laboratories followed either NMKL 86:2013 (30 %), ISO 4833-1:2013 (24 %) or used Petrifilm AC (20 %). A smaller number of laboratories instead stated the use of the older versions NMKL 86:2006 or ISO 4833:2003. Six laboratories used TEMPO AC, which is based on MPN (Most Probable Number). With this method, the sample is incubated in a card that contains wells with different volumes. A substrate in the wells emits fluorescence when hydrolysed by the microorganisms. The concentration is determined by the number and size of the fluorescent wells.

Both NMKL 86:2013 and ISO 4833-1:2013 prescribe a pour-plate method with PCA, with incubation at 30 °C for 72 h. However some laboratories instead use surface-spreading. Users of Petrifilm AC can use different times/temperatures for incubation, depending on which method is followed. For example, AOAC® 990.12 prescribes incubation at 35 °C for 48 h while AFNOR 3M 01/1-09/89 prescribes 30 °C for 72 h. In addition to the previously mentioned media, the use of TSA and TGE was also reported.



Results from analysis of aerobic microorganisms, 30 °C

Medium	Sample A						Sample B						Sample C					
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >
All results	161	155	4.516	0.184	0	1 5	160	159	4.164	0.433	0	0 1	161	152	4.621	0.136	0	3 6
PCA	84	83	4.490	0.157	0	0 1	84	84	4.014	0.315	0	0 0	84	80	4.586	0.140	0	2 2
Petrifilm AC	31	28	4.631	0.168	0	0 3	31	31	4.602	0.459	0	0 0	31	27	4.713	0.108	0	1 3
MPCA	20	20	4.480	0.205	0	0 0	19	19	3.947	0.243	0	0 0	20	20	4.598	0.106	0	0 0
TSA	11	11	4.550	0.198	0	0 0	11	11	4.390	0.418	0	0 0	11	11	4.659	0.109	0	0 0
TEMPO AC	5	3	-	-	0	1 1	5	4	-	-	0	0 1	5	4	-	-	0	0 1
TGE	4	4	-	-	0	0 0	4	4	-	-	0	0 0	4	4	-	-	0	0 0
Other	6	6	4.540	0.156	0	0 0	6	6	4.309	0.647	0	0 0	6	6	4.675	0.128	0	0 0



## Psychrotrophic microorganisms

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### Sample A

The strain of *C. cladosporioides* was main target organism and was present at around  $\log_{10}$  2.5 cfu ml<sup>-1</sup> in the sample. At the National Food Agency quality control, this normally forms very small colonies on PCA after 10 days incubation at 6.5 °C. A magnifying lens is therefore often required when counting the colonies, which may help explain why 13 of the 20 laboratories that performed the analysis reported a false negative result.

*Due to the difficulties with the analysis, the results for sample A are not evaluated. No z-scores have therefore been calculated, and the results are also not included in the tables under the box plots.*

### Sample B

The strain of *B. thermosphacta* was present in the highest concentration (approximately  $\log_{10}$  4.7 cfu ml<sup>-1</sup>) and was therefore the main target organism. The sample also contained, in somewhat lower concentrations, *B. cereus* and *S. putrefaciens*. These however grow less well than *B. thermosphacta* at lower temperatures. No outliers and no false negative results were reported.

### Sample C

All strains in the sample were target organisms. One false negative result was reported. One results was considerably lower than the rest, and was therefore considered an outlier. Two results (larger than or equal to  $\log_{10}$  5.0 cfu ml<sup>-1</sup>) differed from the remaining values, but could not be considered as outliers due to the low number of total results.

### General remarks

In total, 20 laboratories performed the analysis. The majority of these incubated on PCA, but MPCA and Petrifim AC were also used. As in previous proficiency testing rounds, there were considerable variations in the conditions for incubation. This is at least partly due to differences in the methods used by the laboratories. NMKL 86:2013 prescribes 10 days at 6.5 °C, but 20 h followed by 17 °C for 3 days at 7 °C can also be used. For enumeration of psychrotrophic microorganisms in milk, ISO 6730:2005/IDF 101:2005 stipulates incubation at 6.5 °C. The other method for milk, ISO 8552:2004/IDF 132:2004, instead estimates the number of psychrotrophic microorganisms in a rapid method based on incubation at 21 °C. Due to these differences, the results are difficult to evaluate, especially since several laboratories used temperatures and/or incubation times that differed from the stated method. As a general rule, the temperature stated by the laboratory is shown in the table/figures below. However in some cases an assumption has been made regarding which temperature that was used.

In general, 20-22 °C was used together with 24 h incubation, whereas 6.5 °C was used with 10 days incubation. 17 °C / 7 °C was generally used with incubation for 20 h at 17 °C, followed by 3 days at 7 °C.

One laboratory stated the use of ISO 13720:2010 (Enumeration of presumptive *Pseudomonas* spp.) and incubation at 25 °C, which seems incorrect for the analysis.

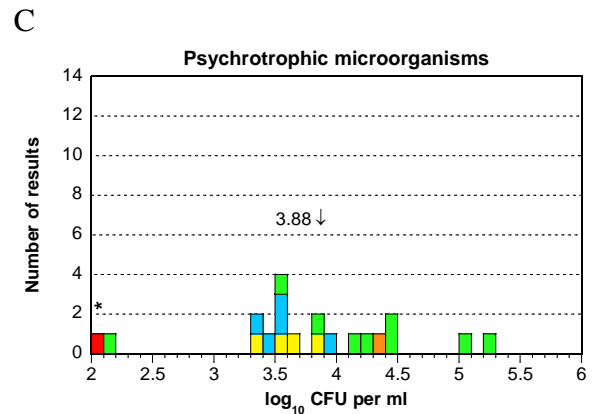
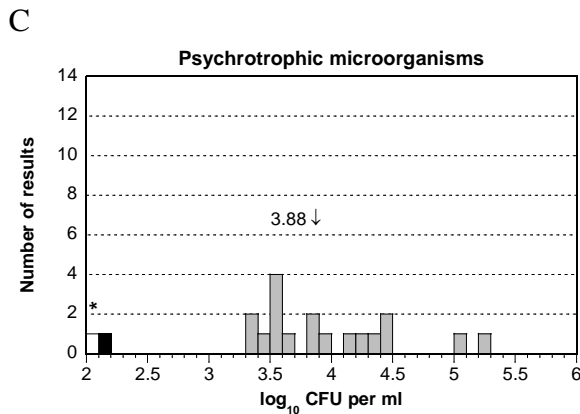
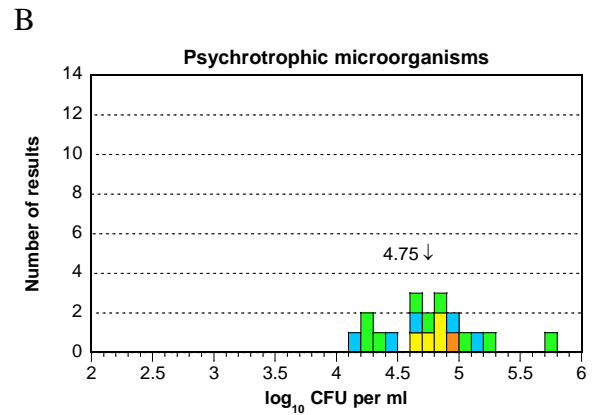
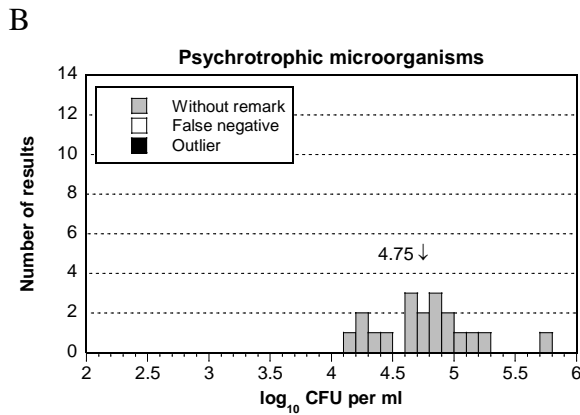
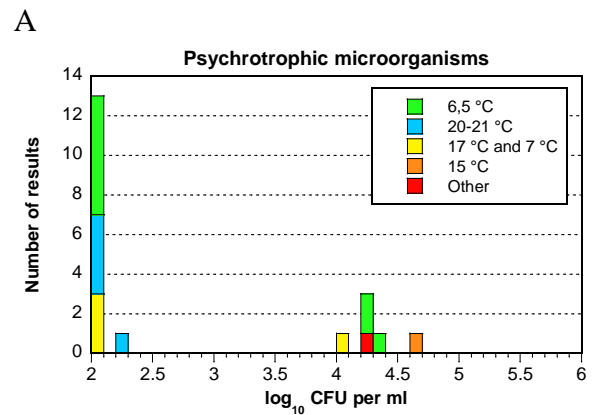
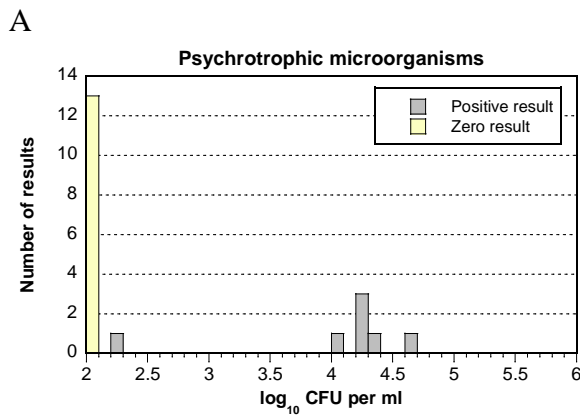
Due to the low number of participants, the median is provided instead of the mean value in tables and figures.

Results from analysis of psychrotrophic microorganisms

Temperature	Sample A*						Sample B						Sample C					
	N	n	Med**	s	F	< >	N	n	Med**	s	F	< >	N	n	Med**	s	F	< >
All results	20	7	-	-	13	- -	19	19	4.75	0.39	0	0 0 0	20	18	3.88	0.56	1	1 0
20-22 °C	9	3	-	-	6	- -	9	9	4.74	0.48	0	0 0 0	9	8	4.31	0.57	0	1 0
6,5 °C	5	1	-	-	4	- -	5	5	4.61	0.41	0	0 0 0	5	5	3.51	0.24	0	0 0 0
17 °C / 7 °C	4	1	-	-	3	- -	4	4	-	-	0	0 0 0	4	4	-	-	0	0 0 0
15 °C	1	1	-	-	0	- -	1	1	-	-	0	0 0 0	1	1	-	-	0	0 0 0
25 °C	1	1	-	-	0	- -	0	0	-	-	0	0 0 0	1	0	-	-	1	0 0 0

\* The results are not evaluated

\*\* Med = median



## Enterobacteriaceae

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### Sample A

Sample A contained to target organism for the analysis. In previous proficiency testing rounds, *P. aeruginosa* that is present in the sample has formed atypical, small beige colonies on VRBG. This may help explain why eight laboratories reported a false positive result. *P. aeruginosa* is however oxidase positive, and can therefore be distinguished from Enterobacteriaceae during confirmation.

The majority of the false positive results were reported by users of NMKL 144:2005 and VRBG, which were at the same time the most commonly used method and medium, respectively. Only two of the laboratories that reported false positive results stated that they performed a confirmation. Both of these however stated that they used an oxidase test.

### Sample B

No target organism for the analysis was present in the sample. No false positive results were reported.

### Sample C

The strains of *E. coli* and *H. alvei* were target organisms and were present in similar concentrations (approximately  $\log_{10}$  4.2 cfu ml<sup>-1</sup> and  $\log_{10}$  4,0 cfu ml<sup>-1</sup>, respectively). At the National Food Agency quality control of the sample mixture, these formed typical pink/red colonies with a bile precipitation zone on VRBG. Smaller colonies without a precipitation zone were also observed on the plates. Upon confirmation, these were oxidase positive, and were therefore not considered as Enterobacteriaceae. They can therefore likely be assumed to be *A. hydrophila*, that was also present in the sample. One low and one high outlier were reported.

### General remarks

As in previous proficiency testing rounds, most laboratories followed either NMKL 144:2005 (47 %) or a method with Petrifilm EB (22 %), whereas the ISO methods (different versions) were used by in total 21 % of the laboratories. The number of users of the new ISO 21528-2:2017 was now clearly higher than ISO 21528-2:2004 (11 % and 5 %, respectively). The new ISO 21528-1:2017 was however only used by one laboratory (1 %), whereas six laboratories (4 %) used the older ISO 21528-1:2004.

ISO 21528-2:2017 is based on colony-count, while ISO 21528-1:2017 is based on MPN (Most Probable Number). The latter method is recommended when the expected level of Enterobacteriaceae is lower than 100 cfu g<sup>-1</sup>. As in the analysis of aerobic microorganisms, a few laboratories used methods based on detection of fluorescence (TEMPO® EB).

Enterobacteriaceae are gram-negative and oxidase-negative bacteria that ferment glucose with the production of acid by-products. On VRBG, which is used by both NMKL 144 and ISO 21528-2, they therefore form pink/red colonies, with or without a bile precipitation zone. The appearance is similar on Petrifilm EB, which also includes a colour indicator for acid by-products and a plastic film for detection of gas production. With NMKL 144:2005, presumptive colonies on VRBG are confirmed with an oxidase test. With ISO 21528-2:2017, colonies are instead confirmed both with an oxidase test and with a test for glucose fermentation. Oxidase-negative colonies that also ferment glucose in glucose oxidation/fermentation (OF) medium are confirmed as Entero-

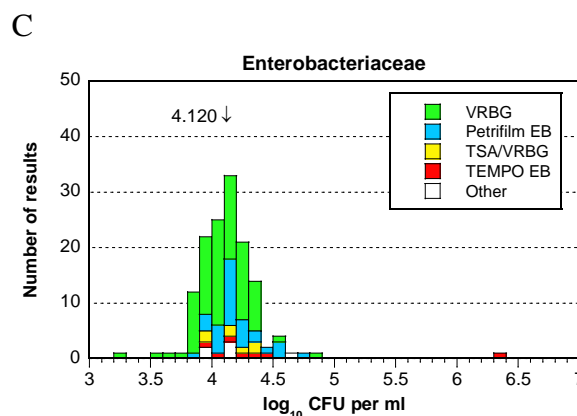
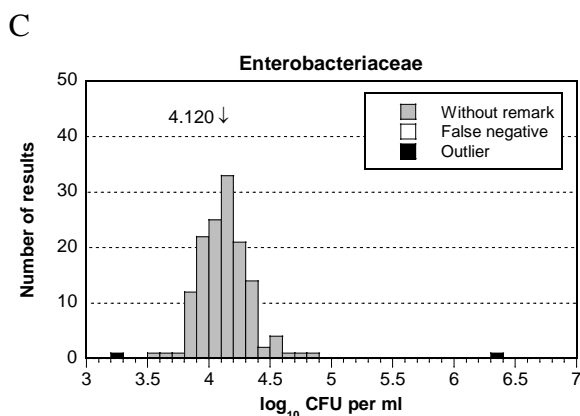
bacteriaceae. In total, 67 % of the laboratories stated that they performed some kind of confirmation. The vast majority of these specified that this consisted of an oxidase test.

