Laboratory evaluation of portable water quality testing kits

Portable testing kit	Aquagenx Gel EC CFU Kit
Manufacturer	Aquagenx, LLC PO Box 17181 Chapel Hill, NC 27516 USA Phone: +1-919-590-0343 Email: info@aquagenx.com Website: https://www.aquagenx.com/
Evaluation procedure	Independent laboratory evaluation, Phase 1 and Phase 2
JMP report issue date	October, 2022

Summary

This report summarizes the results of an independent laboratory assessment of a portable water quality testing kit called the Aquagenx Gel EC CFU kit. The evaluation was carried out at KWR Research laboratory, with support from the WHO/UNICEF Joint Monitoring Programme for Water Supply, Sanitation and Hygiene (JMP) following a protocol established by WHO. The Aquagenx Gel EC CFU kit successfully passed through Phase 1 testing and was challenged with different tests in Phase 2 testing:

- No false positives were found due to non-target bacteria, and no false negatives were found due to competition
- The portable kit results were compared in triplicate against a reference method using five different natural water matrices, and four different levels of *E. coli* contamination. Across the four test waters (excluding sterilized blanks) and five natural water matrices, a total of 60 paired samples were tested.
- When incubated for 20 hours at 35 °C, in 83% of tests, the semi-quantitative risk class matched the expected value. Matches were lowest for natural water N2 (58%) and for the high-risk stock (11-100 CFU/100 mL; 67% matching). If used as a presence-absence test, the kit correctly identified the presence or absence of E. coli in 93% of cases with a threshold of 1 CFU/100 mL. With thresholds of 10 CFU/100 mL or 100 CFU/100 mL the kit matched expected results 92% and 98% of the time, respectively.
- When incubated for 48 hours at 25 °C, in 95% of tests, the semi-quantitative risk class matched the expected value. Matches were lowest for natural water N1 (83%) and for the medium risk stock (1-10 CFU/100 mL; 87% matching). If used as a presence-absence test, the kit correctly identified the presence or absence of E. coli in 97% of cases with a threshold of 1 CFU/100 mL. With thresholds of 10 CFU/100 mL or 100 CFU/100 mL the kit matched expected results 100% and 98% of the time, respectively.

Executive Summary

The primary concern regarding drinking water quality is that contamination of drinking water could lead to disease. A large number of pathogens can cause water-borne disease. The majority of these pathogens are fecal in origin, but it is not practical to test drinking water for all potential pathogens. Instead, measurement of fecal indicators is preferred. There is widespread agreement that *Escherichia coli* (*E. coli*) is the best currently available indicator of fecal contamination in drinking water.

A large number of test kits are available to quantify the presence of *E. coli* in water. The objective of this project has been to test and compare a range of kits against a certified reference method, which was chosen to be the IDEXX Quantitray 2000 method using Colilert medium. This report summarizes a set of laboratory assessments of different waters with different compositions and levels of contamination and presents the results of both the Aquagenx Gel and the reference method.

The Aquagenx Gel was compared to the reference method using cultivated *E. coli* in laboratory water with a phosphate-buffered saline matrix, as well as using wastewater treatment plant effluent diluted in five different sterilized natural waters (N1-N5). Results were interpreted graphically and through linear regression on both raw data and log-transformed data (see Table 1 and 2).

Table 1: Overview of the regression analysis of Aquagenx Gel experiments at 25°C.

Water matrix	Time (h)	Number of samples	Maximum value	Slope (raw)	Intercept (raw)	Slope (log)	Intercept (log)	Spearman's r
Lab water	20	39	<1	0.00	0.51	0.00	-0.29	0.00
	48	39	>>	0.21	6.21	0.81	-0.14	0.961
N1	20	15	7	0.01	0.50	0.29	-0.34	0.695
	48	15	131	0.28	3.88	0.86	-0.12	0.951
N2	20	15	12	0.04	0.48	0.38	-0.31	0.702
	48	15	>>	0.62	0.51	0.85	0.01	0.922
N3	20	15	10	0.02	0.92	0.34	-0.31	0.693
	48	15	158	0.44	12.4	0.93	0.03	0.876
N4	20	15	16	0.03	0.34	0.37	-0.35	0.703
	48	15	>>	1.05	0.25	0.95	0.06	0.883
N5	20	15	<1	0.00	0.51	0.00	-0.29	0.000
	48	15	>>	0.95	-0.47	0.95	-0.09	0.948

Table 2: Overview of the regression analysis of Aquagenx Gel experiments at 35°C.

Water matrix	Time (h)	Number of samples	Maximum value	Slope (raw)	Intercept (raw)	Slope (log)	Intercept (log)	Spearman's r
Lab water	20	39	>>	0.26	6.08	0.88	-0.25	0.944
	48	39	>>	0.28	7.84	0.86	-0.16	0.945
N1	20	15	159	0.40	1.79	0.89	-0.19	0.932
	48	15	>>	1.24	-1.30	0.98	-0.12	0.891
N2	20	15	123	0.66	-1.2	0.88	-0.14	0.937
	48	15	>>	0.73	0.24	0.93	-0.08	0.909
N3	20	15	310	0.87	16.6	1.02	0.02	0.820
	48	15	310	0.87	16.7	1.01	0.05	0.820
N4	20	15	>>	0.59	0.28	0.84	-0.06	0.915
	48	15	>>	0.79	0.76	0.94	0.04	0.990
N5	20	15	143	0.47	0.78	0.86	-0.12	0.890
	48	15	>>	0.74	-0.03	0.86	-0.03	0.934

The Aquagenx Gel was also assessed for false positives by using concentrated stocks of six non-target bacteria (*Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Pseudomonas aeruginosa* and *Serratia*); and for false negatives by using the same non-target bacteria spiked with low levels of *E. coli*. The Aquagenx Gel did not report any false positive values in the absence of *E. coli* and was able to detect *E. coli* in the presence of each of the non-target bacteria.

Incubation temperature 25°C with an incubation time of 48 hours.

Across the four test waters (excluding sterilized blanks) and five natural water matrices, a total of 60 paired samples were tested. In 95% of these, the semi-quantitative risk class matched the expected value. Matches were lowest for natural water N1 (83%) and for the medium risk stock (1-10 CFU/100 mL; 87% matching).

If used as a presence-absence test, the kit correctly identified the presence or absence of E. coli in 97% of cases with a threshold of 1 CFU/100 mL. With thresholds of 10 CFU/100 mL or 100 CFU/100 mL the kit matched expected results 100% and 98% of the time, respectively.

Incubation temperature 35°C with an incubation time of 20 hours.

Across the four test waters (excluding sterilized blanks) and five natural water matrices, a total of 60 paired samples were tested. In 83% of these, the semi-quantitative risk class matched the expected value. Matches were lowest for natural water N2 (58%) and for the high-risk stock (11-100 CFU/100 mL; 67% matching).

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Abbreviations

Colony Forming Unit	CFU
Defined Substrate Technology	DST
Ground water	GW
Lower Quantification Limit	LQL
Surface water	SW
Upper Quantification Limit	UQL

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1 Background information

WHO and UNICEF both support national counterparts in monitoring and surveillance of drinking water quality in a variety of settings. In many countries where WHO and UNICEF work, logistical challenges mean that testing drinking water quality in laboratories is often not feasible, due to long distances and travel times required to transport samples. This has led to an interest in portable water quality testing kits, especially for measures of faecal contamination. Both WHO and UNICEF regularly procure portable water quality testing kits for national counterparts and share an interest in ensuring that the equipment procured can produce results that are reliable and match within reasonable margins the results from standard reference methods. In addition, both organizations wish to catalyse the continuous improvement of existing portable water quality testing products, and the development of innovative new products which might allow more efficient, accurate, or low-cost testing of drinking water quality in the field.

2 Rapid Water Quality Testing project

UNICEF, in collaboration with WHO, has developed a Rapid Water Quality Testing project to catalyse the continuous improvement of existing portable water quality testing products, and the development of innovative new products which might allow more efficient, accurate, or low-cost testing of drinking water quality in the field. The project has produced a Target Product Profile to describe the desired characteristics of a field test kit, and UNICEF has requested WHO to provide technical guidance on how to assess the performance of innovative products that result from the Rapid Water Quality Testing project.

There are a number of standards and methods used for measurement of microbiological quality of water, and many of the field test kits purport to follow these standards and methods. However, it can be difficult to conduct assessments with field kits out of a controlled laboratory environment, and some commercially available products, or innovative products recently developed, may in practice not meet all requirements.

In the absence of a clear procedure for assessing field test kits, the WHO Water, Sanitation and Hygiene team developed a template protocol for conducting such an assessment in a laboratory setting. This protocol has been reviewed by an independent technical advisory committee convened by WHO and UNICEF to support the Rapid Water Quality Testing project. The current protocol is focused on culture-based methods of measuring the faecal indicator bacterium *Escherichia coli* (*E. coli*).

The protocol consists of a first phase screening to determine if the assay under evaluation produces results comparable to the reference method over a range of *E. coli* concentrations, under highly controlled conditions. Assays that have passed Phase 1 assessments can proceed to the Phase of 2 of the assessment, which will examine the performance of the test under more challenging conditions (competition from non-target bacteria, use of different natural water matrices and wild *E. coli* strains, and variable temperature incubation if claimed by the manufacturer).

3 Products

3.1 Trial Method: Aquagenx Gel EC CFU kit

The Aquagenx Gel EC CFU kit detects and quantifies *Escherichia coli* based on enzyme-substrate reaction from water samples.

Principle and Interpretation:
The EC growth medium for *E.coli* is a proprietary chromogenic powder growth medium with a substrate mixture that detects β-glucuronidase. When *E.coli* metabolizes Aquagenx Gel media, the colonies will appear as blue/blue-purple (discussion colour is blue green) colonies in the sample. Colonies have the appearance of small dots or circles.

Instructions for use and decontamination are detailed in the User Manual (Appendix 6.2.4).

Figure 1 Different concentration of *E.coli*. Experiment took place in phase 1

The Aquagenx Gel EC kit is available from:

Aquagenx, LLC
PO Box 17181
Chapel Hill, NC 27516 USA
Phone: +1-919-590-0343
Email: info@aquagenx.com
Website: https://www.aquagenx.com/

3.2 Reference Method: IDEXX Quanti-Tray System

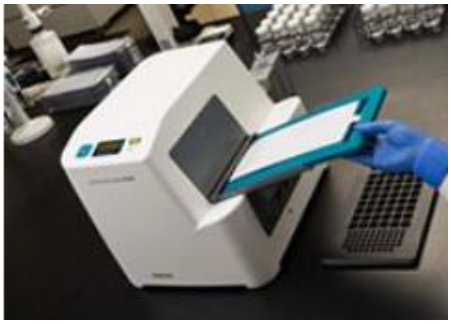
The Colilert Test uses proprietary Defined Substrate Technology (DST) to simultaneously detect coliforms and *E. coli*. Two nutrient-indicators, ONPG and MUG, are the major sources of carbon in Colilert and can be metabolized by the coliform enzyme β-galactosidase and the *E. coli* enzyme β-glucuronidase, respectively.

Step 1
Add reagent to the sample.