As already mentioned, no large differences could be seen between the different methods and media that were used. However, somewhat higher results could be seen for TEMPO EB for sample C, compared to other media. Still, such higher results have been seen for TEMPO EB in several previous proficiency testing rounds, and can therefore be considered normal.

### Results from analysis of Enterobacteriaceae

Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	141	133	-	-	8	- -	139	139	-	-	0	- -	141	139	4.120	0.201	0	1	1
VRBG	88	82	-	-	6	- -	86	86	-	-	0	- -	88	87	4.079	0.195	0	1	0
Petrifilm EB	33	33	-	-	0	- -	33	33	-	-	0	- -	33	33	4.196	0.201	0	0	0
TSA/VRBG	7	7	-	-	0	- -	7	7	-	-	0	- -	7	7	4.140	0.162	0	0	0
TEMPO EB	7	7	-	-	0	- -	7	7	-	-	0	- -	7	6	4.207	0.173	0	0	1
Other*	6	4	-	-	2	- -	6	6	-	-	0	- -	6	6	4.180	0.248	0	0	0

\* The group Other includes e.g. Compact Dry™ ETB and RAPID® Enterobacteriaceae.



## Escherichia coli

### Sample A

No target organism was present in the sample. One false positive result was reported.

### Sample B

No target organism was present in the sample. No false positive results were reported.

### Sample C

The strain of *E. coli* was target organism for the analysis and was present at approximately  $\log_{10}$  4.2 cfu ml<sup>-1</sup> in the sample. The strain is indole positive,  $\beta$ -glucuronidase positive, and forms gas in LTL5B. Five low and one high outliers were reported. Two of the low outliers were reported by users of MPN methods, which are possibly not adapted for detection of the concentration of *E. coli* that was present in the sample. Low outliers were also reported by users of TBX, and by users of TSA in

combination with VRB (TSA/VRB). At the same time, these were the most commonly used media.

### **General remarks**

As before, the use of 3M™ Petrifilm™ was high, and in total 32 % of the laboratories stated the use of “Petrifilm” as method. In comparison, the methods NMKL 125:2005 and ISO 16649-2:2001 were used by 28 % and 15 % of the laboratories, respectively. It should however be added that some of the laboratories that followed NMKL 125:2005 and ISO 16649-2:2001 also stated that they incubated on Petrifilm EC/CC or Petrifilm SEC. The MPN methods ISO 7251:2005 and NMKL 96:2009 were used by four and one laboratory, respectively. It can also be mentioned that NMKL 125 is being revised, and the new version will likely be more similar to ISO 16649-2.

The definition of *E. coli* differs between the methods. ISO 16649-2:2001 defines *E. coli* as bacteria that form typical blue colonies on TBX after 18-24 h at 44 °C. The blue colour is due to *E. coli* β-glucuronidase reacting with an indicator in the medium. No additional confirmation of β-glucuronidase positive colonies is required according to ISO 16649-2:2001. Petrifilm EC/CC and Petrifilm SEC are also based on media that detect *E. coli* β-glucuronidase. Further, the plastic film in these media facilitates detection of gas production due to lactose fermentation. In comparison, NMKL 125:2005 describes the analysis of both thermotolerant coliform bacteria and *E. coli*. Thermotolerant coliform bacteria are defined as those that form typical dark red colonies surrounded by a red precipitation zone on VRB after 24 h at 44 °C. Confirmation is by inoculation into either EC or LTL SB. In both these media, thermotolerant coliform bacteria produce gas as a result of lactose fermentation. *E. coli* are then defined as thermotolerant coliform bacteria that also produce indole in either LTL SB or tryptone broth. In total, 55 % of the laboratories stated they performed some kind of confirmation – normally a test for the production of gas or indole. No clear difference could however be seen between laboratories that performed a confirmation test, and those that did not.

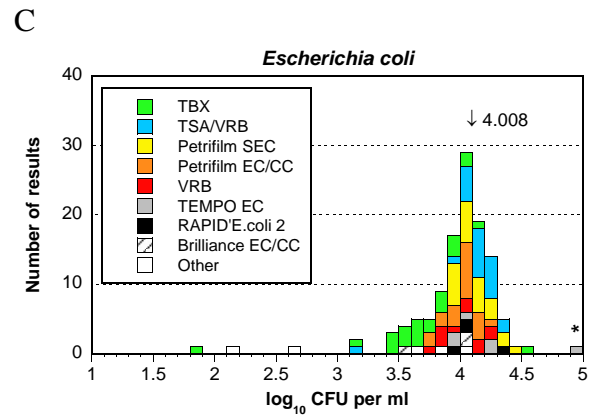
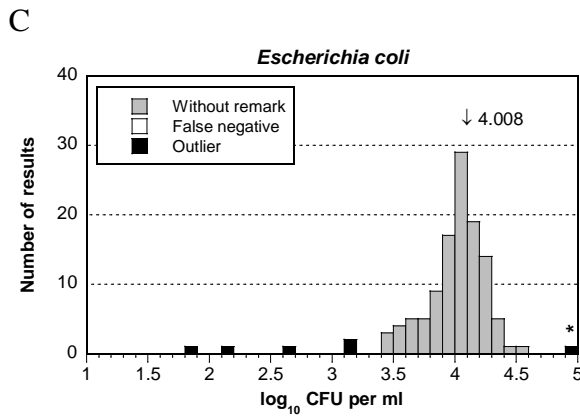
As in previous proficiency testing rounds, for the analysis of *E. coli* there were several methods and media that were only used by a small number of laboratories. As a whole, these however appear to have performed as well as the methods and media used by the majority of the laboratories. Also as before, the mean value for TBX was somewhat lower, and the mean value for TSA/VRB somewhat higher, compared to other media. This has been observed in several previous proficiency testing rounds, and can therefore be considered as normal. The differences are likely due to whether the laboratory has performed a pre-incubation or not. For samples that are suspected to contain stressed microorganisms, ISO 16649-2:2001 prescribes a pre-incubation at 37 °C for 4 h, prior to the final incubation at 44 °C. In comparison, with NMKL 125:2005 a similar pre-incubation is routinely carried out (1-2 h on TSA at 20-25 °C).

Incubation was somewhat more often done at 41.5-44 °C (61 %) than at 35-37 °C (39 %). For sample C, laboratories that incubated at the higher temperature reported five (low) outliers whereas those that incubated at the lower temperature only reported one (high) outlier. However, the mean values for the two temperature did not differ.

*Results from analysis of Escherichia coli*

Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	115	114	-	-	1	- -	114	114	-	-	0	- -	118	112	4.008	0.220	0	5	1
TBX	24	24	-	-	0	- -	24	24	-	-	0	- -	24	22	3.775	0.267	0	2	0
TSA/VRB	22	22	-	-	0	- -	21	21	-	-	0	- -	22	21	4.156	0.103	0	1	0
Petrifilm SEC	22	22	-	-	0	- -	22	22	-	-	0	- -	23	23	4.111	0.142	0	0	0
Petrifilm EC/CC	19	19	-	-	0	- -	19	19	-	-	0	- -	20	20	4.009	0.133	0	0	0
VRB	10	10	-	-	0	- -	10	10	-	-	0	- -	11	11	3.996	0.174	0	0	0
TEMPO EC	6	5	-	-	1	- -	6	6	-	-	0	- -	6	5	4.072	0.146	0	0	1
RAPID'E.coli 2	4	4	-	-	0	- -	4	4	-	-	0	- -	4	4	-	-	0	0	0
Brilliance EC/CC	2	2	-	-	0	- -	2	2	-	-	0	- -	3	3	-	-	0	0	0
Other*	6	6	-	-	0	- -	6	6	-	-	0	- -	5	3	-	-	0	2	0

\* Other media includes EMB, Compact Dry EC/CC, and EC-broth (for the MPN methods)



**Presumptive *Bacillus cereus***

**Sample A**

No target organism for the analysis was present in the sample. Eight of the 118 laboratories reported a false positive result. This was despite the fact that seven of these eight laboratories stated that they performed some kind of confirmation.

**Sample B**

The strain of *B. cereus* was target organism and was present at approximately  $\log_{10}$  4.0 cfu ml<sup>-1</sup> in the sample. One low outlier and one false negative result was reported.

**Sample C**

No target organism for the analysis was present in the sample. Several strains in the sample may however form colonies on BA. In addition, *A. hydrophila* and *S. aureus* may form atypical blue colonies on BcsA during the confirmation. This can help explain why eight laboratories reported a false positive result. Among these eight laboratories, it can be noted that only two stated that they used another medium in addition to BA. Further, only four of the eight laboratories stated that they performed some kind of confirmation. In total, 60 % of the laboratories performed a confirmation test.

## General remarks

As in previous proficiency testing rounds, most laboratories followed either NMKL 67:2010 (57 %) or ISO 7932:2004 (24 %). NMKL 67:2010 is based on incubation on BA. On this medium, *B. cereus* forms large, irregular grey colonies, surrounded by a distinct zone of haemolysis. Colonies are confirmed either on BcsA or on Cereus-Ident agar (a chromogenic medium). On BcsA presumptive *B. cereus* form bluish colonies that are surrounded by a zone of precipitation, due to lecithinase activity on egg yolk present in the medium. On Cereus-Ident agar, presumptive *B. cereus* are blue/turquoise and possibly surrounded by a blue ring. The colour is a result of *B. cereus* phosphatidylinositol phospholipase C (PI-PLC) cleavage of the chromogenic substrate X-myoinositol-1-phosphate present in Cereus-Ident agar. In comparison, ISO 7932:2004 prescribes plating onto MYP, followed by confirmation on BA. On MYP, presumptive *B. cereus* form large pink colonies that are normally surrounded by a zone of precipitation, again as a consequence of lecithinase activity. The ISO method uses haemolysis on BA as the method for confirmation.

Besides BA, BcsA and MYP, the medium CBC was used by nine laboratories. CBC is a chromogenic medium, and cleavage of X-Gluc present in CBC by *B. cereus*  $\beta$ -glucuronidase results in white colonies with a blue/green centre. Other media that were used to a lesser extent were COMPASS® *Bacillus cereus* agar, Compact Dry X-BC, and BACARA®. These have been added to the group “Other”. Two laboratories have stated they used the fluorescence-based TEMPO BC in addition to other media.

As in previous proficiency testing rounds the reporting of method data was in several cases unclear. For example, several laboratories reported that the same medium was used in both steps in the analysis. Other laboratories reported combinations of method and media that were incompatible. As a general rule, the tables and figures below are based on the methods/media stated by the laboratories, regardless if these are compatible or not. Laboratories that have only stated “chromogenic medium” are included in the group “Other”. Despite these uncertainties, the results and mean values for the different methods and media are very similar.

In total 57 % of the laboratories followed NMKL 67:2010. At the same time, it can be noted that 11 of the 14 laboratories (79 %) that reported at least one result with an annotation (false results or outliers) followed this method.

### Results from analysis of presumptive *Bacillus cereus*

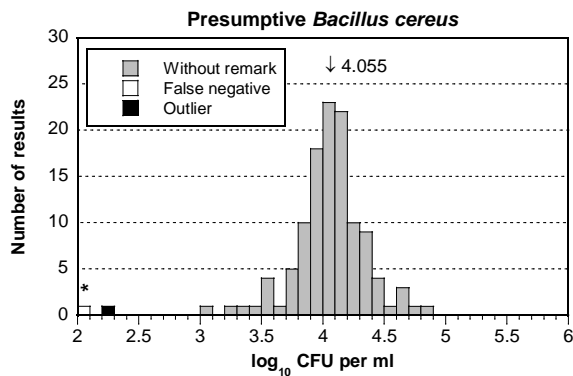
Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	118	110	-	-	8	- -	118	116	4.055	0.285	1	1	0	117	109	-	-	8	- -
BA + BcsA*	33	31	-	-	2	- -	31	31	4.113	0.268	0	0	0	33	32	-	-	1	- -
BA	25	22	-	-	3	- -	27	27	4.074	0.276	0	0	0	25	19	-	-	6	- -
BA + MYP	20	19	-	-	1	- -	20	20	4.034	0.231	0	0	0	19	18	-	-	1	- -
MYP	18	17	-	-	1	- -	18	16	3.894	0.380	1	1	0	18	18	-	-	0	- -
CBC	9	9	-	-	0	- -	9	9	4.150	0.227	0	0	0	9	9	-	-	0	- -
BcsA*	7	6	-	-	1	- -	7	7	4.063	0.319	0	0	0	7	7	-	-	0	- -
Other**	6	6	-	-	0	- -	6	6	4.016	0.281	0	0	0	6	6	-	-	0	- -

\* The use of PEMBA has been interpreted as the use of BcsA.

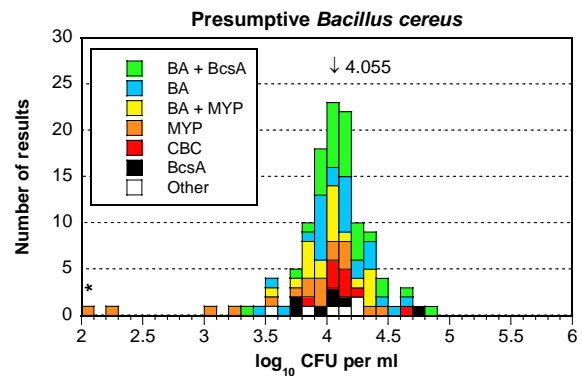
\*\* Other media includes e.g. COMPASS® *Bacillus cereus* agar, Compact Dry X-BC, and BACARA®.



B



B



## Coagulase-positive staphylococci

### Sample A

The strain of *S. aureus* was target organism for the analysis and was present at approximately  $\log_{10}$  3.9 cfu ml<sup>-1</sup> in the sample. Six low outliers and two false negative results were reported.

### Sample B

No target organism for the analysis was present in the sample. Six false positive results were reported.

### Sample C

The strain of *S. aureus* (not identical to the one in sample A) was target organism for the analysis and was present at approximately  $\log_{10}$  3,5 cfu ml<sup>-1</sup> in the sample. Four low and one high outlier were reported.

### General remarks

Similar to previous proficiency testing rounds, most laboratories followed NMKL 66:2009 (46 %). Other common methods were ISO 6888-1:1999 (15 %), ISO 6888-2:1999 (10 %) and Petrifilm Staph (12 %). Both ISO 6888-1:1999 (based on BP) and ISO 6888-2:1999 (based on RPFA) were reviewed by ISO in 2015, and remain current. An alternative confirmation by stab-culture in RPFA has however been added for ISO 6888-1 (ISO 6888-1:1999/Amd 2:2018).