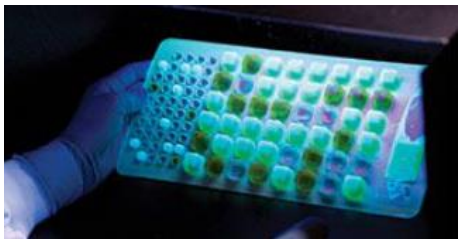
Step 2
Pour into Quanti-Tray/2000 (counts from 1–2,419).

Step 3
Seal in Quanti-Tray Sealer and place in 35°C ± 0.5°C incubator for 24 hours.

(temperature requirement may be different per regulatory requirements in other countries)



Step 4
Yellow wells = total coliforms
Yellow/fluorescent wells = *E. coli*
Count positive wells and refer to MPN table



More information: https://www.idexx.co.uk/en-gb/water/water-products-services/colilert/

4 Test protocol and criteria

4.1 Phase 1

The first phase aimed to determine if the assay under evaluation produced results comparable to the reference method. This was done under highly controlled conditions over a range of *E. coli* concentrations.

A stock solution of a known lab strain of *E. coli* (ATCC 25922) with a concentration of approximately 1000 viable and culturable *E. coli* cells per 100 mL, was prepared (acceptable range: 300 - 3000 cells/100 mL). This was measured and confirmed using the IDEXX Quantitray method in a background of sterile phosphate buffered saline (pH 7.4 ± 0.2). This stock solution was then serially diluted using two-fold dilution with a sterile phosphate buffered saline, for details see Table 3. The resulting stock solutions spanned a range of concentrations which were expected to yield positive results, ranging from zero to above most detection limits, with several critical range stock concentrations in between.

As a blank (A), a sample of stock solution 1 was autoclaved to eliminate any viable and culturable *E. coli*.

Table 3: Visual representation of the two-fold dilution, accounting for acceptable variance in the starting solution.

	Approximate *E. coli* concentration, cells/100 mL		
Stock	Lower acceptable limit	Target concentration	Upper acceptable limit
S1	300	1000	3000
S2	150	500	1500
S3	17	250	750
S4	38	125	375
S5	19	64	188
S6	9	32	94
S7	5	16	47
S8	2	8	23
S9	1	4	12
S10	0.6	2	6
S11	0.3	1	3
S12	0.1	0.5	1.5
A	0	0	0

Two sets of the cultivated *E. coli* stocks were prepared, one was incubated at 25 °C and the other at 35-37 °C. Both sets were evaluated after 20 hours, and again after 48 hours.

The results of the essay under evaluation and the results of the reference method were plotted against each other using a log transformed linear regression of both datasets. Within a given stock, the triplicate samples from the essay under evaluation were “paired” with the triplicate analyses made with the reference method during sample processing (before the incubation period).

Samples below the minimum detection limit were fixed at 50% of the detection limit. Linear regression was made on the datapoints that were within the quantification range, or below the minimum detection limit, for both assays.

An assay proceeded to the Phase 2 assessment if the Spearman’s rank coefficient was at least 0.90, and if the blanks did not show positive results. It was originally intended that tests with a regression slope (before log transformation) significantly different from 1.0 would be excluded from Phase 2 assessment. However, a large number of trial assays had regression slopes significantly different from unity, so this condition was relaxed.

4.2 Phase 2

4.2.1 False Positives due to non-target bacteria

Some tests could potentially generate positive results in the absence of *E. coli* through the growth of non-target organisms. Cultures of six non-target bacteria (*Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Pseudomonas aeruginosa* and *Serratia*) that could potentially cause false positives, were made with a target concentration of 100,000,000 viable and culturable cells/100 mL (acceptable range: 30,000,000 – 300,000,000 cells/100 mL). These cultures were tested using the trial assay without any addition of *E. coli*. Any positive results were considered a false positive. Single tests instead of triplicates were done, and the reference method was not challenged with the non-target organisms. Samples were incubated at 35 °C and evaluated after 20 and 48 hours.

4.2.2 False negative due to competition

The same six cultures of non-target organisms were mixed 1:100 with *E. coli* Stock 1, resulting in an approximate concentration of 30 CFU/100 mL *E. coli* and 30,000 CFU/100 mL of the non-target organism. The resulting stock was tested using the trial kit. Any negative results were considered to indicate that in the presence of competing bacteria, *E. coli* might not be detected by the trial method. Samples were incubated at 35 °C and evaluated after 20 and 48 hours. As for the False Positive experiments, the reference method was not tested and only single tests instead of triplicates were done.

4.2.3 Expanded temperature series

According to the user manual, this trial method can be used at any temperature between 25 and 44.5 °C. From 35-37 °C it is recommended to incubate for 20 hours; for 31-34 °C it is recommended to incubate for 24-30 hours, and for 25-30 °C it is recommended to incubate for 40-48 hours.

A stock solution of a known lab strain of *E. coli* (ATCC 25922) with a concentration of approximately 30 *E. coli* cells per 100 mL, was prepared in a background of sterile phosphate buffered saline (pH 7.4 ± 0.2). Aliquots of this stock were incubated in triplicate at six temperatures (20, 25, 30, 35, 40, and 45°C), and evaluated after 24, 48 and 72 hours. The reference method was incubated at 35 °C and evaluated after 24 hours.

4.2.4 Natural waters

The water matrix, as well as the strain of *E. coli* used, may affect the performance of the trial method. To assess this possibility, five different natural waters were selected. These included at least two surface water (SW) and two groundwater (GW) sources. Full list of requirements for the natural waters can be found in Table 4.

Table 4: Criteria for the natural waters.

Natural water	Source	Turbidity	pH	Alkalinity
N1	GW or SW	> 10	Any	
N2	GW or SW	< 10	< 6.5	At least one of the
N3	GW or SW	< 10	> 8.0	waters should have a
N4	GW or SW	Any	6.5 – 8.0	low <50 mg/L $CaCO_3$
N5	GW or SW	Any	Any	

The natural waters were sterilised and then spiked with effluent from a wastewater treatment plant to reach a target concentration of 300 *E. coli* per 100 mL (acceptable range: 100 – 1000 cells/100 mL). Pre-testing of the effluent was required to determine the concentration in order to properly dilute it into the natural waters. The stock solutions of

effluent in natural water were serially diluted using ten-fold dilutions with the sterilised natural waters three times. The resulting stock solutions spanned a range of concentrations which would be expected to yield at least one stock in each of the risk classes listed below in Table 5. The blank (A) was made by autoclaving the natural waters.

Table 5: Ten-fold dilution of effluent stock solution in sterilised waste water, accounting for the acceptable variance in starting solution.

		Approximate *E. coli* concentration, cells/100 mL		
Stock	Risk class	Lower acceptable limit	Target concentration	Upper acceptable limit
N*S1	Very high	100	300	1000
N*S2	High	10	30	100
N*S3	Medium	1	3	10
N*S4	Low	0.1	0.3	1
N*A	Not applicable	0	0	0

All natural water stocks were tested in triplicate with the trial method, using three different sets of equipment per triplicate: 5 water stocks (N1-5) * 5 dilution stocks (N*S1-A) * 3 replicates using different equipment, for a total of 75 analyses in all (60 stocks and 15 blanks). The same was done for the reference method.

Samples below the minimum detection limit were fixed at 50% of the detection limit. Linear regression was made on the datapoints that were within the quantification range, or below the detection limit, for both assays. Statistical tests were made as in Phase 1.

Two sets of natural water stocks were prepared; one was incubated at 25 °C and the other at 35 °C. Both sets were evaluated after 20 hours, and again after 48 hours. The reference method was incubated at 35 °C and evaluated after 24 hours.

5 Results

5.1 Phase 1

Tests were performed by one technician. The stock dilutions were made the day of testing.

Results were compared to the reference method over a wide range of *E. coli* concentrations, under highly controlled conditions (see **Table 6** - **Table 9**).

Table 6: Results of the CFU testing using the reference method and trial method over multiple dillutions at 25°C after 20 hours.

	Reference method (CFU/100 mL)			Trial method (CFU/100 mL)		
Stock	1	2	3	1	2	3
S1	> 2419.6	> 2419.6	> 2419.6	<1	<1	<1
S2	1553.1	1553.1	1732.9	<1	<1	<1
S3	727	920.8	686.7	<1	<1	<1
S4	325.5	435.2	547.5	<1	<1	<1
S5	172.2	127.4	153.9	<1	<1	<1
S6	111.2	115.3	114.5	<1	<1	<1
S7	47.1	64.4	81.3	<1	<1	<1
S8	21.6	17.1	23.3	<1	<1	<1
S9	7.5	12.1	9.8	<1	<1	<1
S10	3.1	5.2	4.1	<1	<1	<1
S11	1	2	2	<1	<1	<1
S12	1	1	2	<1	<1	<1
Blank	< 1.0	< 1.0	< 1.0	<1	<1	<1

Table 7: Results of the CFU testing using the reference method and trial method over multiple dillutions at 25°C after 48 hours.

	Reference method (CFU/100 mL)			Trial method (CFU/100 mL)		
Stock	1	2	3	1	2	3
S1	>2419,6	>2419,6	>2419,6	>>	>>	>>
S2	1553.1	1553.1	1732.9	>>	>>	>>
S3	727	920.8	686.7	195	179	155
S4	325.5	435.2	547.5	80	73	76
S5	172.2	127.4	153.9	52	66	71
S6	111.2	115.3	114.5	40	59	24
S7	47.1	64.4	81.3	19	13	19
S8	21.6	17.1	23.3	16	9	18
S9	7.5	12.1	9.8	3	1	2
S10	3.1	5.2	4.1	2	1	3
S11	1	2	2	1	1	1
S12	1	1	2	1	2	<1
Blank	< 1.0	< 1.0	< 1.0	<1	<1	<1

Table 8: Results of the CFU testing using the reference method and trial method over multiple dillutions at 35-37°C after 20 hours.

	Reference method (CFU/100 mL)			Trial method (CFU/100 mL)		
Stock	1	2	3	1	2	3
S1	>2419,6	>2419,6	>2419,6	>>	>>	>>
S2	1553.1	1553.1	1732.9	>>	>>	>>
S3	727	920.8	686.7	234	206	201
S4	325.5	435.2	547.5	112	115	126
S5	172.2	127.4	153.9	66	64	68
S6	111.2	115.3	114.5	62	39	43
S7	47.1	64.4	81.3	15	13	39
S8	21.6	17.1	23.3	6	5	7
S9	7.5	12.1	9.8	9	<1	8
S10	3.1	5.2	4.1	<1	1	4
S11	1	2	2	1	<1	<1
S12	1	1	2	2	<1	<1
Blank	< 1.0	< 1.0	< 1.0	<1	<1	<1

Table 9: Results of the CFU testing using the reference method and trial method over multiple dillutions at 35-37°C after 48 hours.