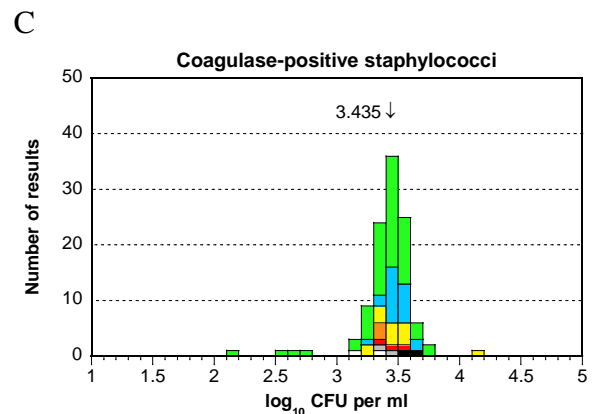
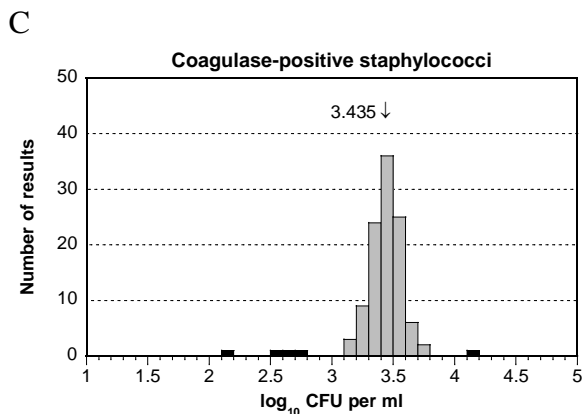
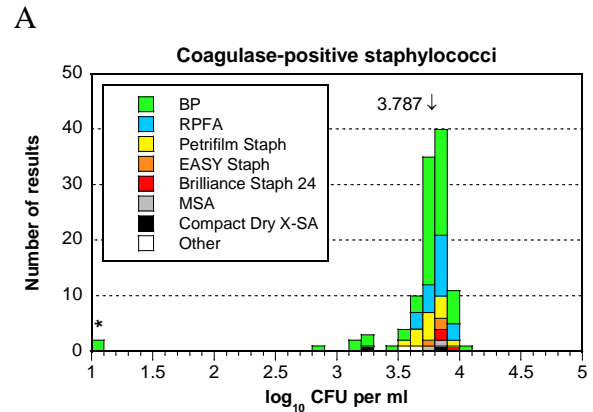
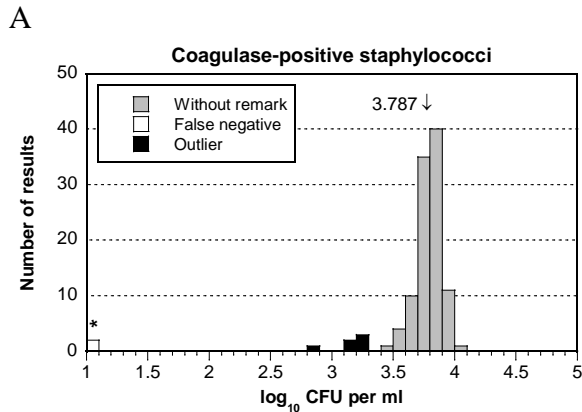
NMKL 66:2009 prescribes incubation on BP and/or RPFA. On BP, *S. aureus* forms characteristic convex, shiny colonies that have a grey/black colour due to reduction of tellurite in the medium. Proteolysis of egg yolk in the medium (due to lecithinase activity) normally causes a clear zone around the colonies. An opaque halo may also form near the colony, due to precipitation caused by lipase activity. The colonies are confirmed by a positive result in a coagulase test. When using RPFA, the coagulase activity is instead tested directly in the medium, and no further confirmation is required. In comparison, ISO 6888-1:1999 stipulates surface spreading on BP followed by confirmation with a coagulase test, whereas 6888-2:1999 stipulates the use of RPFA. Petrifilm Staph is based on a modified Baird-Parker agar. It also contains a chromogenic indicator that causes *S. aureus* to form red/purple colonies.

In summary, the results were very similar for the most common media BP, RPFA and Petrifilm Staph, for all three samples. Somewhat more results with an annotation were reported by users of BP, but at the same time this was the medium used by most laboratories. Slightly lower mean values have in previous proficiency testing rounds been observed for Petrifilm Staph, but this time no such trend could be seen. Several media were used by only a small number of laboratories, which make them difficult to evaluate. However altogether, only two results with an annotation were reported by the laboratories that used either of the media MSA, EASY Staph®, Brilliance™ Staph 24 and Compact Dry™ X-SA.

Traditionally, coagulase-positive staphylococci are confirmed by detection of extracellular or bound coagulase (tube coagulase test and slide coagulase test respectively). Another common confirmation is a latex agglutination test. This is based on latex particles coated either with fibrinogen or with IgG that binds to protein A on the bacterial cell surface. Antibodies targeted against polysaccharides on the bacterial cell surface are also used in variations of this test. With Petrifilm Staph, confirmation is instead often carried out with Petrifilm Disk. This test detects extracellular DNase, which is produced by the majority of coagulase-positive *S. aureus*, but also by the coagulase-positive staphylococci *S. intermedius* and *S. hyicus*. Toluidin blue O in the disks visualises DNase activity as a pink zone around the colonies. Confirmation of some kind was here performed by 76 % of the laboratories as a whole, and by 95 % of the laboratories that incubated on BP. The most common method was a tube coagulase test, but latex agglutination tests and Petrifilm Disk were also common. As a whole, the results with an annotation appear to be proportionally distributed between laboratories that performed a confirmation test, and those that did not.

*Results from analysis of coagulase-positive staphylococci*

Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	110	102	3.787	0.104	2	6 0	108	102	-	-	6	- -	110	105	3.435	0.118	0	4	1
BP	62	55	3.791	0.102	2	5 0	60	57	-	-	3	- -	62	58	3.429	0.128	0	4	0
RPFA	22	22	3.808	0.091	0	0 0	22	21	-	-	1	- -	22	22	3.482	0.091	0	0	0
Petrifilm Staph	14	14	3.731	0.106	0	0 0	14	14	-	-	0	- -	14	13	3.412	0.096	0	0	1
EASY Staph	3	3	-	-	0	0 0	3	3	-	-	0	- -	3	3	-	-	0	0	0
Brilliance Staph 24	3	3	-	-	0	0 0	3	2	-	-	1	- -	3	3	-	-	0	0	0
MSA	2	2	-	-	0	0 0	2	2	-	-	0	- -	2	2	-	-	0	0	0
Compact Dry X-SA	2	1	-	-	0	1 0	2	2	-	-	0	- -	2	2	-	-	0	0	0
Other	2	2	-	-	0	0 0	2	1	-	-	1	- -	2	2	-	-	0	0	0



## Lactic acid bacteria

### Sample A

The strain of *L. plantarum* was target organism for the analysis and was present at approximately  $\log_{10} 4.2$  cfu ml<sup>-1</sup> in the sample. One low and one high outlier were reported.

### Sample B

No target organism for the analysis was present in the sample. Despite this, 18 of the 60 laboratories reported a false positive result. The 10 laboratories that used MRS-aB appeared to be somewhat over-represented among the false results, while none of the six laboratories that used Rogosa reported a false positive result. At the National Food, no colonies were observed on MRS-aB during quality control of the sample mixture. Performing a confirmation test does not appear to have had an impact on the outcome for the laboratories that reported a false positive result.

### Sample C

The strain of *L. plantarum* (not identical to the one in sample A) was target organism for the analysis and was present at approximately  $\log_{10} 4.3$  cfu ml<sup>-1</sup> in the sample. At the National Food Agency, it formed typical round, white colonies on MRS-aB. As expected, the strain was catalase negative upon confirmation.

Two high outliers were reported. This may be due to detection of *S. aureus*. The strain of *S. aureus* in the sample has in previous proficiency testing rounds been able to form small colonies on MRS and MRS-aB.

### General remarks

The majority of the laboratories stated that they followed NMKL 140, either NMKL 140:2007 (41 %), or the older NMKL 140:1991 (13 %). The older method prescribes spreading onto MRS-S, whereas the new method prescribes MRS-aB. On both media, lactic acid bacteria form 1.5-2 mm large grey-white colonies. In comparison, ISO 15214:1998 uses a pour-plate method with MRS. This method was used by 15 % of the laboratories. ISO 15214:1998 was reviewed by ISO in 2015, and remains current. NMKL 140 is however considered for revision, and changes will likely be made to the confirmation tests.

Lactic acid bacteria constitute a heterogeneous group of microorganisms, and therefore have different optimal medium, pH and incubation conditions. For example MRS-aB (pH 6.2), which is used in NMKL 140:2007, is a less selective medium that allows the growth of a more wide range of lactic acid bacteria. This may however also result in the appearance of more false positive colonies compared to the more acid medium MRS-S (pH 5.7). Such differences between media and incubation conditions underline the importance of performing a confirmation test in uncertain cases, especially when using a less selective medium. Possibly, this might have contributed to the false positive results for MRS-aB in sample B.

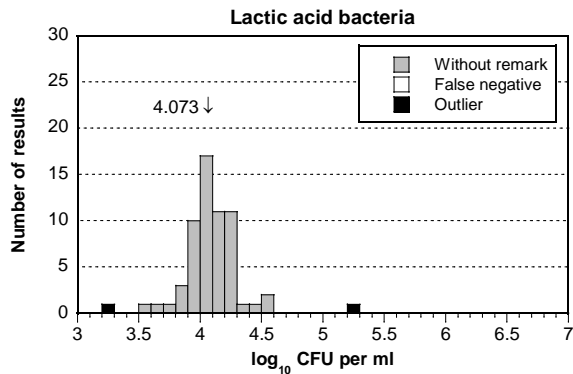
Both the ISO and the NMKL methods recommend confirmation of uncertain colonies by Gram staining and/or with a catalase test. Lactic acid bacteria are Gram positive and normally catalase negative. Confirmation was in this proficiency testing performed by roughly half (54 %) of the laboratories. Usually, it consisted of a catalase test, but Gram staining was also common. As a whole, the use of a confirmation test does not appear to have had an impact on the result. The results with an annotation were also distributed proportionally between laboratories that performed a confirmation test, and those that did not. This was true for all three samples.

### Results from analysis of lactic acid bacteria

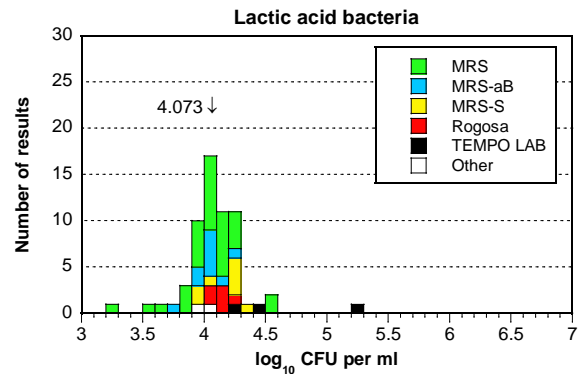
Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	61	59	4.073	0.179	0	1	1	60	42	-	-	18	-	-	61	59	4.222	0.140	0	0	2
MRS	32	31	4.052	0.210	0	1	0	31	22	-	-	9	-	-	32	31	4.195	0.113	0	0	1
MRS-aB	10	10	4.029	0.114	0	0	0	10	5	-	-	5	-	-	10	10	4.213	0.215	0	0	0
MRS-S	8	8	4.141	0.151	0	0	0	8	6	-	-	2	-	-	8	8	4.265	0.142	0	0	0
Rogosa	6	6	4.127	0.066	0	0	0	6	6	-	-	0	-	-	6	6	4.273	0.118	0	0	0
TEMPO LAB*	3	2	-	-	0	0	1	3	3	-	-	0	-	-	3	2	-	-	0	0	1
Other	2	2	-	-	0	0	0	2	0	-	-	2	-	-	2	2	-	-	0	0	0

\* TEMPO® Lactic Acid Bacteria

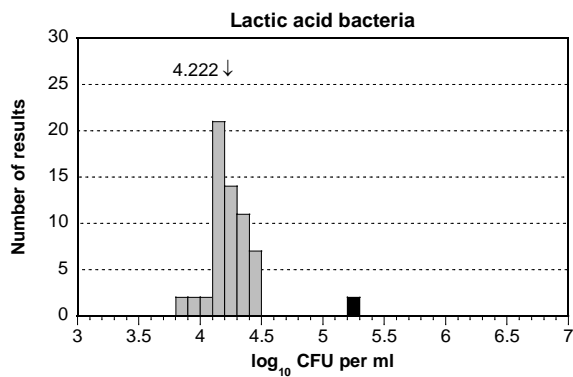
A



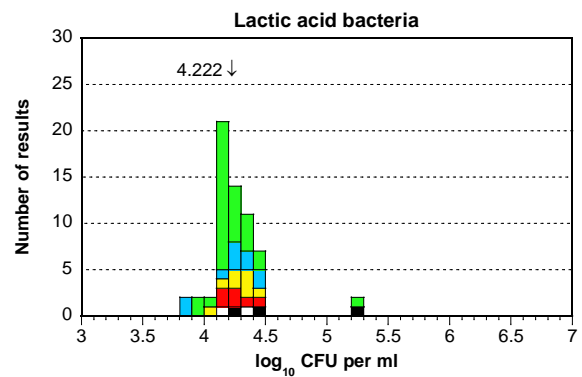
A



C



C



## *Clostridium perfringens*

### Sample A

The strain of *C. perfringens* was target organism for the analysis and was present at approximately  $\log_{10} 2.8$  cfu  $\text{ml}^{-1}$  in the sample. Five low outliers and four false negative results were reported. In previous proficiency testing rounds (e.g. PT April 2016) such low results have been attributed to the use of mCP. In the current proficiency testing round mCP was only used by one laboratory, which however did report a low outlier. The remaining four low outliers were reported by laboratories that incubated on TSC.

### Sample B

The strain of *C. perfringens* (identical to the one in sample A) was target organism for the analysis and was present at approximately  $\log_{10} 2,7$  cfu  $\text{ml}^{-1}$  in the sample. Two low outliers and one false negative result were reported.

### Sample C

No target organism for the analysis was present in the sample. It did however contain a strain of *C. bifermentans*, which is false negative for the analysis, at approximately  $\log_{10} 3.2$  cfu  $\text{ml}^{-1}$ . The strain can easily be distinguished from *C. perfringens* upon confirmation, for example since *C. bifermentans* is motile. Not performing a confirmation, or problems with the confirmation, may explain why eight of the 60 laboratories reported a false positive result. Five of the eight laboratories stated that they performed a confirmation.