	Reference method (CFU/100 mL)			Trial method (CFU/100 mL)		
Stock	1	2	3	1	2	3
S1	>2419,6	>2419,6	>2419,6	>>	>>	>>
S2	1553.1	1553.1	1732.9	>>	>>	>>
S3	727	920.8	686.7	240	215	214
S4	325.5	435.2	547.5	123	122	133
S5	172.2	127.4	153.9	82	69	75
S6	111.2	115.3	114.5	66	42	46
S7	47.1	64.4	81.3	16	15	42
S8	21.6	17.1	23.3	6	11	10
S9	7.5	12.1	9.8	11	<1	9
S10	3.1	5.2	4.1	<1	2	6
S11	1	2	2	2	1	<1
S12	1	1	2	2	<1	<1
Blank	< 1.0	< 1.0	< 1.0	<1	<1	<1

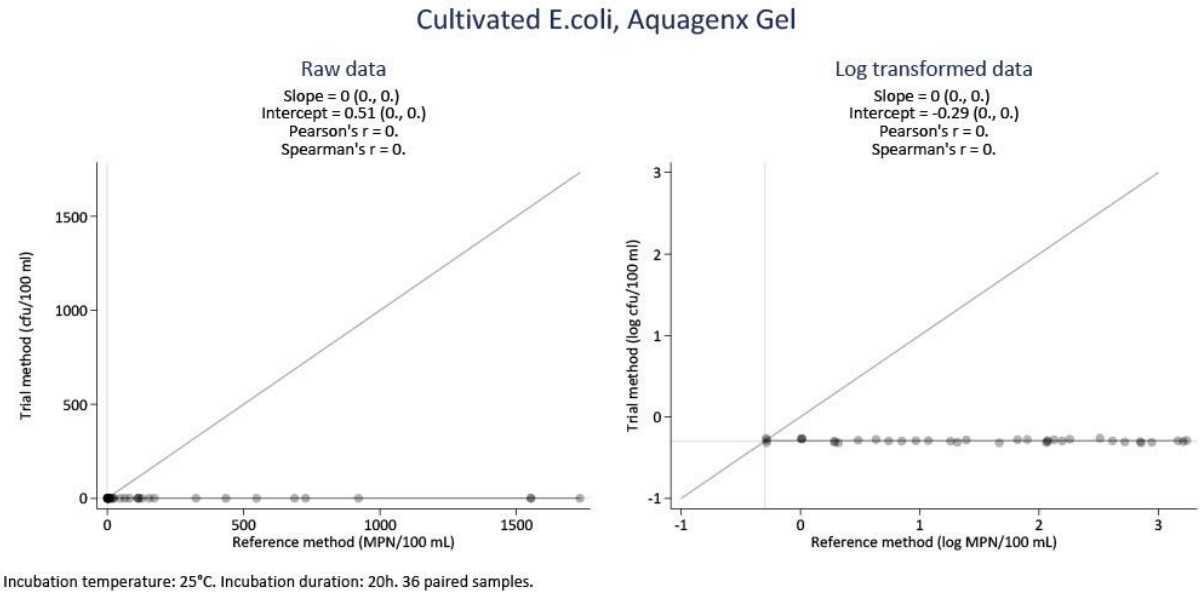


Figure 2 Statistical analysis of Phase 1 results after 20 hours at 25°C.

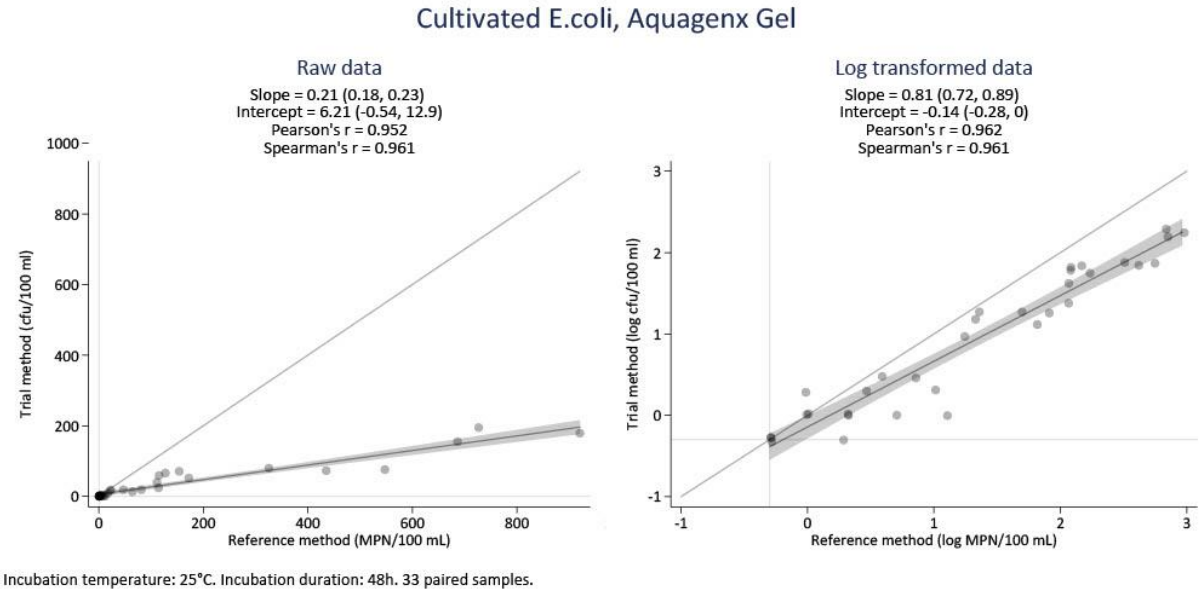
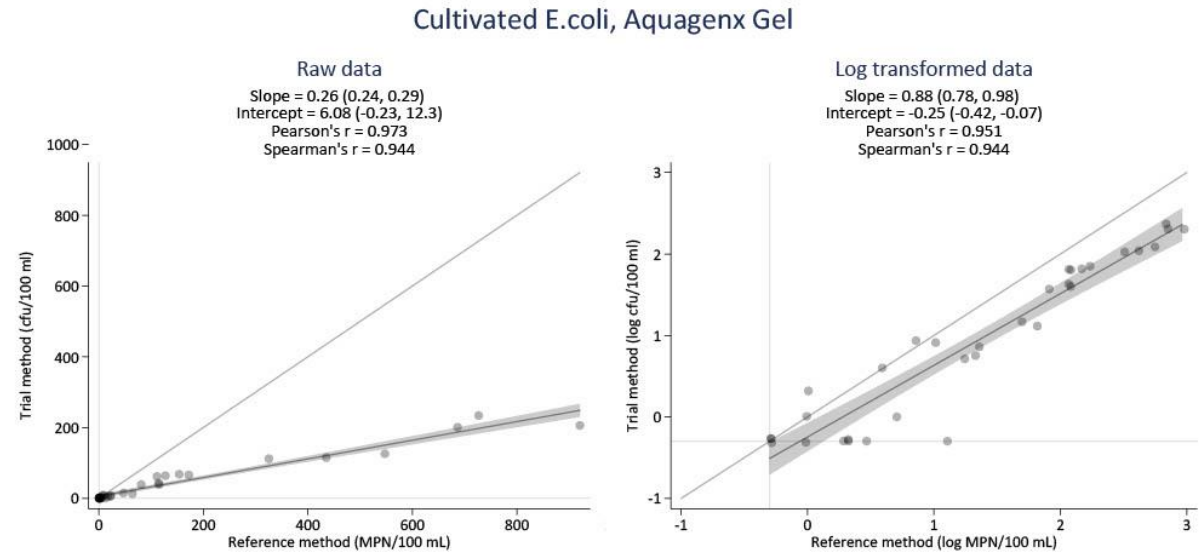
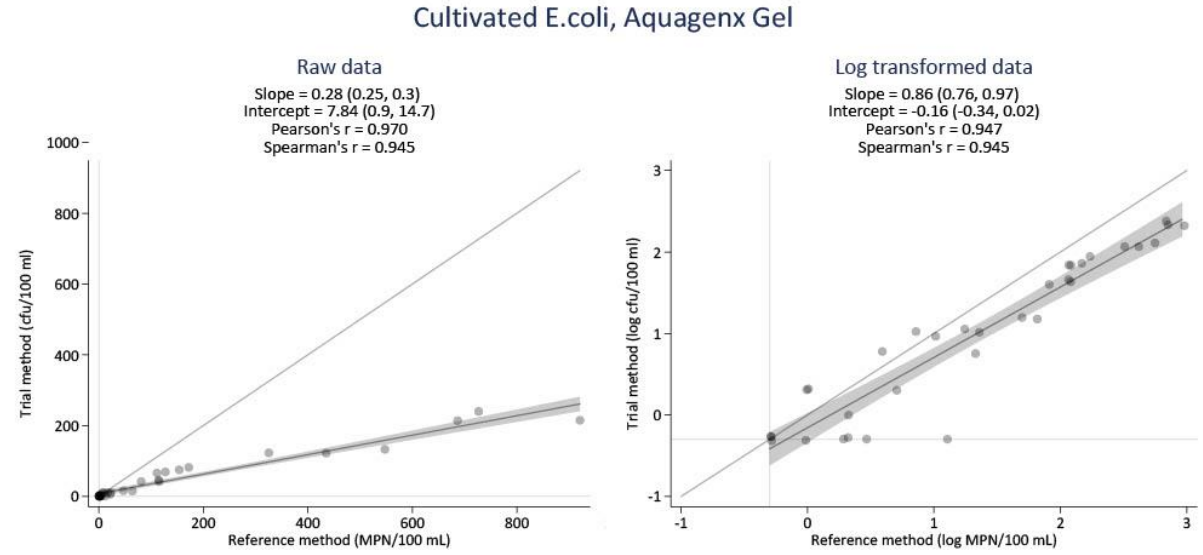


Figure 3 Statistical analysis of Phase 1 results after 48 hours at 25°C.



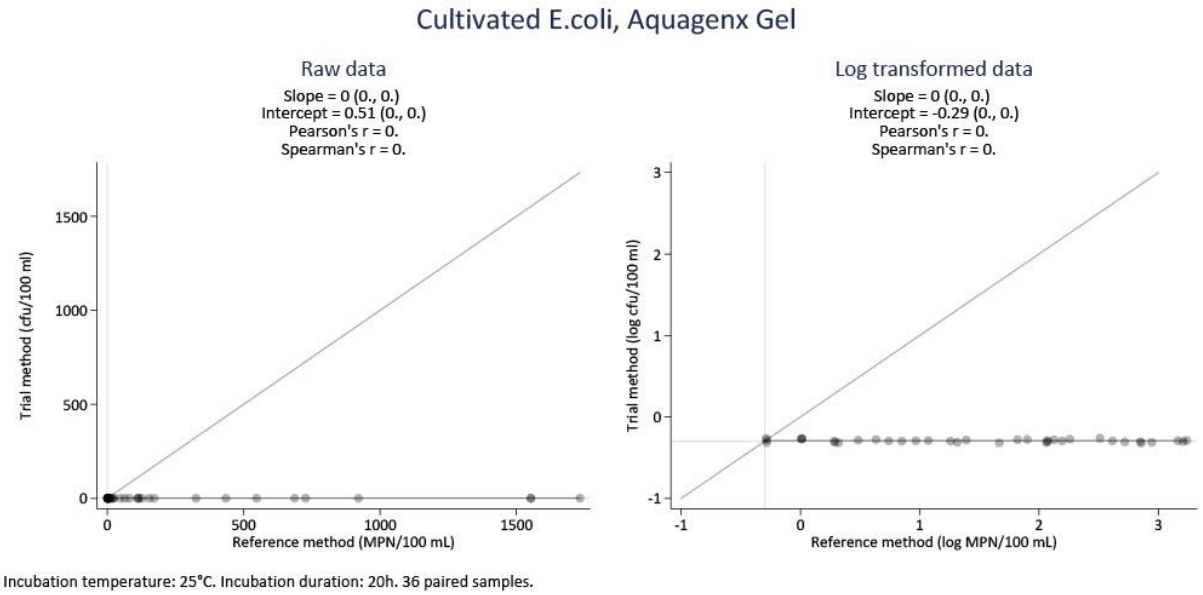
Incubation temperature: 35-37°C. Incubation duration: 20h. 33 paired samples.

Figure 4 Statistical analysis of Phase 1 results after 20 hours at 35-37°C.



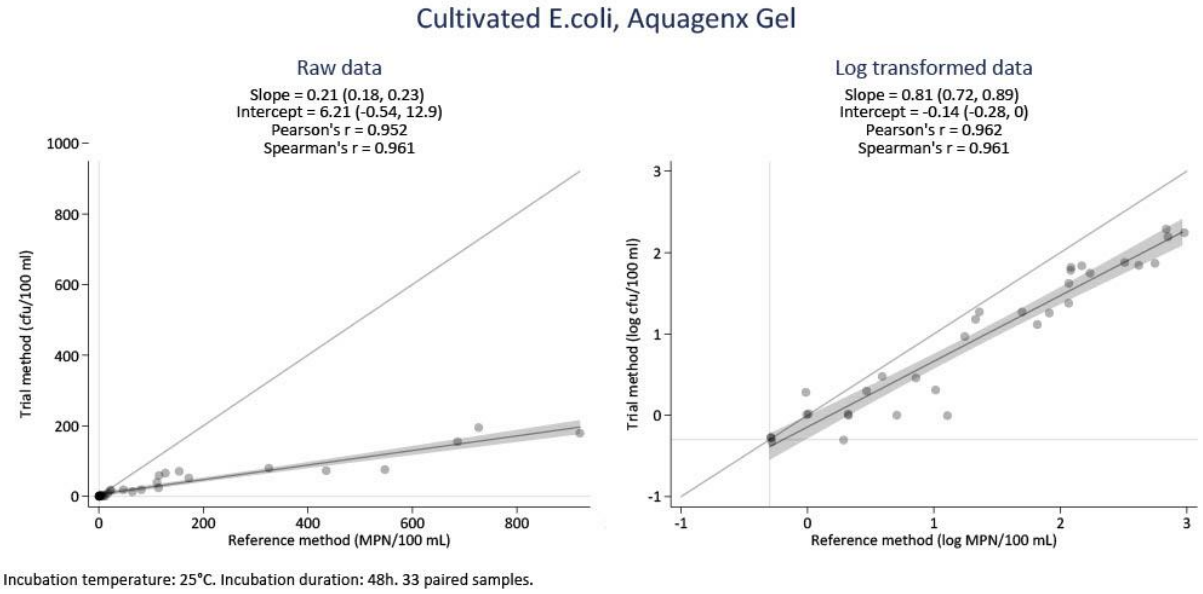
Incubation temperature: 35-37°C. Incubation duration: 48h. 33 paired samples.

Figure 5 Statistical analysis of Phase 1 results after 48 hours at 35-37°C.



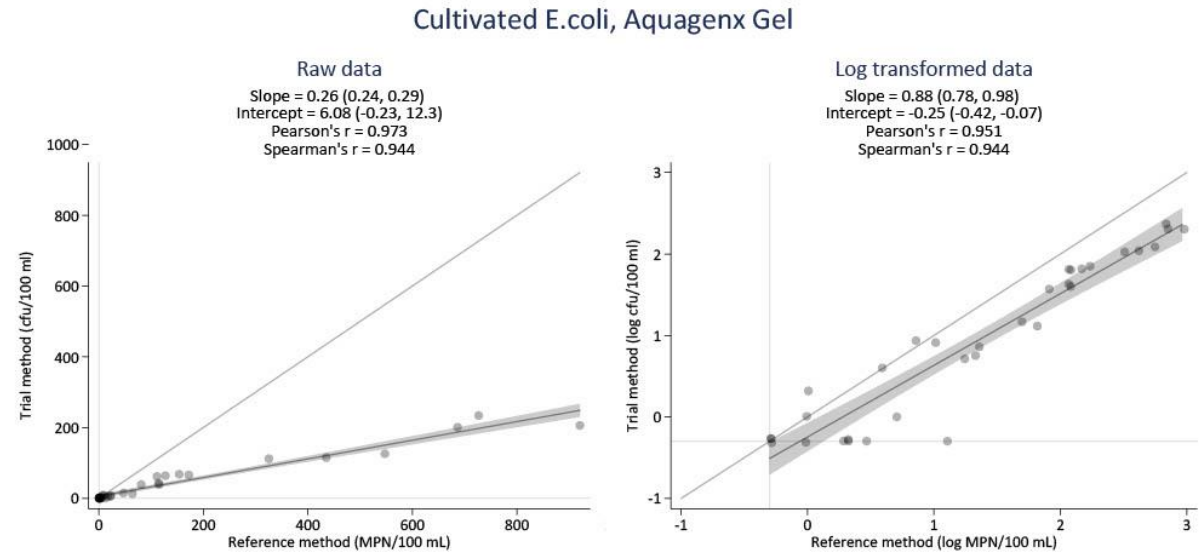
Incubation temperature: 25°C. Incubation duration: 20h. 36 paired samples.

Figure 6 Statistical analysis of Phase 1 results after 20 hours at 25°C.



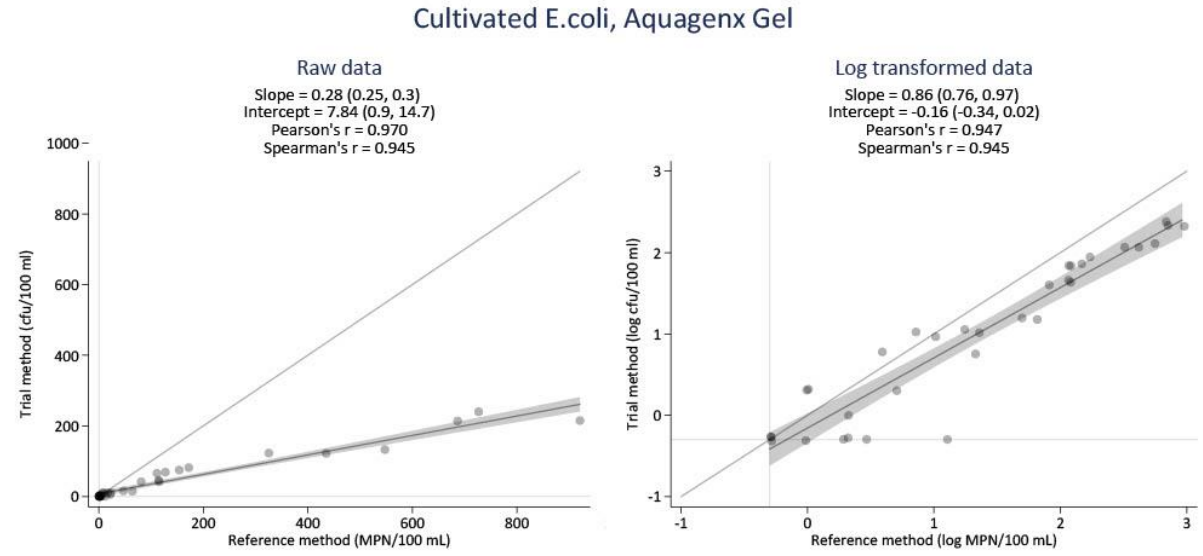
Incubation temperature: 25°C. Incubation duration: 48h. 33 paired samples.

Figure 7 Statistical analysis of Phase 1 results after 48 hours at 25°C.



Incubation temperature: 35-37°C. Incubation duration: 20h. 33 paired samples.

Figure 8 Statistical analysis of Phase 1 results after 20 hours at 35-37°C.



Incubation temperature: 35-37°C. Incubation duration: 48h. 33 paired samples.

Figure 9 Statistical analysis of Phase 1 results after 48 hours at 35-37°C.

The Pearson's rank coefficient is higher than 0,9 and meets the criterion. WHO agreed to proceed to Phase 2 assessments with the Aquagenx Gel.

5.2 Phase 2

5.2.1 False positive due to non-target bacteria.

The results of the false positives test can be found in Table 10 below. The full list of numerical results can be found in Appendix 6.2.1

Table 10: Results of the false positives test

Non-target bacteria (100,000,000 CFU/100 mL)	Target bacteria (30 CFU/100 mL)	Test results
Aeromonas		negative
Citrobacter		negative
Enterobacter		negative
Klebsiella		negative
Pseudomonas		negative
	E. coli *	positive

* *E. coli* has been analysed as a positive control to ensure growth conditions.

5.2.2 False negatives due to competition

The results of the false positives test can be found below in Tabel 11. The full list of numerical results can be found in Appendix 6.2.2.

Table 11: Results of the false negatives test.

Non-target bacteria (30,000 CFU/100 mL)	Target bacteria (30 CFU/100 mL)	Test results
Aeromonas	*E. coli*	positive
Citrobacter	*E. coli*	positive
Enterobacter	*E. coli*	positive
Klebsiella	*E. coli*	positive
Pseudomonas	*E. coli*	positive
	E. coli	positive

5.2.3 Expanded temperature series

The results of a stock solution tested with the trial method when incubated at different temperatures can be found in Table 27 below.

Table 27: Results in CFU/100 mL for the expanded temperature range assessment experiments.

		Time		
Temp	Replicate	24h	48h	72h
20°C	1	<1	<1	24
	2	<1	<1	16
	3	<1	<1	19
25°C	1	<1	34	35
	2	<1	36	37
	3	<1	29	29
30°C	1	40	54	55
	2	30	38	39
	3	29	30	29
35°C	1	47	51	51
	2	47	48	48
	3	50	50	52
40°C	1	41	41	41
	2	48	49	49
	3	49	49	49
45°C	1	<1	<1	<1
	2	<1	<1	<1
	3	<1	<1	<1

Result: reference method 32 CFU/100 mL

5.2.4 Natural waters

pH, turbidity, and alkalinity of all natural water samples were tested and matched with the criteria from Table 4. Since autoclaving the water samples caused changes in the pH and turbidity, some samples were sterilised by filtering them through 0.22 µm filters in order to meet the (see below in Table 12).

Table 12: Selection of the natural water samples and their required and tested specifications.