## General remarks

The majority of the laboratories (61 %) followed NMKL 95:2009. Two and one laboratory followed the older versions NMKL 95:2006 and NMKL 95:1997, respectively. ISO 7937:2004 was used by 28 % of the laboratories. Two other laboratories stated that they followed NMKL 56 (Sulphite-reducing Clostridia). This method includes detection of *C. perfringens* by referring to the confirmation tests in NMKL 95. ISO 7937:2004 was reviewed by ISO in 2015 and remains current. It is however scheduled to be replaced by ISO 15213-2 ("Enumeration of *Clostridium perfringens* by colony-count technique"), which is currently under development. No differences could be seen in the results from the different methods.

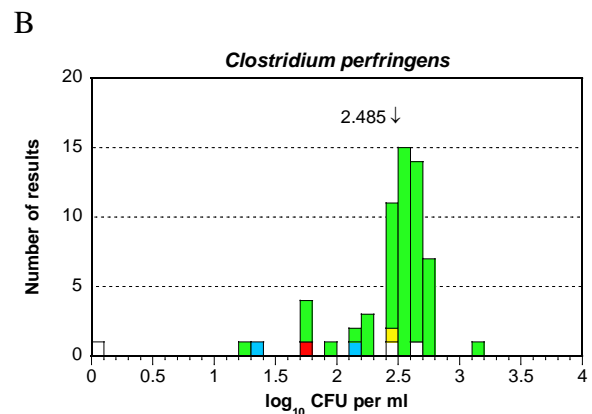
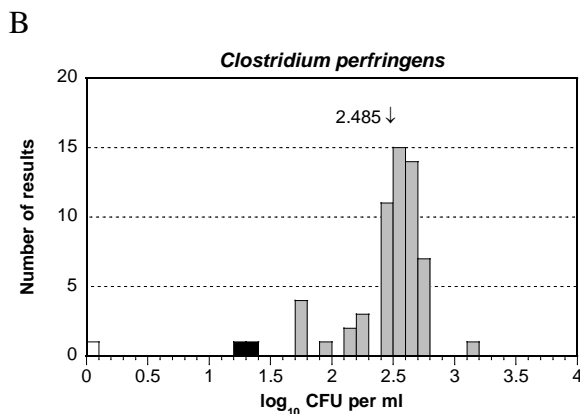
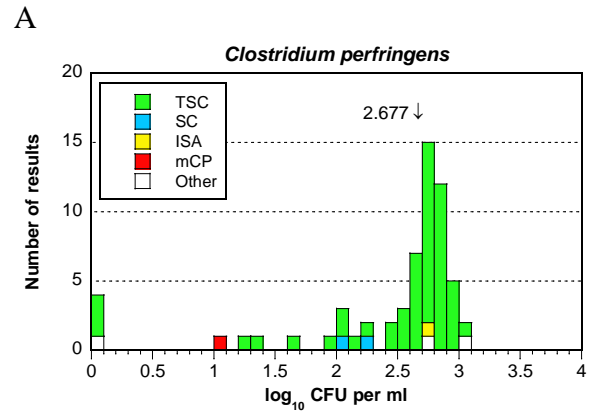
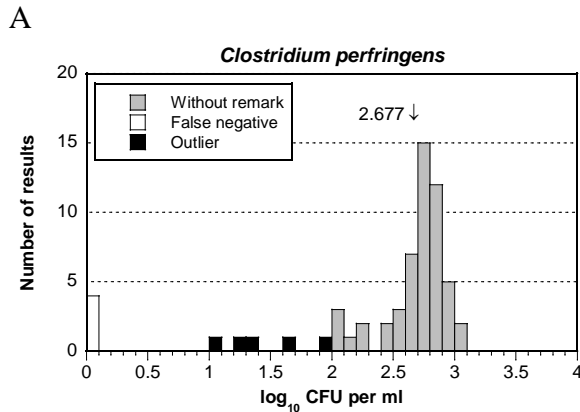
ISO 7937:2004 prescribes a pour-plate method with TSC, while NMKL 95 prescribes surface-spreading on mCP and/or pour-plating with TSC. Here, the majority of the laboratories (89 %) stated the use of TSC. On TSC, *C. perfringens* form black colonies after anaerobic incubation at 37 °C. In addition to TSC, mCP, SC and JSA were used by a few laboratories. Since the number of users was low, comparisons with TSC are difficult to make. Still, it could be mentioned two studies that recommend TSC for the analysis of *C. perfringens* in food samples (2, 3).

Suspected and typical colonies are in NMKL 95:2009 confirmed with a motility test and a test for lactose fermentation. *C. perfringens* are non-motile and form acid and gas as a consequence of lactose fermentation. The method for confirmation is similar in ISO 7937:2004. In total 95 % of the laboratories stated that they performed some kind of confirmation. Normally, this consisted of a motility test, usually in combination with a test of lactose fermentation.

The majority of the laboratories (92 %) incubated at 37 °C. The remaining five laboratories incubated at 44 °C. *C. perfringens* normally grows at both 37 °C and 44 °C, and even if only a few laboratories incubated at 44 °C, the incubation temperature does not appear to have had an impact on the outcome. Four of the five laboratories that incubated at 44 °C did actually report results with an annotation. But for two of these, their false positive results for sample C are likely explained by the fact that they did not perform a confirmation test. The other two laboratories received results with an annotation that are with some certainty explained by mixing together of two samples, and by incubation on mCP, respectively.

### Results from analysis of *Clostridium perfringens*

Medium	Sample A					Sample B					Sample C				
	N	n	m	s	F < >	N	n	m	s	F < >	N	n	m	s	F < >
All results	61	52	2.677	0.243	4 5 0	61	58	2.485	0.273	1 2 0	60	52	-	-	8 - -
TSC	54	47	2.691	0.220	3 4 0	54	53	2.504	0.260	0 1 0	53	46	-	-	7 - -
SC	2	2	-	-	0 0 0	2	1	-	-	0 1 0	2	2	-	-	0 - -
ISA	1	1	-	-	0 0 0	1	1	-	-	0 0 0	1	1	-	-	0 - -
mCP	1	0	-	-	0 1 0	1	1	-	-	0 0 0	1	1	-	-	0 - -
Other	3	2	-	-	1 0 0	3	2	-	-	1 0 0	3	2	-	-	1 - -



## Anaerobic sulphite-reducing bacteria

### Sample A

The strain of *C. perfringens* was target organism for the analysis and was present at approximately log<sub>10</sub> 2.8 cfu ml<sup>-1</sup> in the sample. Three low and one high outlier were reported, as well as two false negative results.

### Sample B

The strain of *C. perfringens* (identical to the one in sample A) was target organism for the analysis and was present at approximately log<sub>10</sub> 2.7 cfu ml<sup>-1</sup> in the sample. Two low outliers were reported, as well as two false negative results.

### Sample C

The strain of *C. bifermentans* was target organism for the analysis and was present at approximately log<sub>10</sub> 3.2 cfu ml<sup>-1</sup> in the sample. Four low and one high outlier were reported, as well as three false negative results.

### General remarks

As in previous proficiency testing rounds, the majority of the laboratories followed a version of NMKL 56. The proportion of users of the new NMKL 56:2015 was however still low, and it was only used by 10 % of the laboratories. Most laboratories instead stated that they followed NMKL 56:2008 (50 %) or the considerably older NMKL 56:1994 (4 %). ISO 15213:2003 was in comparison followed by 16 % of the

laboratories. It was last reviewed by ISO in 2015 and remains current. It is however scheduled to be replaced by ISO 15213-1 ("Enumeration of sulphite-reducing *Clostridium* spp. by colony-count technique"), which is currently under development. Three laboratories followed ISO 7937:2004 ("Horizontal method for the enumeration of *Clostridium perfringens*"), which will be replaced by the future ISO 15213-2 ("Enumeration of *Clostridium perfringens* by colony-count technique"). No obvious differences between the methods could however be identified.

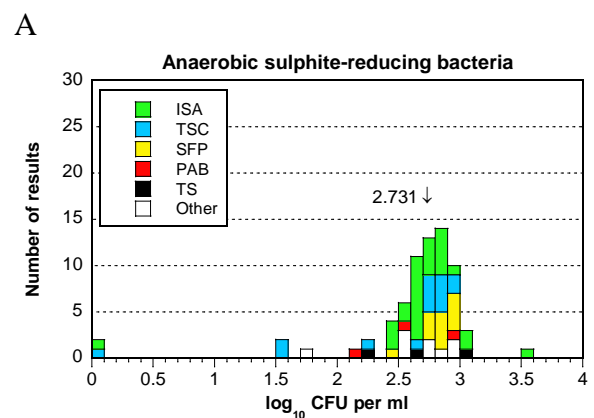
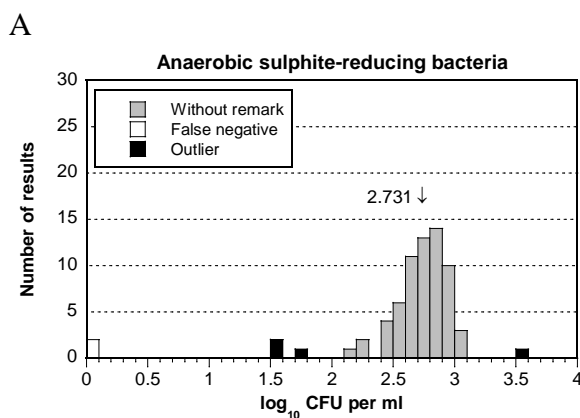
Both NMKL 56:2015 and ISO 15213:2003 prescribe pour-plate methods with ISA, which was also the medium most frequently used by the laboratories. Black colonies on ISA (possibly surrounded by a black zone) are considered as sulphite-reducing bacteria. The black colour of the colonies comes from iron sulphide, which is formed as a precipitate of Fe<sup>3+</sup> in the medium and H<sub>2</sub>S that is produced by the reduction of sulphite. Growth of anaerobic bacteria that only produce hydrogen (and not H<sub>2</sub>S) may sometimes result in a diffuse and unspecific blackening of the medium.

In addition to ISA, laboratories also reported using TSC, SFP, PAB and TS. These media are often used when identifying *C. perfringens*, and it should therefore be mentioned that for that purpose, colonies should be confirmed using the methods in for example NMKL 95. Use of these media did not cause any obvious problems here. Though the use of SFP was associated with relatively many low outliers for sample C, the number of users was also low, and it can therefore not be ruled out that this was simply due to chance.

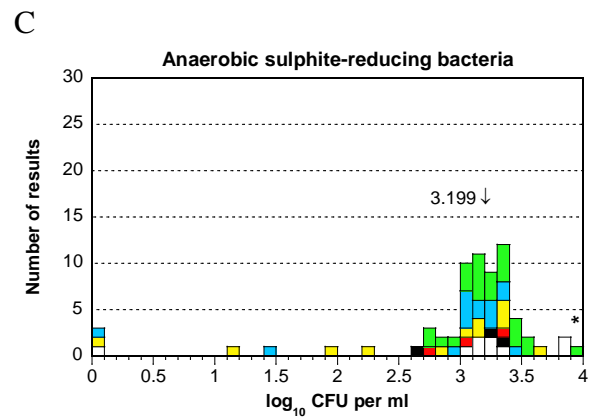
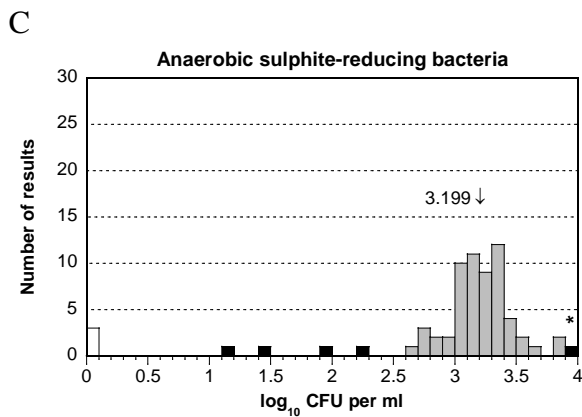
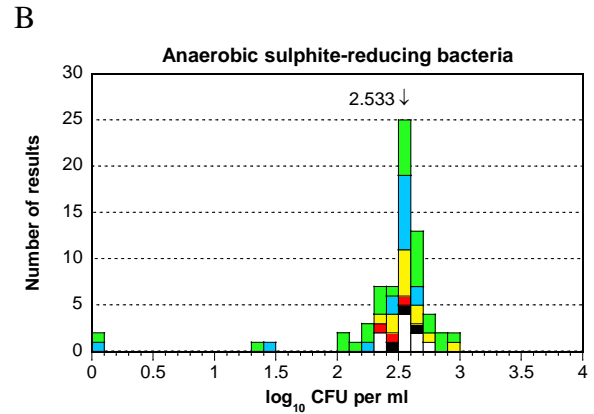
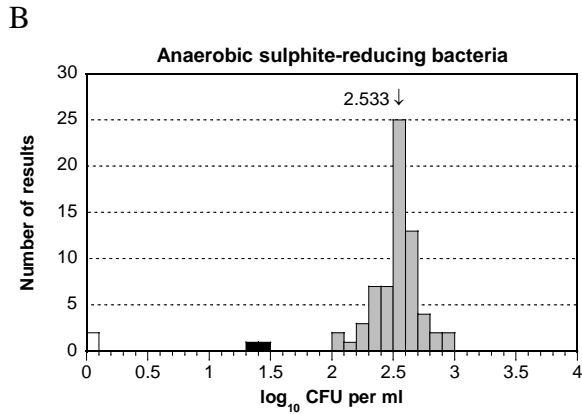
*Results from analysis of anaerobic sulphite-reducing bacteria*

Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	70	64	2.731	0.190	2	3	1	70	66	2.533	0.180	2	2	0	67	59	3.199	0.241	3	4	1
ISA	28	26	2.708	0.163	1	0	1	28	26	2.514	0.242	1	1	0	25	24	3.191	0.230	0	0	1
TSC	15	12	2.755	0.169	1	2	0	15	13	2.525	0.113	1	1	0	15	13	3.169	0.149	1	1	0
SFP	12	12	2.825	0.145	0	0	0	12	12	2.580	0.158	0	0	0	12	8	3.237	0.230	1	3	0
PAB	3	3	-	-	0	0	0	3	3	-	-	0	0	0	3	3	-	-	0	0	0
TS	3	3	-	-	0	0	0	3	3	-	-	0	0	0	3	3	-	-	0	0	0
Other*	9	8	2.730	0.168	0	1	0	9	9	2.558	0.120	0	0	0	9	8	3.351	0.307	1	0	0

\* The group Other mainly includes laboratories with unclear or ambiguous reporting of the medium.







## Aerobic microorganisms in fish and fish products, 20-25 °C

### Sample A

The strains of *P. aeruginosa*, *S. aureus* and *L. plantarum* were present in the highest concentrations and were thus the main target organisms. No outliers or false negative results were reported.

### Sample B

The strains of *B. thermosphacta*, *B. cereus* and *S. putrefaciens* were present in the highest concentrations and were thus the main target organisms. One low outlier was reported.

### Sample C

The strains of *E. coli*, *L. plantarum* and *A. hydrophila* were present in the highest concentrations and were thus the main target organisms. Two low outliers were reported. Both of these were from laboratories that followed NMKL 86 (different versions) and incubated on PCA.

### General remarks

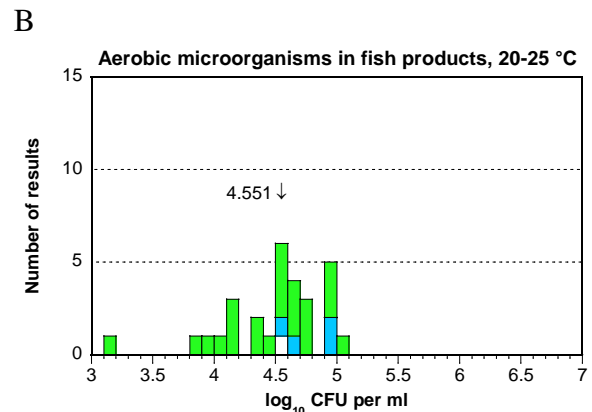
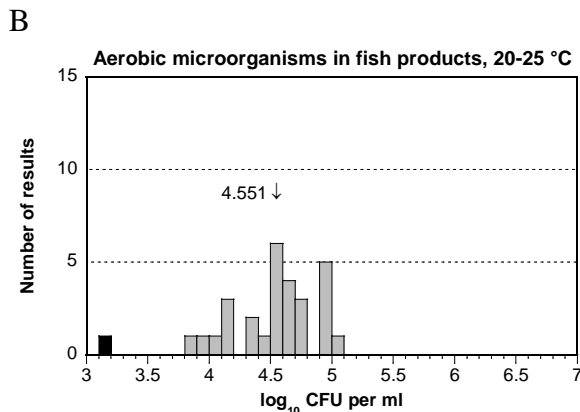
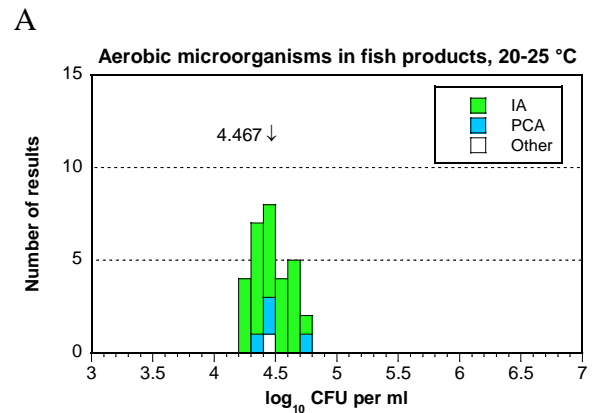
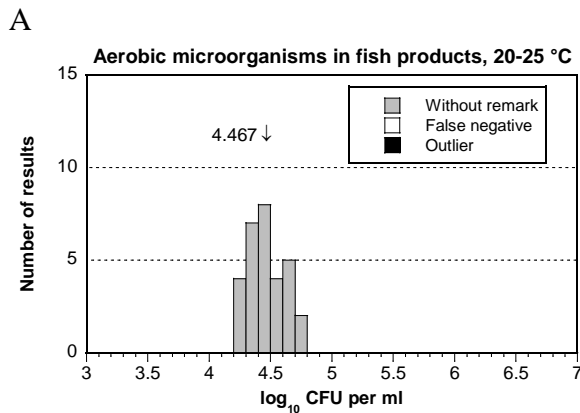
The majority of the laboratories (83 %) followed the method for aerobic microorganisms and specific spoilage organisms in fish and fish products, NMKL 184:2006. This prescribes a pour-plate method with IA, which was also the medium

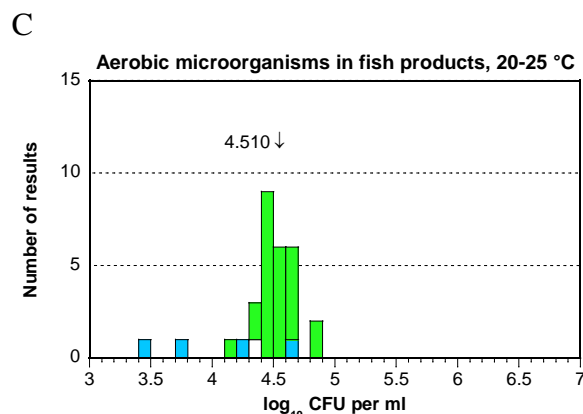
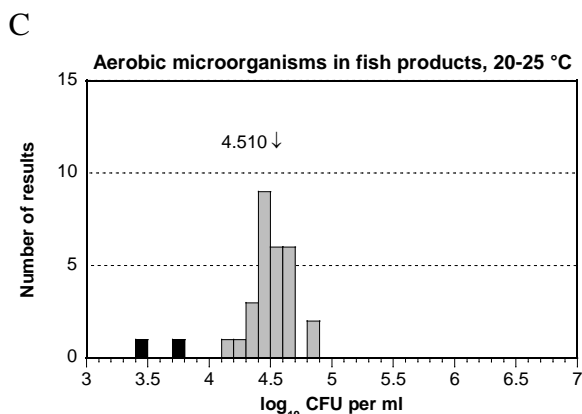
most frequently used by the laboratories (83 %). One laboratory followed ISO 4833-1:2013 (“Aerobic microorganisms”) and thus incubated on PCA. Two laboratories followed NMKL 86 (“Aerobic microorganisms in food”). Though this method is adapted for use in all types of food, it also refers to NMKL 184:2006 when analysing fish and fish products. One laboratory followed NMKL 96:2003, which uses the same method for total aerobic count as NMKL 184:2006. However, NMKL 96:2003 has been replaced by NMKL 96:2009 (“Coliform bacteria, thermotolerant coliform bacteria and *E. coli*”) which refers to NMKL 184:2006 for the analysis of total aerobic count in fish and seafood.

It could here be mentioned that NMKL 184:2006 also describes incubation on Long & Hammer agar for the detection of psychrotrophic and heat-sensitive microorganisms. With this medium, incubation is done at 15 °C, which may be advantageous when analysing fresh minced fish meat or lightly preserved fish products.

*Results from analysis of aerobic microorganisms in fish and fish products*

Medium	Sample A						Sample B						Sample C					
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >
All results	30	30	4.467	0.144	0	0 0 0	29	28	4.551	0.324	0 1 0	0 1 0	30	28	4.510	0.157	0 2 0	0 2 0
IA	25	25	4.465	0.147	0	0 0 0	24	23	4.513	0.335	0 1 0	0 1 0	25	25	4.521	0.151	0 0 0	0 0 0
PCA	4	4	-	-	0	0 0 0	4	4	-	-	0 0 0	0 0 0	4	2	-	-	0 2 0	0 2 0
Other	1	1	-	-	0	0 0 0	1	1	-	-	0 0 0	0 0 0	1	1	-	-	0 0 0	0 0 0





## H<sub>2</sub>S-producing bacteria in fish products

### Sample A

No target organism for the analysis was present in the sample. One false positive result was reported.

### Sample B

The strain of *S. putrefaciens* was target organism for the analysis and was present at approximately log<sub>10</sub> 3.6 cfu ml<sup>-1</sup> in the sample. Two false negative results were reported.

### Sample C

The strain of *H. alvei* was target organism for the analysis and was present at approximately log<sub>10</sub> 4.0 cfu ml<sup>-1</sup> in the sample. One low outlier and one false negative result were reported.

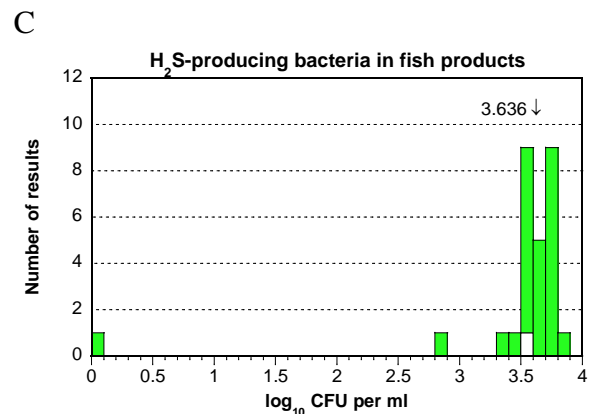
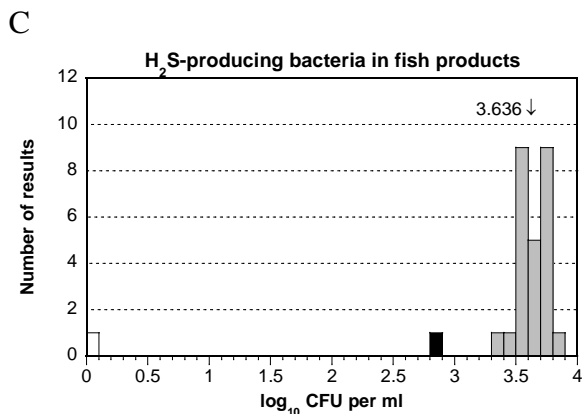
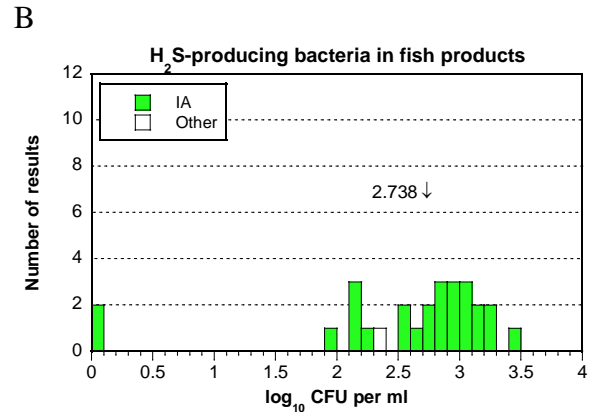
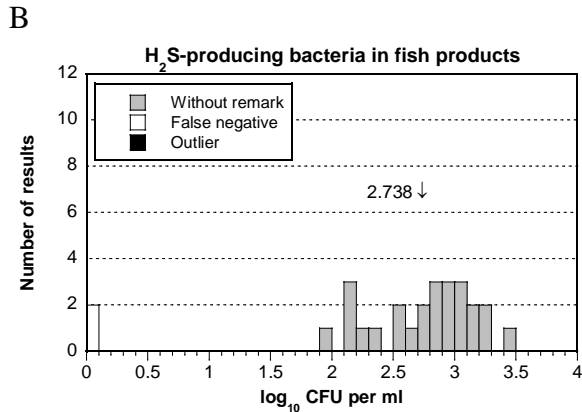
### General remarks

The majority of the laboratories (96 %) followed the method for aerobic microorganisms and specific spoilage organisms in fish and fish products, NMKL 184:2006. This prescribes a pour-plate method with IA, which was also the medium most frequently used by the laboratories (96 %). One laboratory followed NMKL 96:2003 ("Bacterial examinations in fresh and frozen seafood"), which includes the analysis of H<sub>2</sub>S-producing bacteria. The laboratory however incubated in lauryl sulphate broth, which is not correct. Further, NMKL 96:2003 has been replaced by NMKL 96:2009 which in turn refers to NMKL 184:2006 for the analysis of aerobic microorganisms and spoilage organisms in fish and fish products.

Since the majority of the laboratories followed NMKL 184:2006 and incubated on IA, no differences between methods and media have been identified.

### Results from analysis of H<sub>2</sub>S-producing bacteria in fish products

Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	28	27	-	-	1	-	-	27	25	2.738	0.406	2	0	0	28	26	3.636	0.107	1	1	0
IA	27	26	-	-	1	-	-	26	24	2.756	0.404	2	0	0	27	25	3.641	0.105	1	1	0
Other	1	1	-	-	0	-	-	1	1	-	-	0	0	0	1	1	-	-	0	0	0



## Yeasts and moulds

### Sample A

The strain of *C. glabrata* was target organism for the analysis of yeasts and was present at approximately  $\log_{10}$  2.4 cfu ml<sup>-1</sup> in the sample. Six low and 14 high outliers were reported, as well as two false negative results.

The strain of *C. cladosporioides* was target organism for the analysis of moulds and was present at approximately  $\log_{10}$  2.5 cfu ml<sup>-1</sup> in the sample. Four low and one high outlier were reported, as well as 15 false negative results.

The many high outliers for yeasts – and the false negative results for moulds – are difficult to explain. Only two of the 15 laboratories that reported false negative results for moulds, simultaneously reported high outliers for yeasts. In general, it therefore appears unlikely that the laboratories have mistaken *C. cladosporioides* for a yeast. Other microorganisms present in the sample should normally not form colonies on the media, as long as a suitable amount of antibiotics has been added. At the National Food Agency, no colonies except *C. glabrata* and *C. cladosporioides* were observed, neither on DG18 nor on DRBC after 7 days incubation at 25 °C. Further, none of the deviating results appear to be due to mixing together of samples. Also, the deviating results appear to be relatively evenly distributed between the different media. The exception is that none of the laboratories that incubated on both DG18 and DRBC reported false negative results. The majority of the laboratories incubated for 5-7 days, which is enough time to detect both *C. cladosporioides* and *C. glabrata*. Finally, only two of the

high outliers for yeast come from laboratories that used TEMPO YM, which does not distinguish between yeast and moulds.

### **Sample B**

The strain of *H. uvarum* was target organism for the analysis of yeasts and was present at approximately  $\log_{10}$  2.4 cfu ml<sup>-1</sup> in the sample. One low and one high outlier were reported, as well as two false negative results.

The strain of *A. flavus* was target organism for the analysis of moulds and was present at approximately  $\log_{10}$  2.4 cfu ml<sup>-1</sup> in the sample. One high outlier was reported, as well as one false negative result.

### **Sample C**

No target organism was present in the sample, neither for the analysis of yeasts nor for moulds. Three false positive results were reported for yeasts, and two false positive results for moulds.

### **General remarks**

In principle, the same laboratories analysed yeasts and moulds, and they generally reported identical methods for both analyses. The methods mainly consisted of NMKL 98:2005, ISO 6611:2004 / IDF 94:2004, 3M™ Petrifilm™ and ISO 21527-1:2008 / ISO 21527-2:2008. A few laboratories followed ISO 7954:1987 ("General guidance for enumeration of yeasts and moulds"), which has been replaced by ISO 21527-1:2008 and ISO 21527-2:2008.