Waters	Sample point coding	Matrix	Sterilization	Specifications	Required	Tested
N1	Supply channel after Bethune polder pumping station	SW	Autoclave	pH	any	8.4
				Turbidity (FTU)	> 10	89
				Alkalinity (mg/L)	any	210
N2	Pumping station Archemberg joint raw groundwater	GW	Filtration 0.22 µm	pH	< 6.5	6.2
				Turbidity (FTU)	< 10	< 0.1
				Alkalinity (mg/L)	any	18
N3	Surface water intake point on the Petrusplaat	SW	Autoclave	pH	> 8	8.3
				Turbidity (FTU)	< 10	3.4
				Alkalinity (mg/L)	any	50
N4	Pumping station Nijmegen joint raw ground water	GW	Filtration 0.22 µm	pH	6.5 - 8.0	7.5
				Turbidity (FTU)	any	< 0.1
				Alkalinity (mg/L)	any	55
N5	Pumping station Vessum joint raw ground water	GW	Filtration 0.22 µm	pH	any	6.6
				Turbidity (FTU)	any	5.7
				Alkalinity (mg/L)	< 50	22

5.2.5 Natural waters spiked with effluent.

In **Table -Table 16**, the results for the measurement of colony forming units using both the reference and the trial method can be found. This was done for all the natural water sample with different effluent concentrations. A total of 15 paired samples were analysed for each natural water, for a grand total of 75 paired samples, including 15 blanks. No *E. coli* was detected in any of the blank samples, using either the trial or reference method.

Table 13: Results in CFU/100 mL of the natural waters spiked with effluent from the wastewater treatment plant for both the reference and trial method after 20 hours at 25°C.

		N1		N2		N3		N4		N5	
Stock	Replicate	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial
S1	1	344.1	7	191.8	5	109.5	6	135.4	16	204.6	<1
	2	325.5	7	167	7	435.2	4	195.6	10	290.9	<1
	3	410.6	2	119.8	12	193.5	10	214.3	10	325.5	<1
S2	1	24.6	<1	18.9	<1	22.3	<1	13.5	<1	28.8	<1
	2	21.6	<1	18.7	<1	27.9	<1	26	<1	23.8	<1
	3	21.8	<1	23.1	<1	26.5	<1	30.9	<1	21.3	<1
S3	1	5.2	<1	3.1	<1	2	<1	1	<1	4.1	<1
	2	3.1	<1	3.1	<1	1	<1	3.1	<1	2	<1
	3	4.1	<1	3	<1	1	<1	< 1	<1	3.1	<1
S4	1	< 1	<1	< 1	<1	1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	2	<1	< 1	<1	< 1	<1
	3	1	<1	< 1	<1	< 1	<1	1	<1	< 1	<1
A	1	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	3	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1

Table 4: Results in CFU/100 mL of the natural waters spiked with effluent from the wastewater treatment plant for both the reference and trial method after 48 hours at 25°C.

		N1		N2		N3		N4		N5	
Stock	Replicate	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial
S1	1	344.1	131	191.8	>>	109.5	144	135.4	>>	204.6	>>
	2	325.5	72	167	>>	435.2	158	195.6	>>	290.9	>>
	3	410.6	110	119.8	>>	193.5	149	214.3	>>	325.5	>>
S2	1	24.6	19	18.9	12	22.3	34	13.5	48	28.8	26
	2	21.6	19	18.7	16	27.9	27	26	44	23.8	29
	3	21.8	25	23.1	12	26.5	26	30.9	50	21.3	14
S3	1	5.2	1	3.1	2	2	2	1	5	4.1	3
	2	3.1	<1	3.1	2	1	2	3.1	6	2	<1
	3	4.1	4	3	4	1	2	< 1	5	3.1	1
S4	1	< 1	<1	< 1	<1	1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	2	<1	< 1	<1	< 1	<1
	3	1	<1	< 1	1	< 1	1	1	<1	< 1	<1
A	1	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	3	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1

Table 5: Results in CFU/100 mL of the natural waters spiked with effluent from the wastewater treatment plant for both the reference and trial method after 20 hours at 35-37°C.

		N1		N2		N3		N4		N5	
Stock	Replicate	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial
S1	1	344.1	139	191.8	123	109.5	258	135.4	>>	204.6	121
	2	325.5	159	167	106	435.2	310	195.6	>>	290.9	143
	3	410.6	142	119.8	90	193.5	297	214.3	>>	325.5	131
S2	1	24.6	15	18.9	10	22.3	28	13.5	30	28.8	10
	2	21.6	12	18.7	7	27.9	28	26	26	23.8	13
	3	21.8	17	23.1	3	26.5	33	30.9	24	21.3	10
S3	1	5.2	1	3.1	3	2	<1	1	3	4.1	<1
	2	3.1	1	3.1	<1	1	4	3.1	1	2	1
	3	4.1	<1	3	1	1	3	< 1	5	3.1	1
S4	1	< 1	<1	< 1	<1	1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	2	<1	< 1	<1	< 1	<1
	3	1	<1	< 1	<1	< 1	<1	1	<1	< 1	1
A	1	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	3	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1

Table 16: Results in CFU/100 mL of the natural waters spiked with effluent from the wastewater treatment plant for both the reference and trial method after 48 hours at 35-37°C.

		N1		N2		N3		N4		N5	
Stock	Replicate	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial	Ref	Trial
S1	1	344.1	>>	191.8	141	109.5	258	135.4	>>	204.6	>>
	2	325.5	>>	167	>>	435.2	310	195.6	>>	290.9	>>
	3	410.6	>>	119.8	>>	193.5	297	214.3	>>	325.5	>>
S2	1	24.6	35	18.9	20	22.3	28	13.5	38	28.8	20
	2	21.6	21	18.7	16	27.9	28	26	33	23.8	22
	3	21.8	25	23.1	12	26.5	33	30.9	37	21.3	13
S3	1	5.2	2	3.1	3	2		1	3	4.1	2
	2	3.1	2	3.1	<1	1	5	3.1	6	2	2
	3	4.1	<1	3	2	1	4	< 1	8	3.1	1
S4	1	< 1	<1	< 1	<1	1	<1	< 1	1	< 1	<1
	2	< 1	<1	< 1	<1	2	<1	< 1	<1	< 1	<1
	3	1	<1	< 1	<1	< 1	1	1	<1	< 1	1
A	1	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	2	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1
	3	< 1	<1	< 1	<1	< 1	<1	< 1	<1	< 1	<1

5.2.6 Statistical analysis Natural Waters.

Graphical interpretation and overview of results on both raw data and log-transformed data for all five natural water matrices can be found below in Figure 10 - Figure 29.

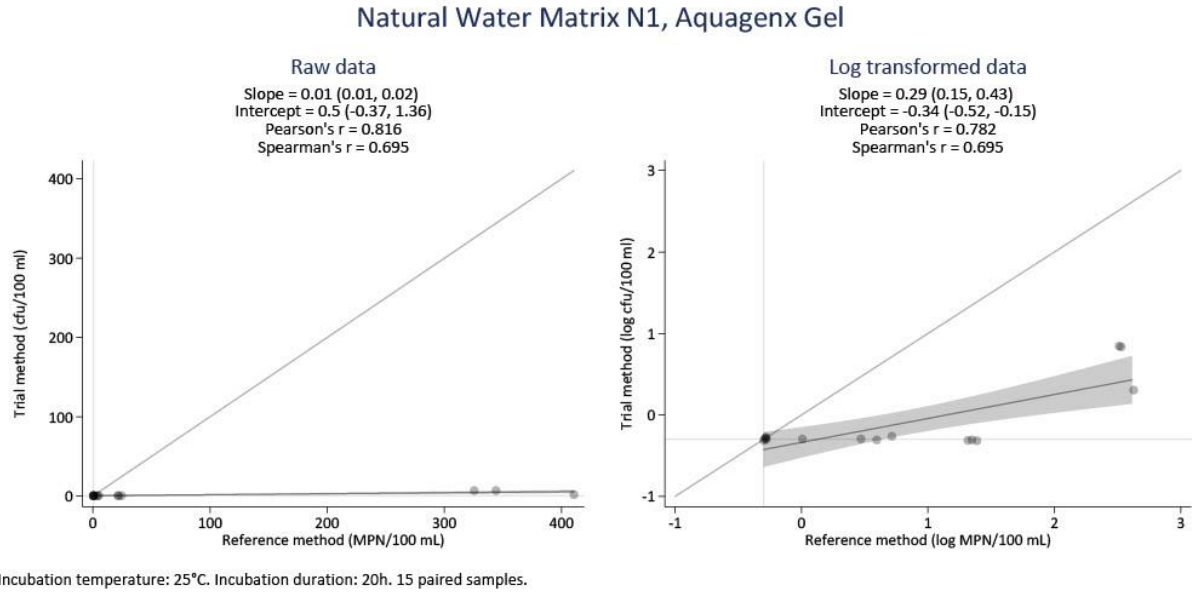


Figure 10: Statistical analysis Natural Matrix N1 after 20 hours at 25°C.

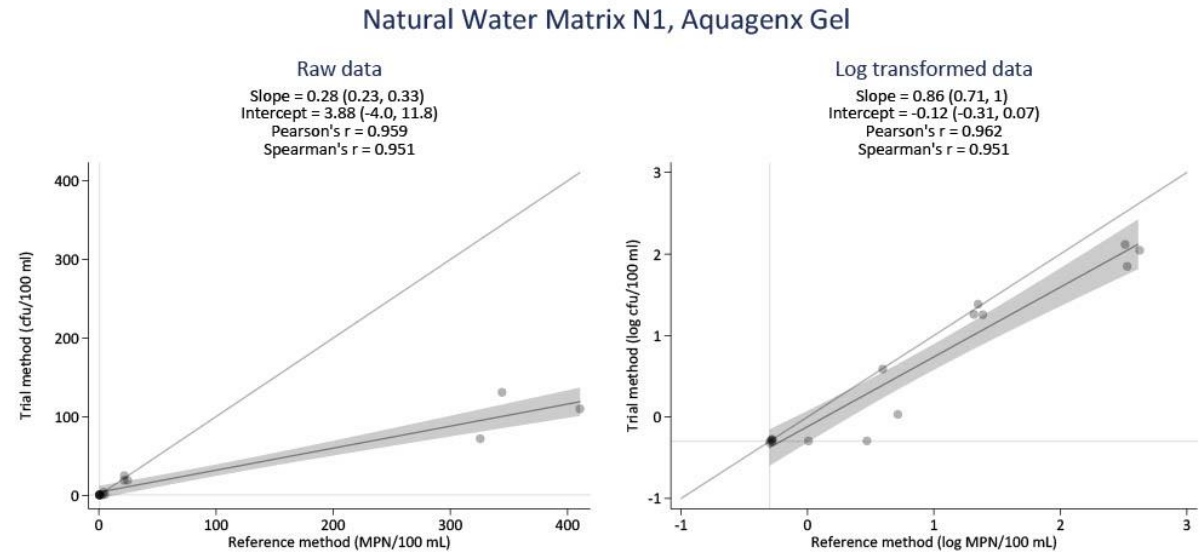


Figure 11: Statistical analysis Natural Matrix N1 after 48 hours at 25°C.