NMKL 98:2005 prescribes the use of either DRBC, DG18 or OGYE. ISO 6611:2004/IDF 94:2004 describes the enumeration of yeasts and moulds in milk and milk products, and is based on a pour-plate method with OGYE or YGC. With ISO 21527, different media are used depending on the water activity ( $a_w$ ) of the food that is analysed, and ISO 21527-1:2008 therefore uses DRBC while ISO 21527-2:2008 uses DG18. In general, DRBC is recommended for fresh foods with  $a_w > 0.95$  (e.g. fruit, vegetables, meat and milk products) and DG18 for foods with  $a_w \leq 0.95$  (e.g. dried fruits, dried meats, grains, nuts). OGYE is recommended if only yeasts are to be analysed.

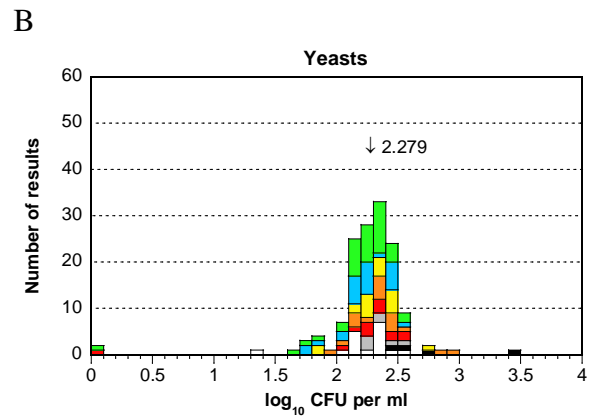
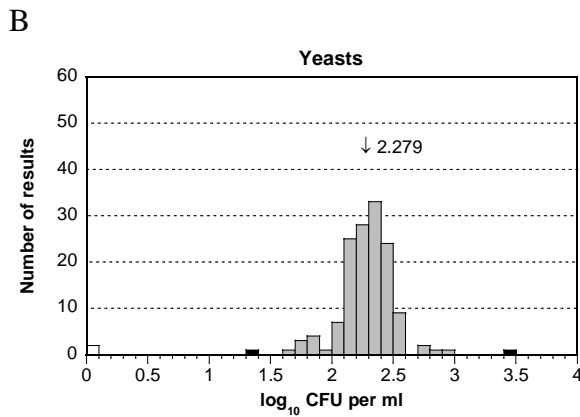
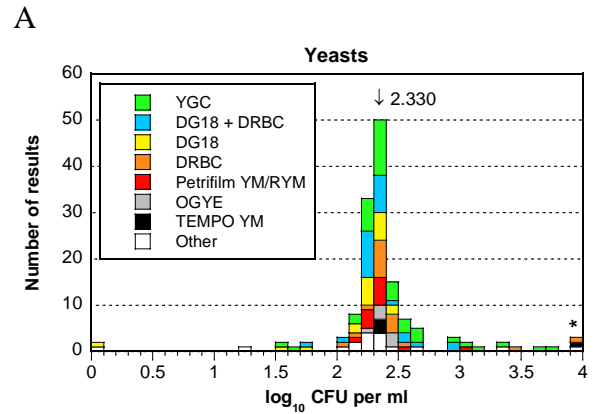
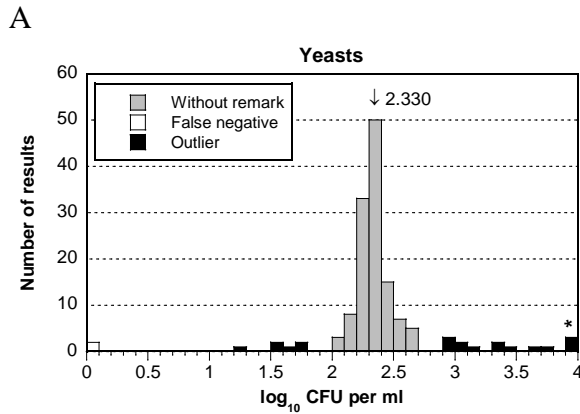
In addition to what has already been mentioned, outliers and false results were relatively evenly distributed between the main methods and media that were used. The mean values of the different groups were also in general similar. Several methods and media were however used only by a few laboratories. It is therefore difficult to make certain conclusions on potential differences in the results for these.

Five laboratories to various extents reported the use of TEMPO YM, sometimes in combination with other methods/media. The results from these laboratories have been included in the evaluation, but in some cases they have likely been determined as outliers of false results since the method in TEMPO YM give a combined value for yeast and moulds. Reporting of a combined value for yeasts and moulds can currently not be handled in the statistical analysis – such results therefore need to be evaluated by the laboratories themselves.

Results from analysis of yeasts

Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	143	121	2.330	0.117	2	6	14	143	139	2.279	0.204	2	1	1	140	137	-	-	3	-	-
YGC	39	31	2.373	0.132	0	2	6	39	38	2.235	0.201	1	0	0	38	38	-	-	0	-	-
DG18 + DRBC	26	23	2.321	0.117	0	1	2	26	26	2.208	0.209	0	0	0	26	25	-	-	1	-	-
DG18	19	16	2.283	0.096	1	2	0	19	19	2.281	0.208	0	0	0	18	18	-	-	0	-	-
DRBC	18	16	2.343	0.111	0	0	2	18	18	2.355	0.253	0	0	0	17	16	-	-	1	-	-
Petrifilm YM/RYM	13	12	2.308	0.082	0	0	1	13	12	2.316	0.154	1	0	0	13	12	-	-	1	-	-
OGYE	7	7	2.374	0.054	0	0	0	7	7	2.368	0.100	0	0	0	7	7	-	-	0	-	-
TEMPO YM	4	3	-	-	0	0	1	4	3	-	-	0	0	1	4	4	-	-	0	-	-
Other*	17	13	2.280	0.150	1	1	2	17	16	2.284	0.135	0	1	0	17	17	-	-	0	-	-

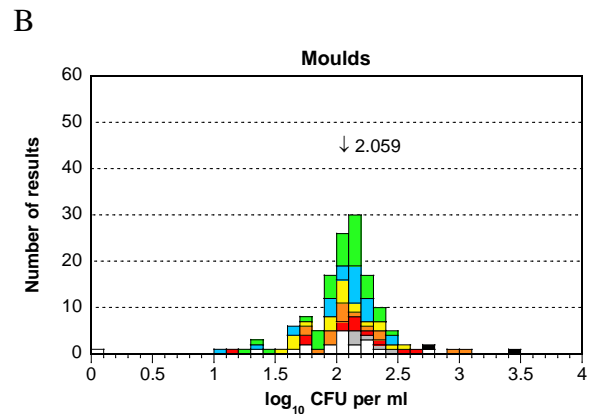
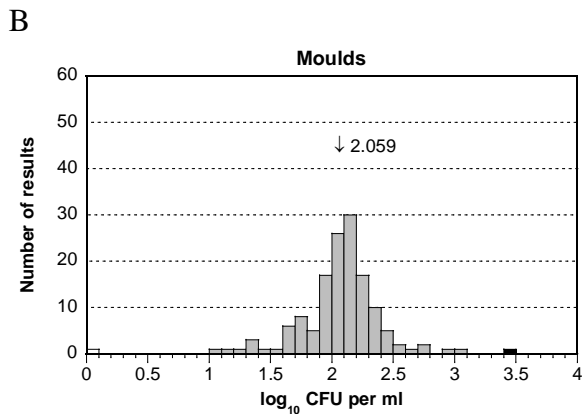
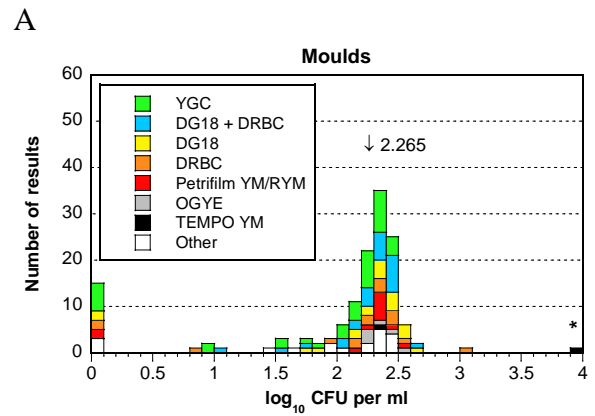
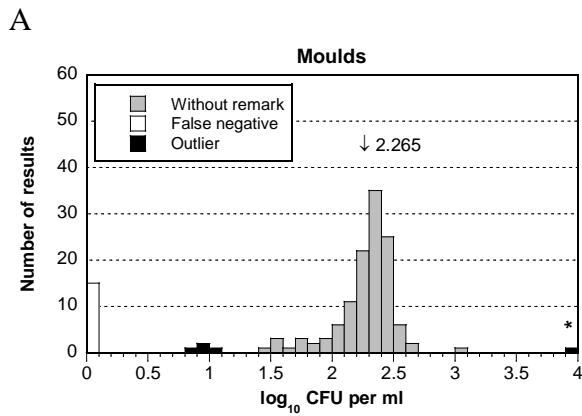
\* The group Other mainly includes laboratories that used a unique medium or medium combination.



Results from analysis of moulds

Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	141	121	2.265	0.237	15	4	1	141	139	2.059	0.304	1	0	1	139	137	-	-	2	-	-
YGC	40	32	2.187	0.227	6	2	0	40	40	2.031	0.251	0	0	0	40	40	-	-	0	-	-
DG18 + DRBC	26	25	2.277	0.236	0	1	0	26	26	2.029	0.314	0	0	0	26	25	-	-	1	-	-
DG18	20	18	2.314	0.231	2	0	0	20	20	2.020	0.275	0	0	0	19	19	-	-	0	-	-
DRBC	16	13	2.359	0.251	2	1	0	16	16	2.139	0.374	0	0	0	15	15	-	-	0	-	-
Petrifilm YM/RYM	12	10	2.344	0.091	2	0	0	12	12	2.063	0.404	0	0	0	12	12	-	-	0	-	-
OGYE	6	6	2.340	0.148	0	0	0	6	6	2.240	0.133	0	0	0	6	6	-	-	0	-	-
TEMPO YM	2	1	-	-	0	0	1	2	1	-	-	0	0	1	2	2	-	-	0	-	-
Other*	19	16	2.188	0.303	3	0	0	19	18	2.035	0.297	1	0	0	19	18	-	-	1	-	-

\* The group Other mainly includes laboratories that used a unique medium or medium combination.



## **Outcome of the results of individual laboratory - assessment**

### **Reporting and evaluation of results**

The reported results of all participating laboratories are listed in Annex 1, together with the minimum and maximum accepted values for each analysis. Results that received a remark (false results and outliers) are highlighted in yellow, with bold font.

It is the responsibility of the participating laboratories to correctly report results according to the instructions. When laboratories incorrectly report their results, for example by stating “pos” or “neg” for quantitative analyses, the results cannot be correctly processed. Such incorrectly reported results are normally excluded. Inclusion and further processing of such results may still be done, after manual assessment in each individual case.

Z-scores (see below) for individual analyses are shown in Annex 2 and can be used as a tool by laboratories when following up on the results.

The laboratories are not grouped or ranked based on their results. The performance of a laboratory as a whole can be evaluated from the number of false results and outliers that are listed in Annex 1 and below the box plots.

Information on the results processing and recommendations for follow-up work are given in the Scheme Protocol (4). Samples for follow-up can be ordered, free of charge via our website: [www.livsmedelsverket.se/en/PT-extra](http://www.livsmedelsverket.se/en/PT-extra)

### **Z-scores, box plots and deviating results**

In order to allow comparison of the results from different analyses and mixtures, all results are transformed into standard values (z-scores). For quantitative analyses, a z-score is either positive or negative, depending on whether the individual result is higher or lower than the mean value calculated from all laboratory results for each analysis.

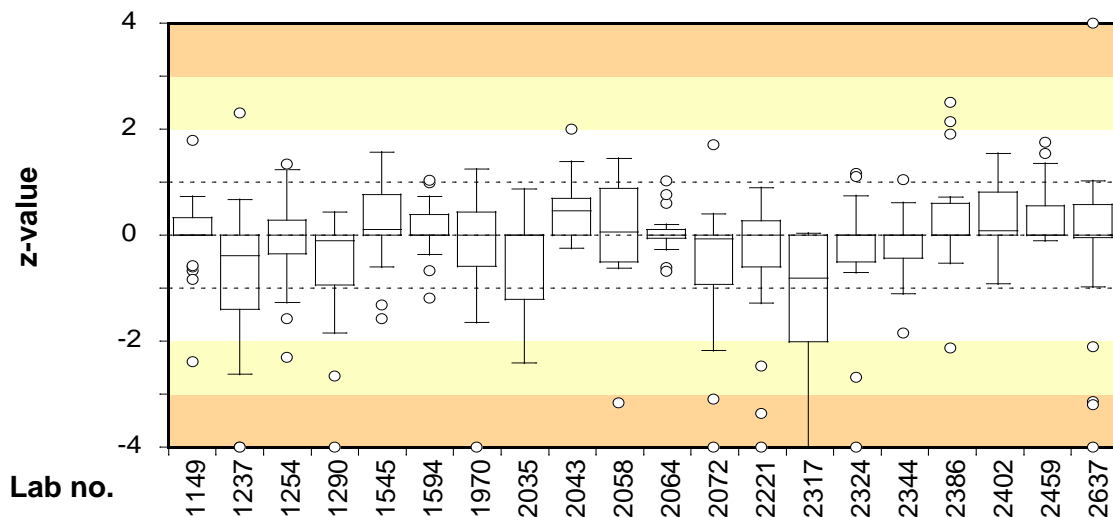
The box plots are based on the z-scores listed in Annex 2, and give a comprehensive view of the achievement of each laboratory. A small box, centred around zero, indicates that the results of the individual laboratory, with false results excluded, are close to the general mean values calculated for all laboratory results. The range of z-scores is indicated by the size of the box and, for most laboratories, by lines and/or circles above and beneath the box. For each laboratory, the number of false results and outliers are also listed in the tables below the box plots.



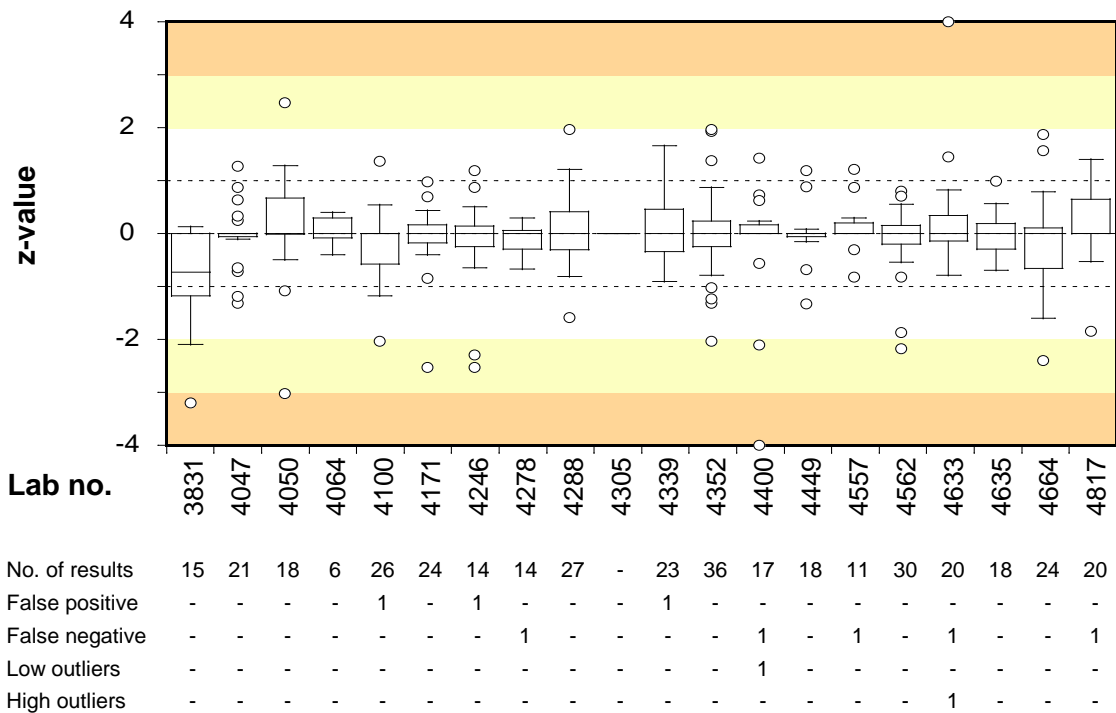
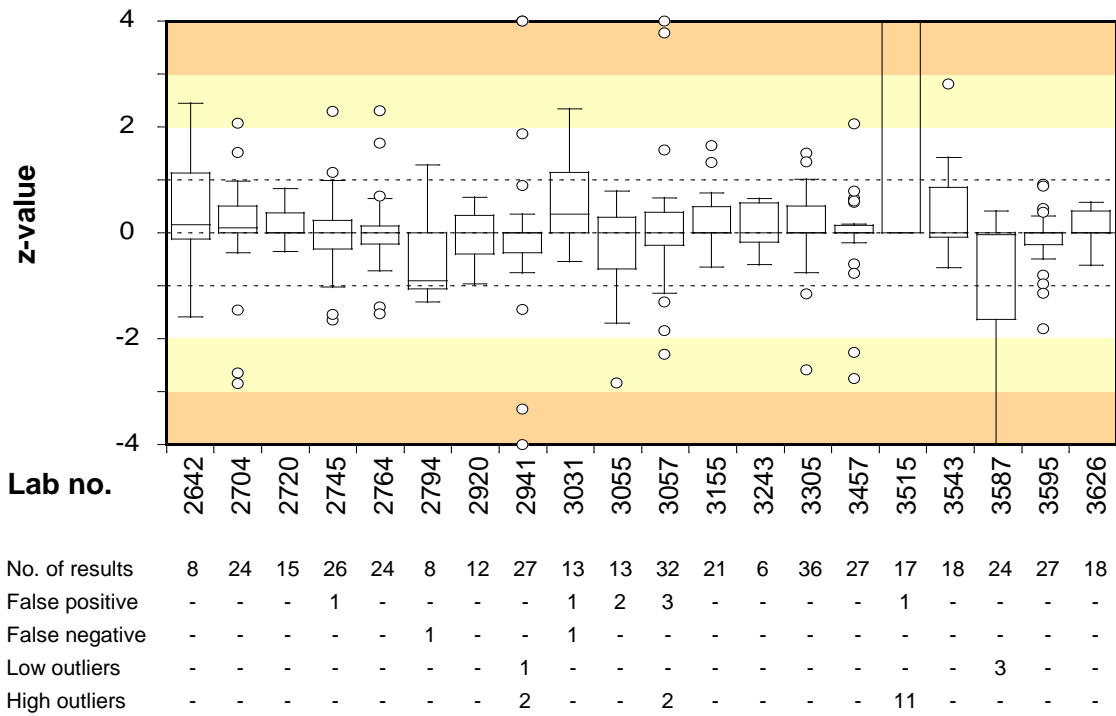
## Box plots and numbers of deviating results for each laboratory

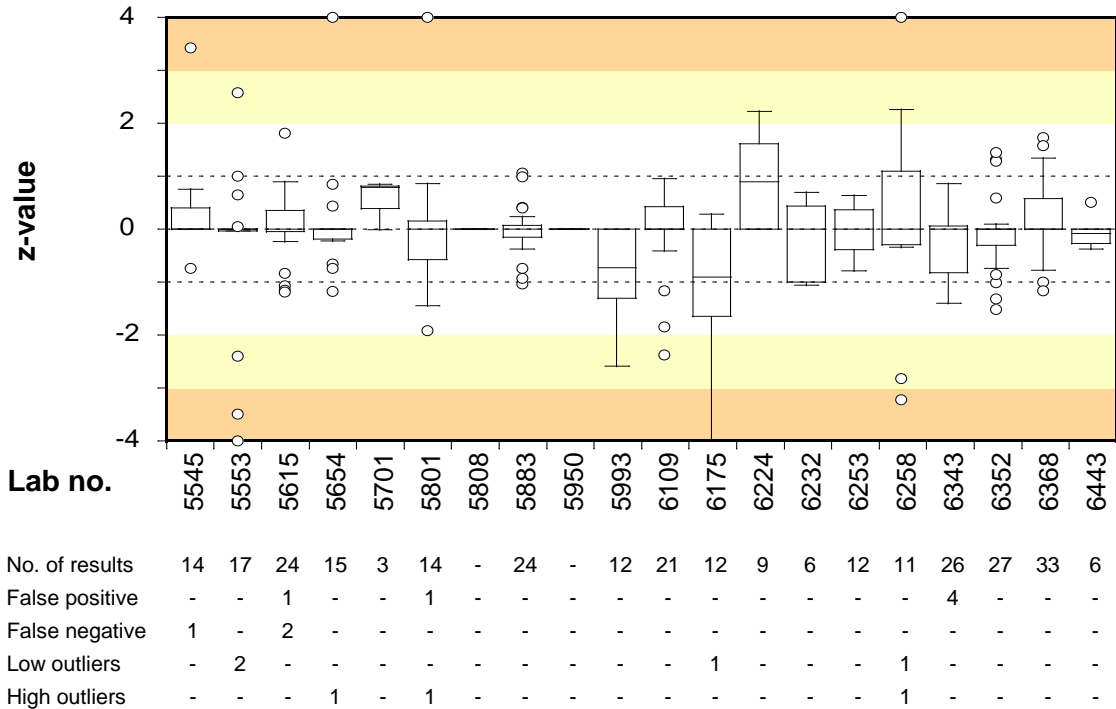
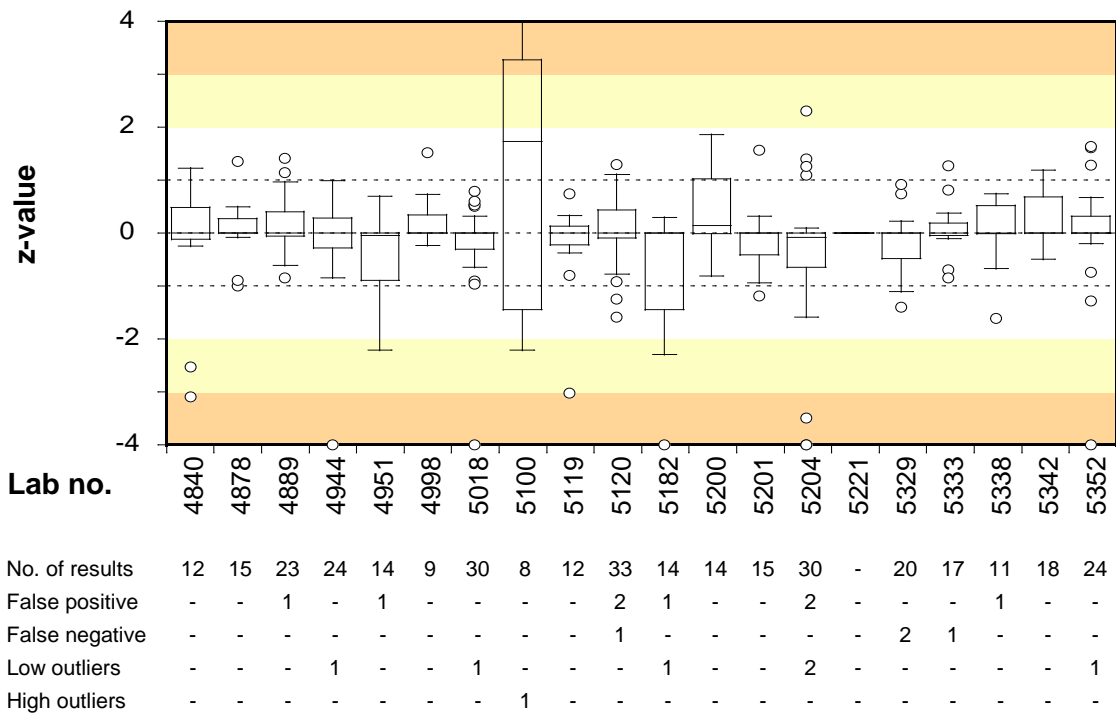
- Z-scores are calculated according to the formula:  $z = (x-m)/s$ , where  $x$  is the result of the individual laboratory,  $m$  is the mean of the results of all participating laboratories, and  $s$  is the standard deviation of the participating laboratories, after removing outliers and false results.
- Outliers are included in the figures after being calculated to z-scores in the same way as for other results.
- False results do not generate any z-scores, and are not included in “No. of results”.
- Correct results for qualitative analyses and correct negative results for quantitative analyses without target organism generate a z-score of 0.
- The laboratory median value is illustrated by a horizontal line in the box.
- The box includes 50 % of a laboratory’s results (25 % of the results above the median and 25 % of the results below the median). The remaining 50 % are illustrated by lines and circles outside the box.
- A circle is for technical reasons shown in the plot when a value deviates to certain degree\* from the other values. This does not by itself indicate that the value is an outlier.
- z-scores  $>+4$  and  $<-4$  are positioned at  $+4$  and  $-4$ , respectively, in the plot.
- The background is divided by lines and shaded fields to simplify identifying the range in which the results are located.

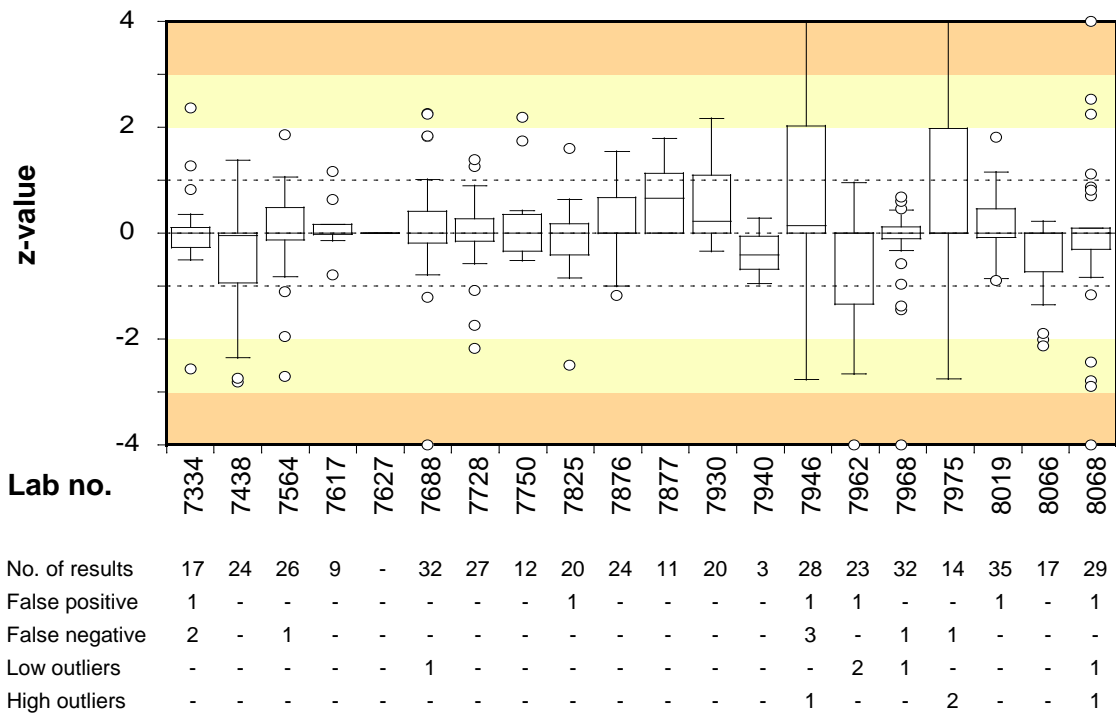
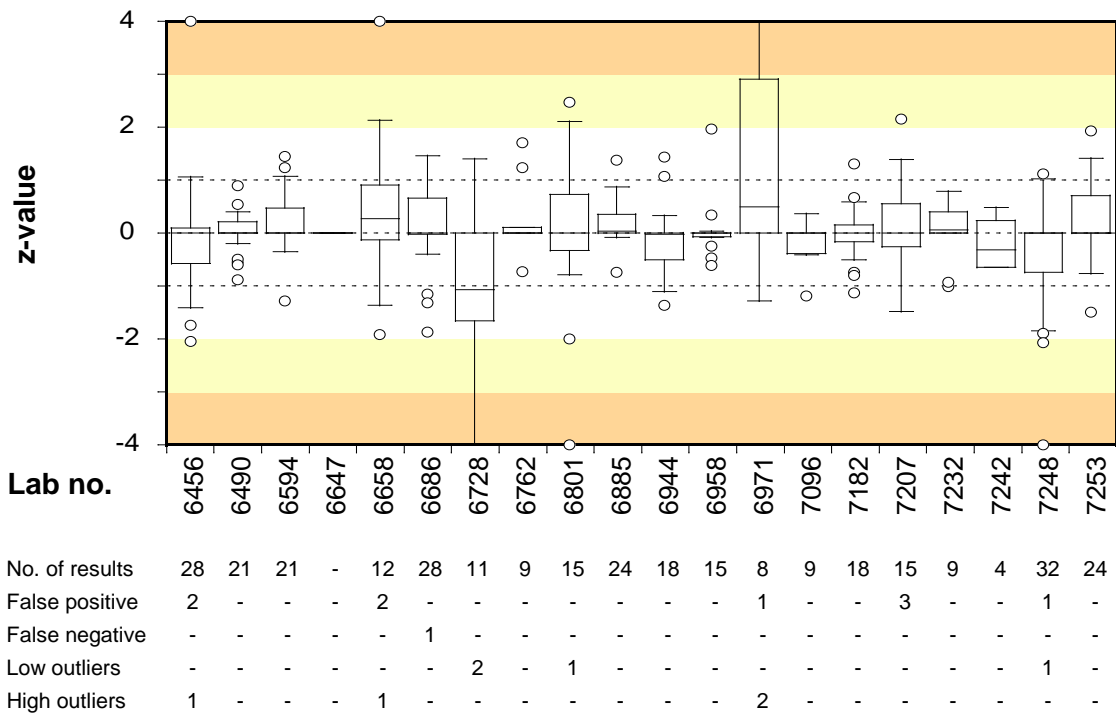
\*  $< [lowest\ value\ in\ the\ box - 1,5 \times (highest\ value\ in\ the\ box - lowest\ value\ in\ the\ box)]$   
or  
 $> [highest\ value\ in\ the\ box + 1,5 \times (highest\ value\ in\ the\ box - lowest\ value\ in\ the\ box)]$ .