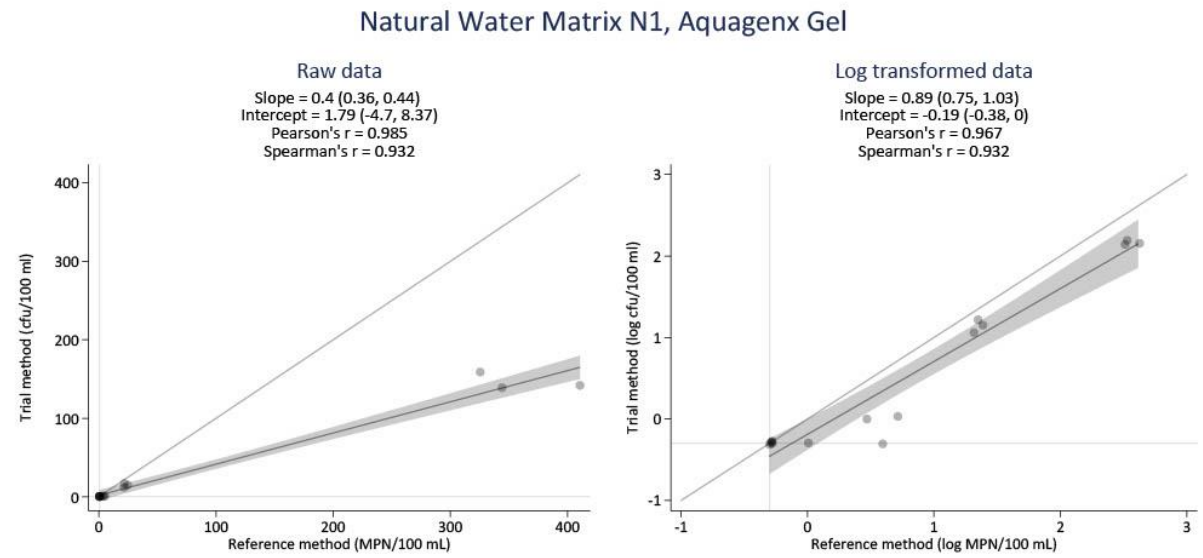
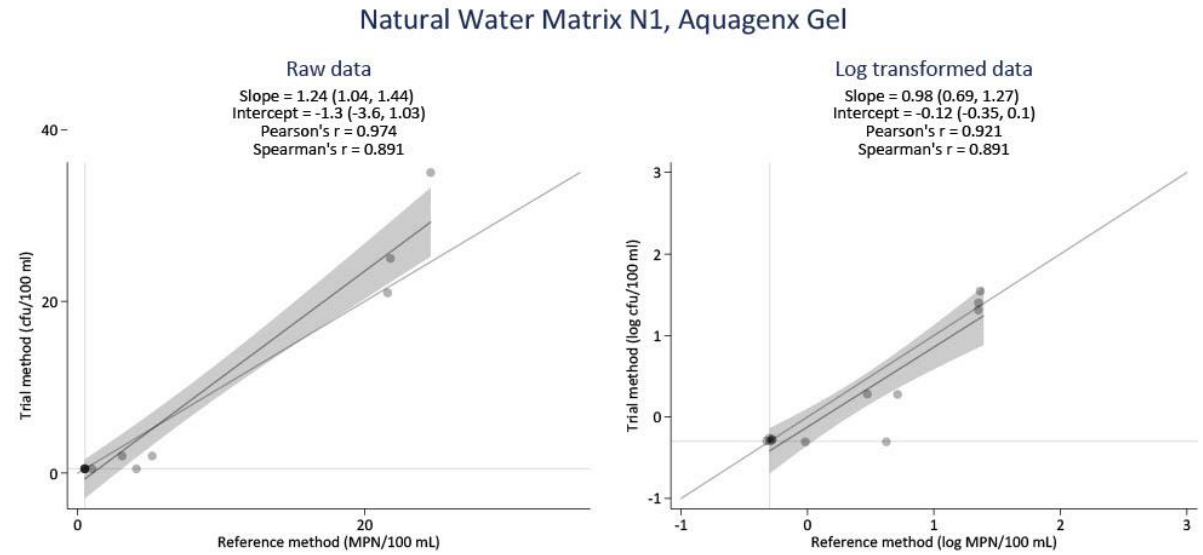
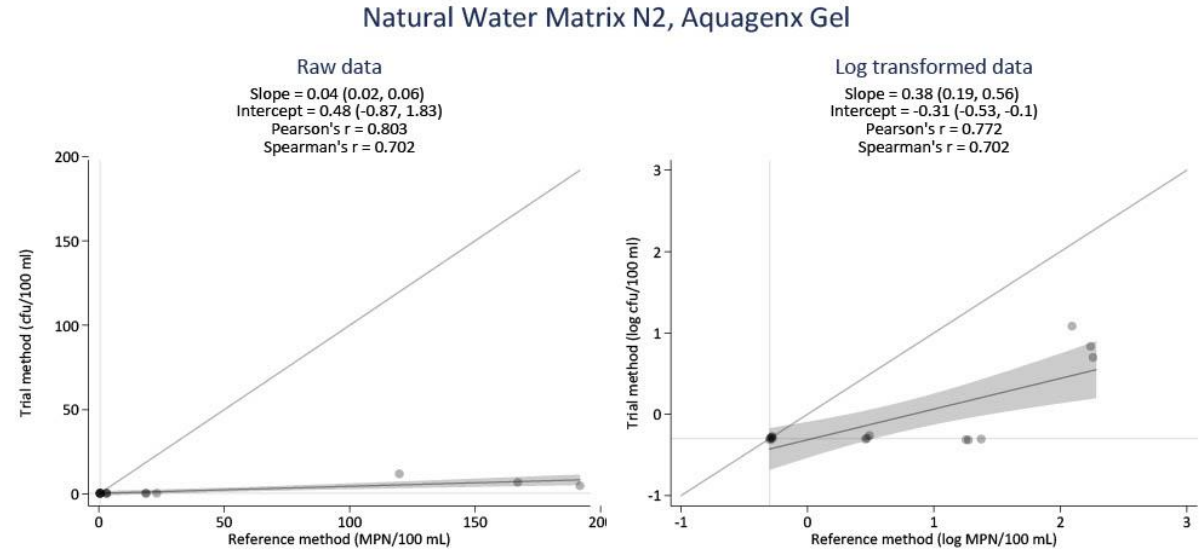


Figure 12: Statistical analysis Natural Matrix N1 after 20 hours at 35-37°C.



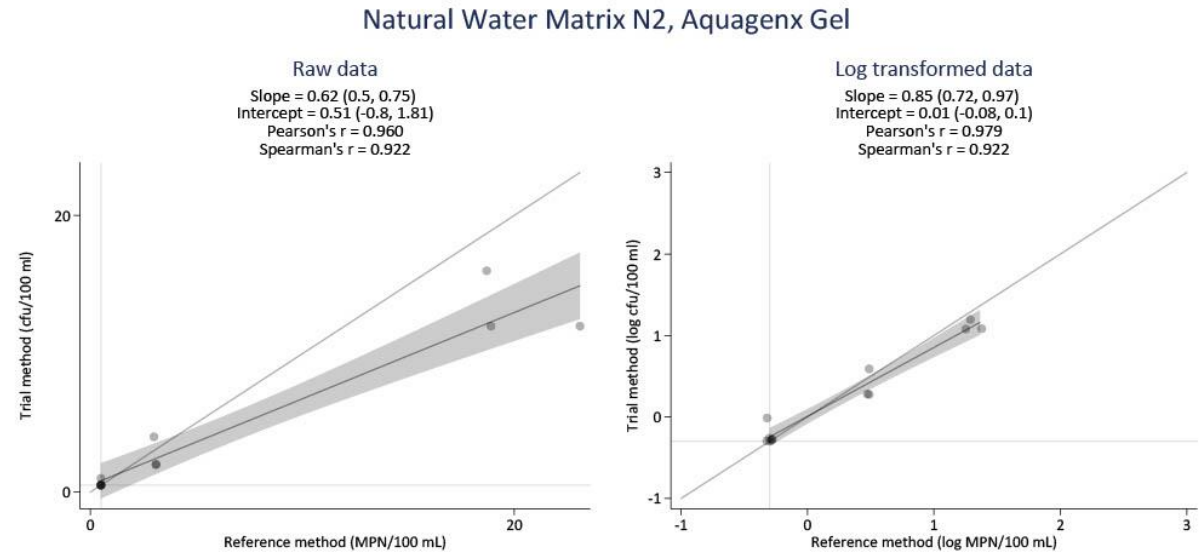
Incubation temperature: 35-37°C. Incubation duration: 48h. 12 paired samples.

Figure 13: Statistical analysis Natural Matrix N1 after 48 hours at 35-37°C.



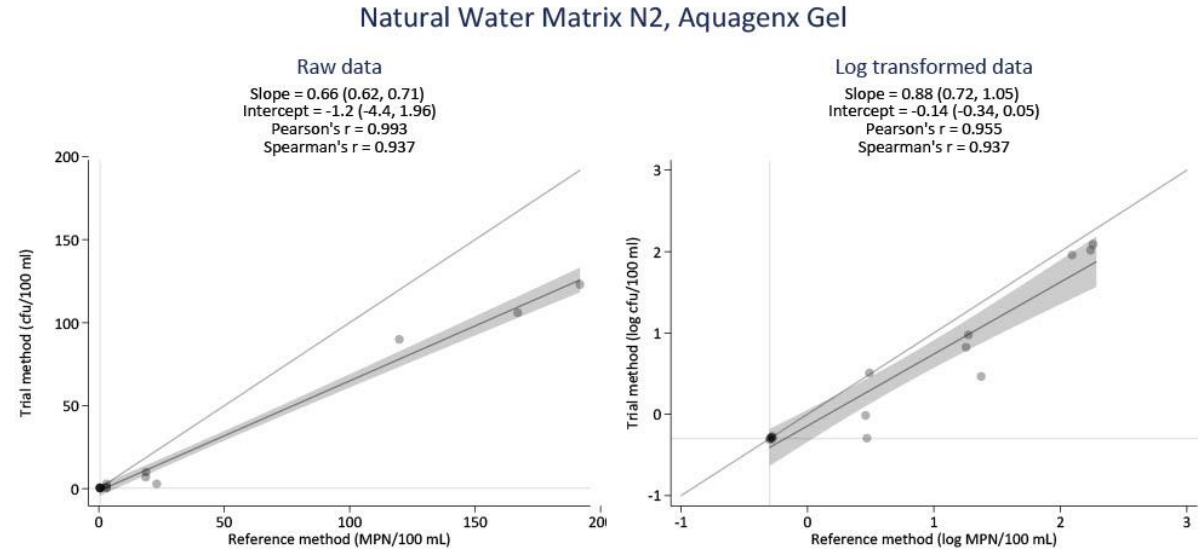
Incubation temperature: 25°C. Incubation duration: 20h. 15 paired samples.

Figure 14: Statistical analysis Natural Matrix N2 after 20 hours at 25°C.



Incubation temperature: 25°C. Incubation duration: 48h. 12 paired samples.

Figure 15: Statistical analysis Natural Matrix N2 after 48 hours at 25°C.



Incubation temperature: 35-37°C. Incubation duration: 20h. 15 paired samples.

Figure 16: Statistical analysis Natural Matrix N2 after 20 hours at 35-37°C.