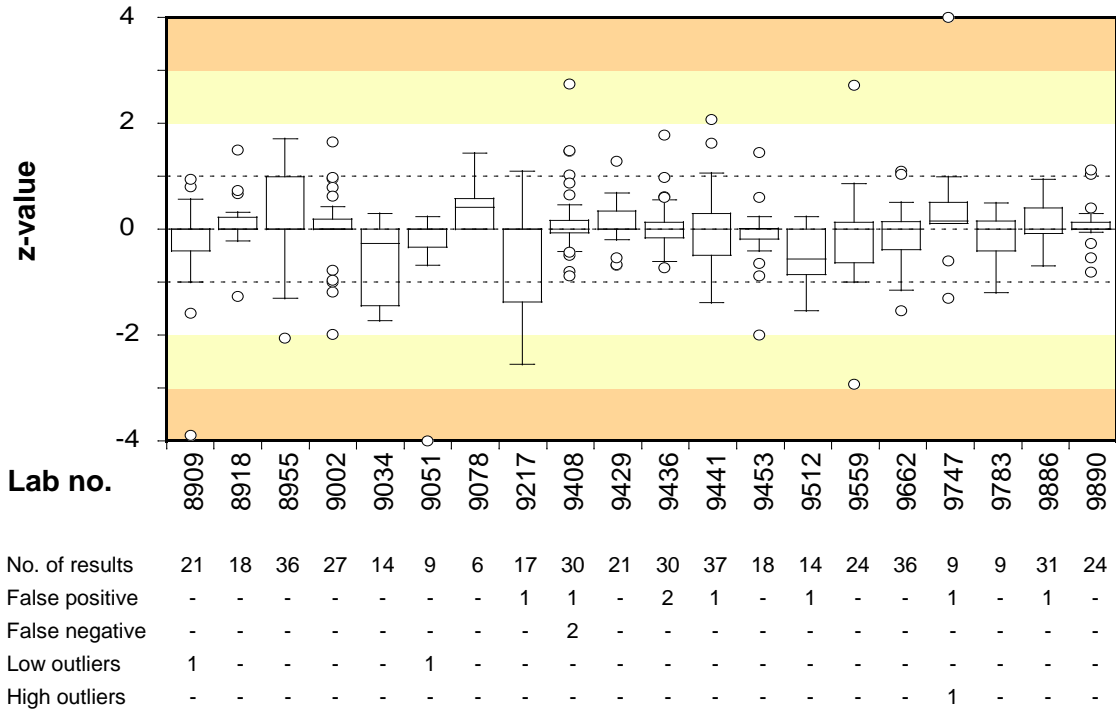
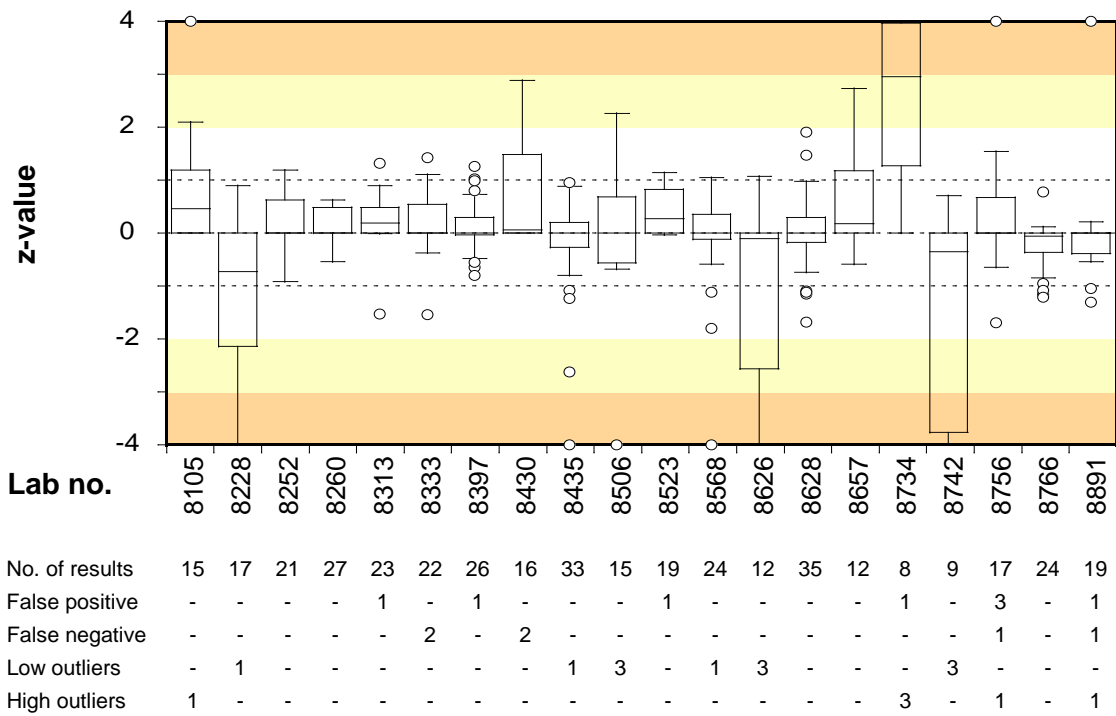


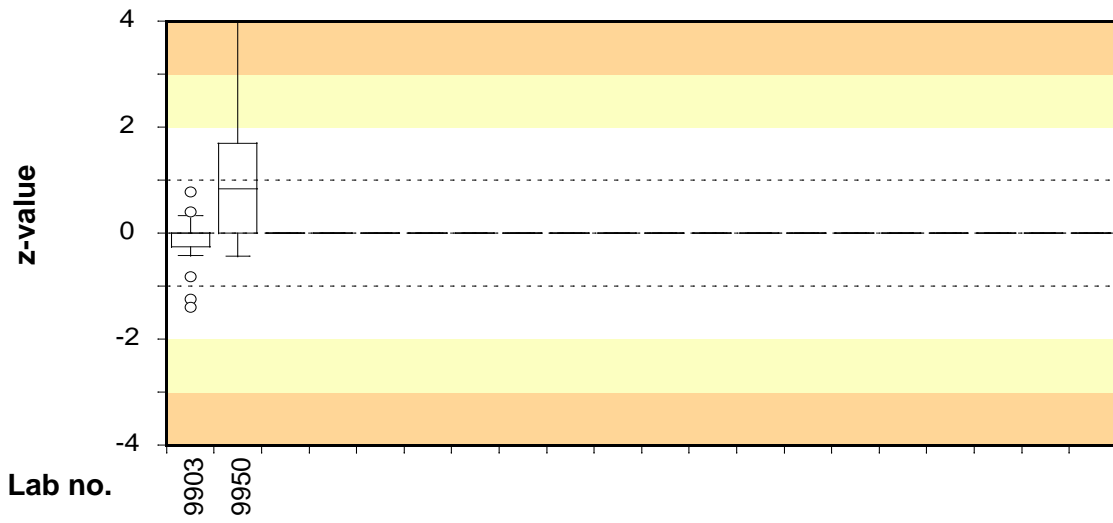
Lab no.	1149	1237	1254	1290	1545	1594	1970	2035	2043	2058	2064	2072	2221	2317	2324	2344	2386	2402	2459	2637
No. of results	21	36	30	23	30	27	38	18	9	11	15	30	32	27	18	23	15	15	21	29
False positive	-	1	-	1	-	-	-	-	-	2	-	1	-	-	-	-	-	-	1	-
False negative	-	1	-	-	-	-	-	-	-	1	-	1	-	-	1	-	-	-	2	1
Low outliers	-	5	-	1	-	-	1	-	-	-	-	2	3	1	1	-	-	-	-	2
High outliers	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1











Lab no.	9903	9950
No. of results	21	13
False positive	-	1
False negative	-	1
Low outliers	-	-
High outliers	-	1

## Test material and quality control

### Test material

Each laboratory received three sample mixtures with freeze-dried microorganisms, designated A-C. The test material was freeze-dried in portions of 0.5 ml in vials, as described by Peterz and Steneryd (5). Before analysing the samples, the contents of each vial had to be dissolved in 254 ml of sterile diluent. The organisms present in the mixtures are listed in Table 2.

**Table 2.** *Microorganisms in the samples*

Sample <sup>1</sup>	Microorganism	Strain	
		SLV no. <sup>2</sup>	Reference <sup>3</sup>
A	<i>Candida glabrata</i>	SLV-052	-
	<i>Cladosporium cladosporioides</i>	SLV-488	CBS 812.96
	<i>Clostridium perfringens</i>	SLV-442	CCUG 43593
	<i>Lactobacillus plantarum</i>	SLV-475	CCUG 30503
	<i>Pseudomonas aeruginosa</i>	SLV-429	-
	<i>Staphylococcus aureus</i>	SLV-280	Egg, 1989
B	<i>Aspergillus flavus</i>	SLV-480	CBS 282.95
	<i>Bacillus cereus</i>	SLV-518	CCUG 44741
	<i>Brandotrix thermosphacta</i>	SLV-220	CCUG 45641
	<i>Clostridium perfringens</i>	SLV-442	CCUG 43593
	<i>Hanseniaspora uvarum</i>	SLV-555	-
	<i>Shewanella putrefaciens</i>	SLV-520	CCUG 46538
C	<i>Aeromonas hydrophila</i>	SLV-467	CCUG 46535
	<i>Clostridium bifermentans</i>	SLV-009	CCUG 43592
	<i>Escherichia coli</i>	SLV-082	CCUG 45097
	<i>Hafnia alvei</i>	SLV-015	CCUG 45642
	<i>Lactobacillus plantarum</i>	SLV-445	ATCC 8014
	<i>Staphylococcus aureus</i>	SLV-350	CCUG 45099

<sup>1</sup> The links between the mixtures and the randomised sample numbers are shown in Annex 1.

<sup>2</sup> Internal strain identification no. at the National Food Agency

<sup>3</sup> Origin or culture collection (CCUG: Culture Collection University of Gothenburg, Sweden ; ATCC: American Type Culture Collection, CBS: Westerdijk Fungal Biodiversity Institute)

### Quality control of the samples mixtures

In order to allow comparison of all freeze-dried samples, it is essential to have aliquots of homogeneous sample mixtures and equal volume in all vials. Quality control is performed on 10 randomly chosen vials in conjunction with manufacturing of the samples or on 5 vials if an “old” sample mixture was used and the last quality control was performed more than 6 months ago. Homogeneity of a sample mixture is approved if, for each analysis, the values obtained for the test of reproducibility (T) and the test “Index of dispersion” between vials ( $I_2$ ) do not simultaneously exceed 2.6 and 2.0, respectively. (For definitions of T and  $I_2$ , see references 6 and 7 respectively.)

**Table 3.** Concentration mean ( $m$ ), T and  $I_2$  values from the quality control of the sample mixtures;  $m$  is expressed in  $\log_{10}$  cfu (colony forming units) per ml of sample.

Analysis and method	A <sup>1</sup>			B <sup>1</sup>			C <sup>2</sup>		
	m	T	$I_2$	m	T	$I_2$	m	T	$I_2$
Aerobic microorganisms, 30 °C NMKL method no. 86:2013	4.65	1.39	1.13	4.09	1.54	0.68	4.66	1.50	1.71
Psychrotrophic microorganisms NMKL method no. 86:2013	2.49	1.50	1.41	4.67	1.72	3.56	3.86	1.36	1.75
Enterobacteriaceae NMKL method no. 144:2005	-	-	-	-	-	-	4.23	1.35	1.95
<i>Escherichia coli</i> NMKL method no. 125:2005	-	-	-	-	-	-	4.16	1.41	2.18
Presumptive <i>Bacillus cereus</i> NMKL method no. 67:2010	-	-	-	4.01	2.30	1.32	-	-	-
Coagulase-positive staphylococci NMKL method no. 66:2009	3.91	1.18	0.57	-	-	-	3.53	1.24	0.37
Lactic acid bacteria NMKL method no. 140:2007	4.19	1.15	0.40	-	-	-	4.27	1.13	0.35
<i>Clostridium perfringens</i> NMKL method no. 95:2009	2.78	1.38	1.69	2.63	1.37	0.99	-	-	-
Anaerobic sulphite-reducing bacteria NMKL method no. 56:2015	2.80	1.68	4.54	2.78	1.34	0.63	3.20	1.52	3.56
Aerobic microorganisms in fish products NMKL method no. 184:2006	4.20	1.66	0.94	4.81	2.12	11.10	4.44	1.43	0.88
H <sub>2</sub> S-producing bacteria in fish products NMKL method no. 184:2006	-	-	-	3.64	3.75	1.40	3.96	2.23	1.72
Yeasts NMKL method no. 98:2005, DRBC	2.40	1.51	1.11	2.40	1.27	0.35	-	-	-
Moulds NMKL method no. 98:2005, DRBC	2.49	1.40	0.95	2.44	2.45	7.70	-	-	-

- No target organism and therefore no value

<sup>1</sup> n = 5 vials analysed in duplicate

<sup>2</sup> n = 10 vials analysed in duplicate



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## **Internal and external control for microbiological analyses of food and drinking water**

All analytical activities require work of a high standard that is accurately documented. For this purpose, most laboratories carry out some form of internal quality assurance, but their analytical work also has to be evaluated by an independent party. Such external quality control of laboratory competence is commonly required by accreditation bodies and can be done by taking part in proficiency testing (PT).

In a proficiency test, identical test material is analysed by a number of laboratories using their routine methods. The organiser evaluates the results and compiles them in a report.

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- External and independent evaluation of laboratories analytical competence.
- Improved knowledge of analytical methods with respect to various types of organisms.
- Expert support.
- Tool for inspections regarding accreditation.
- Free extra material for follow-up analyses.

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