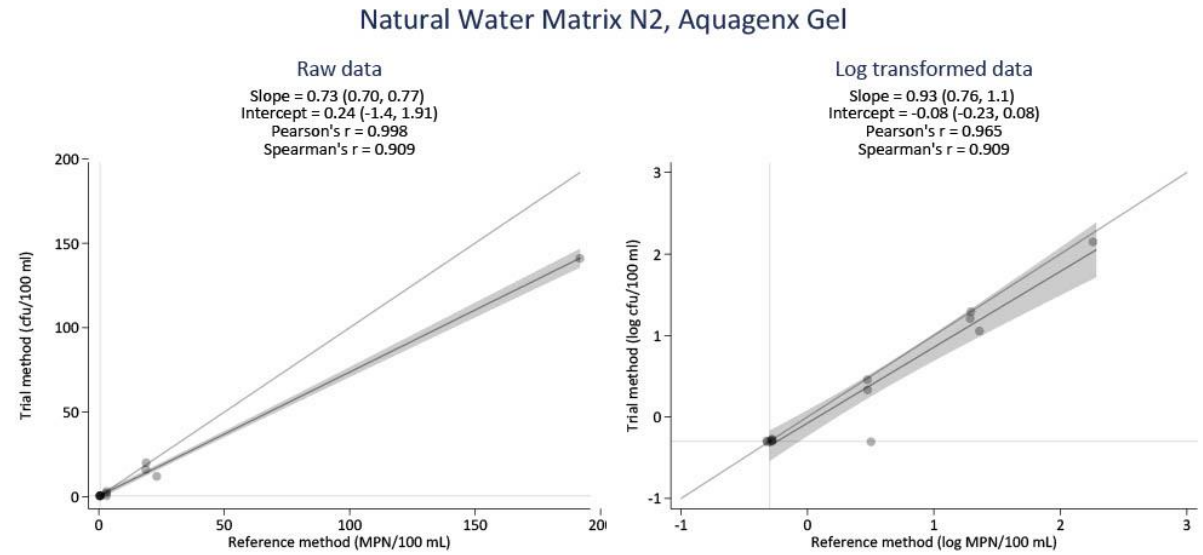


Figure 17: Statistical analysis Natural Matrix N2 after 48 hours at 35-37°C.

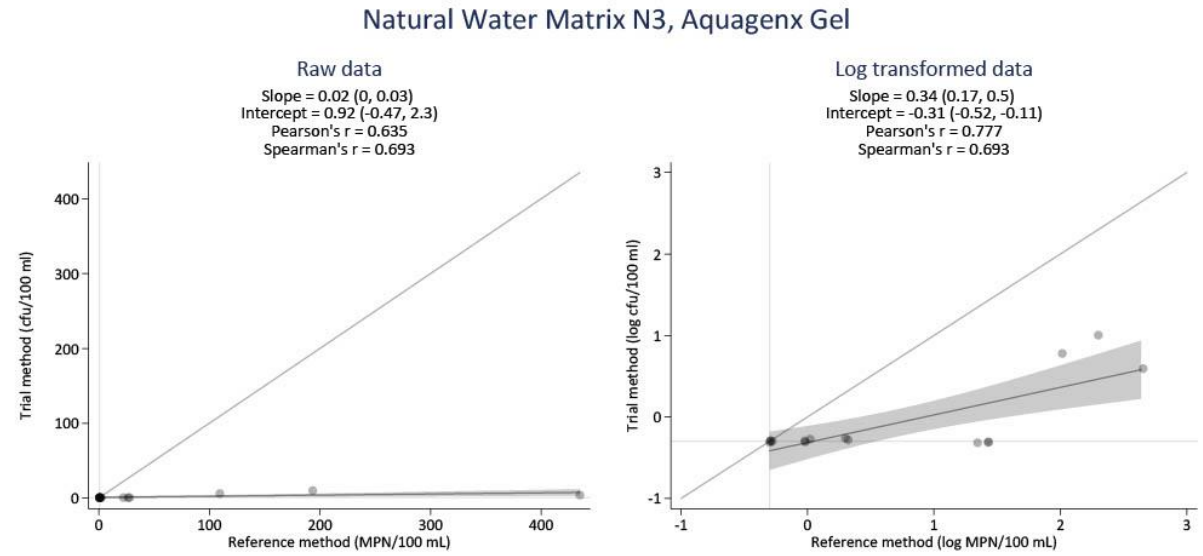
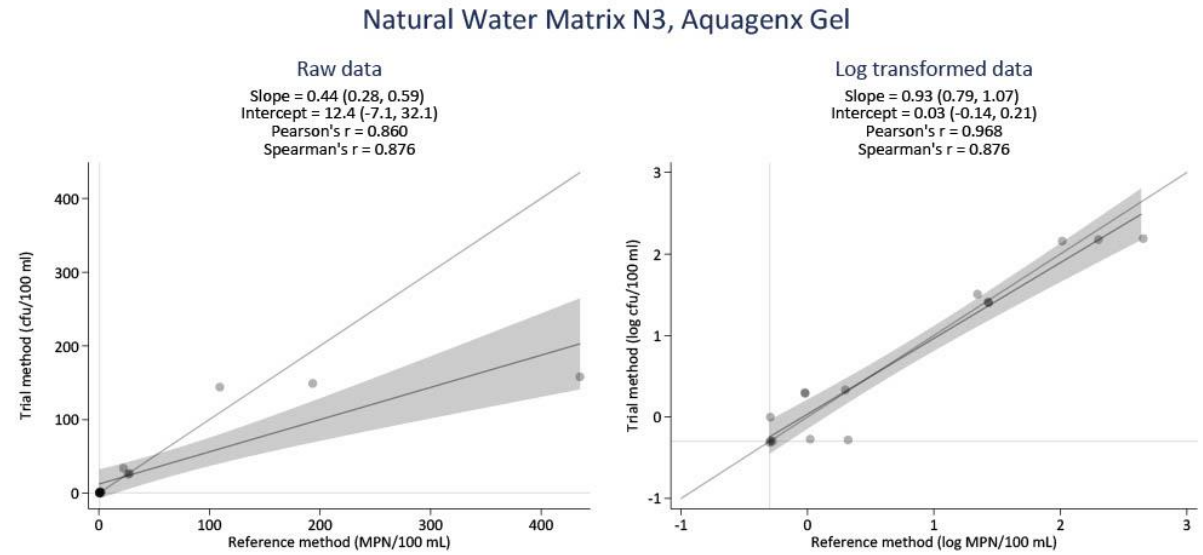
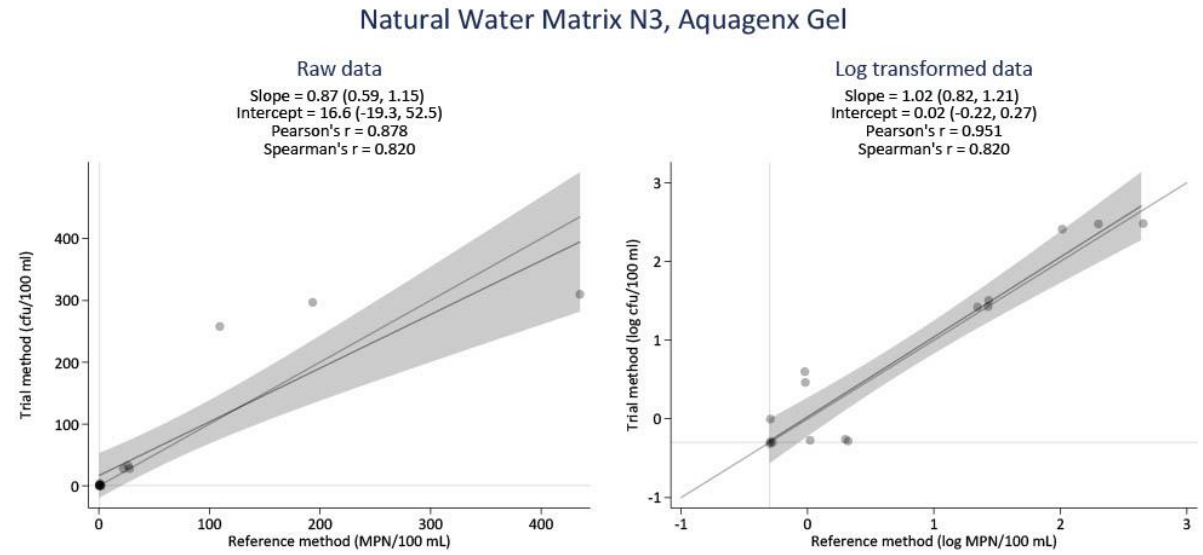


Figure 18: Statistical analysis Natural Matrix N3 after 20 hours at 25°C.



Incubation temperature: 25°C. Incubation duration: 48h. 15 paired samples.

Figure 19: Statistical analysis Natural Matrix N3 after 48 hours at 25°C.



Incubation temperature: 35-37°C. Incubation duration: 20h. 15 paired samples.

Figure 20: Statistical analysis Natural Matrix N3 after 20 hours at 35-37°C.

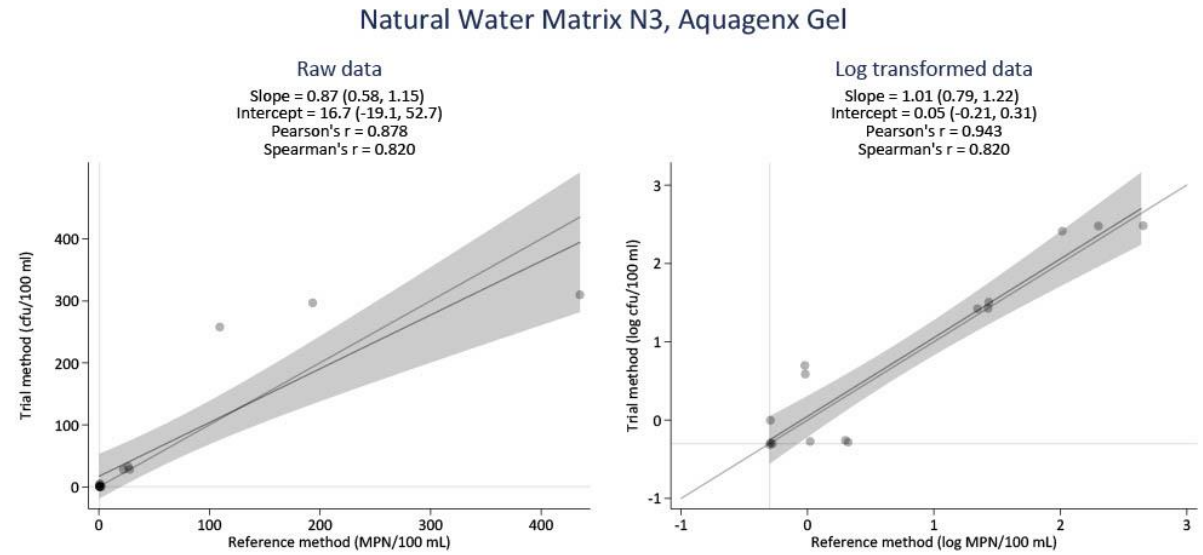


Figure 21: Statistical analysis Natural Matrix N3 after 48 hours at 35-37°C.

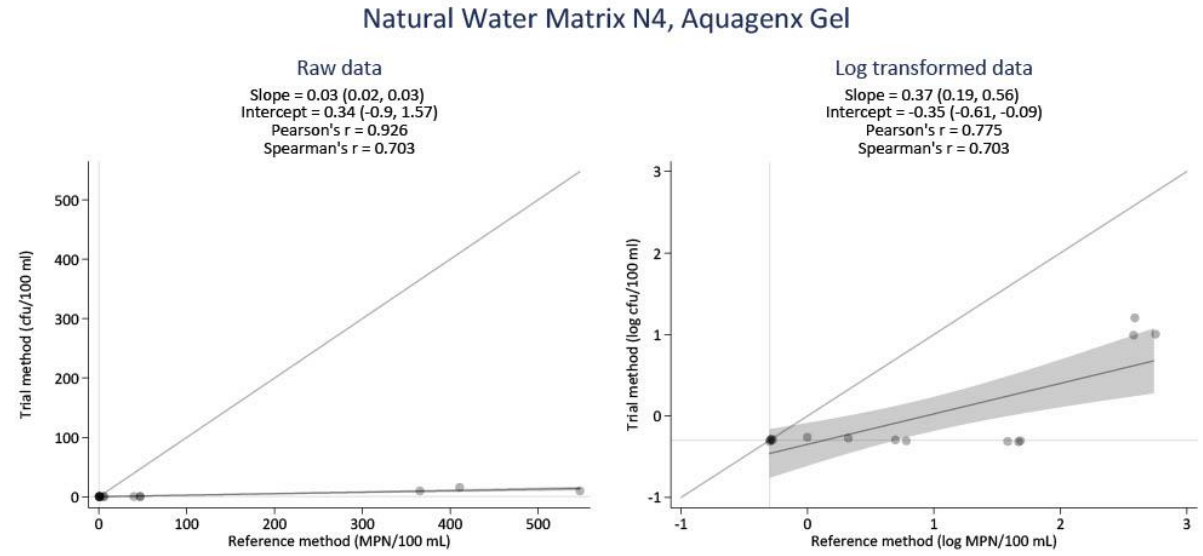


Figure 22: Statistical analysis Natural Matrix N4 after 20 hours at 25°C.

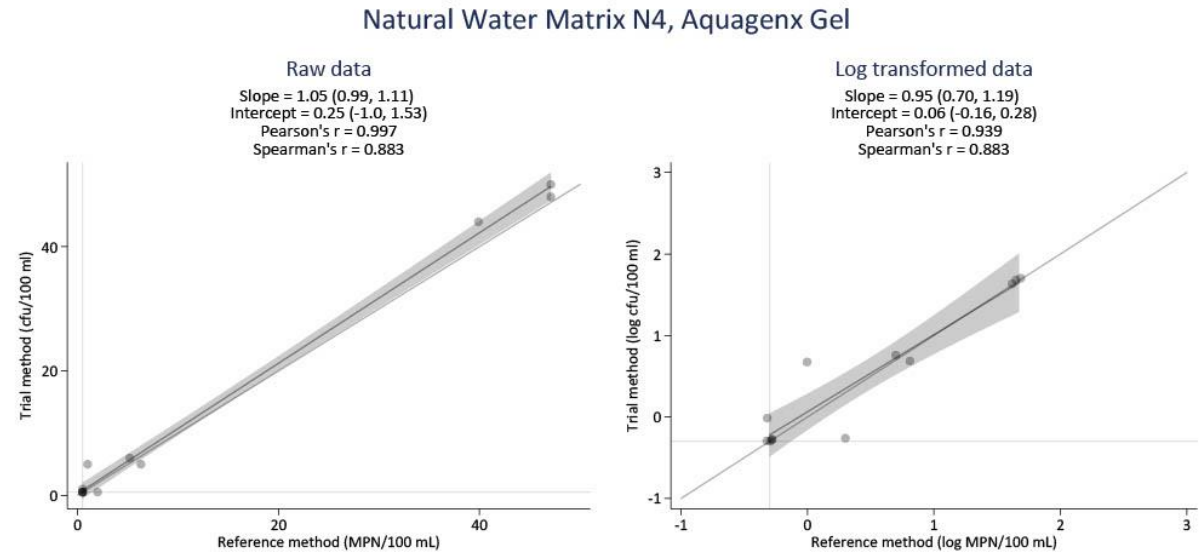


Figure 23: Statistical analysis Natural Matrix N4 after 48 hours at 25°C.

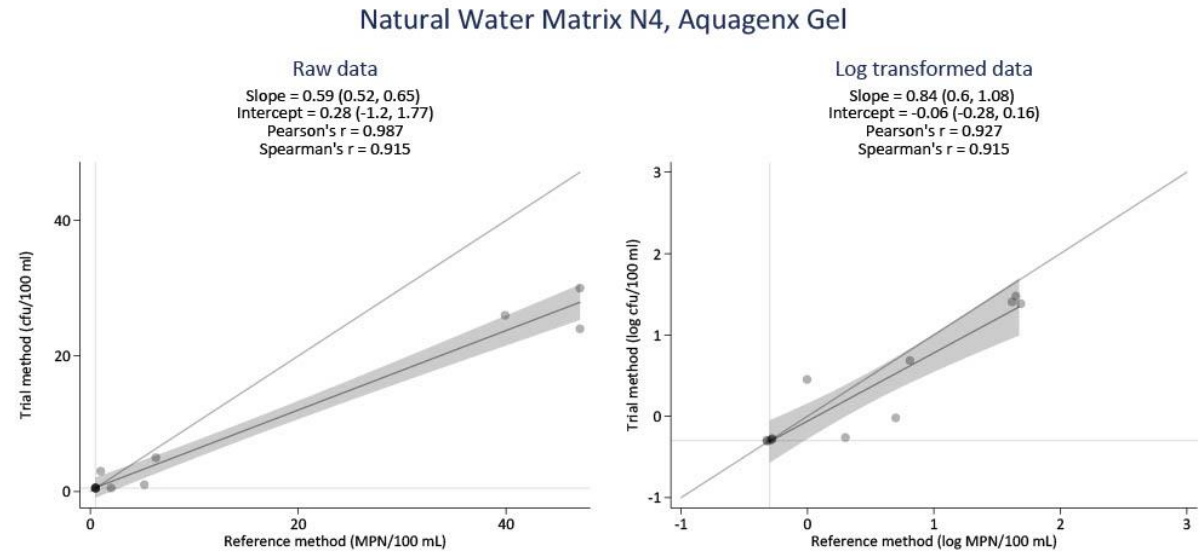


Figure 24: Statistical analysis Natural Matrix N4 after 20 hours at 35-37°C.

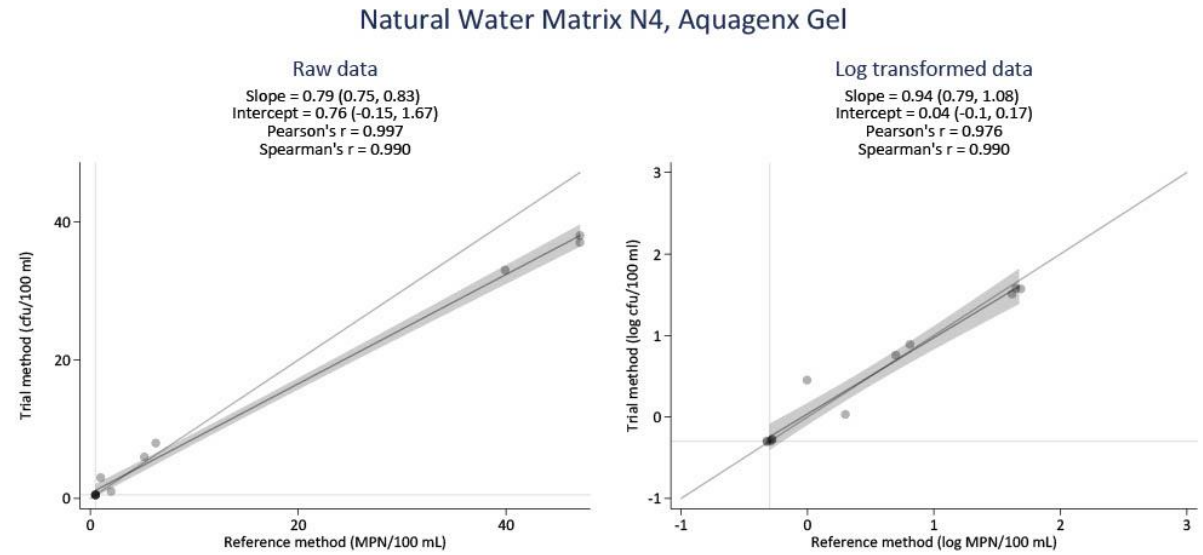


Figure 25: Statistical analysis Natural Matrix N4 after 48 hours at 35-37°C.

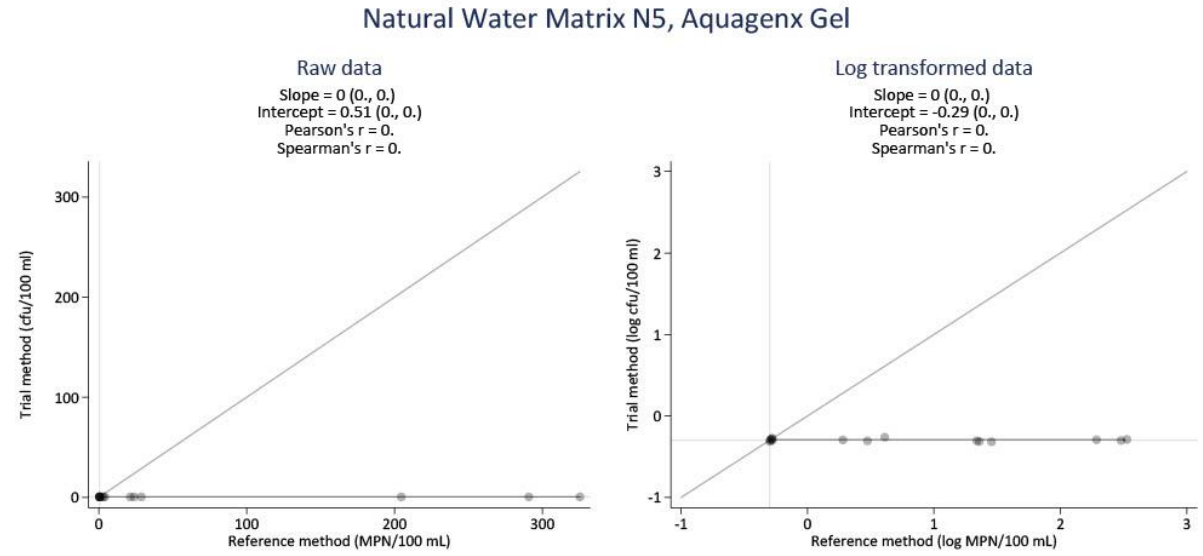
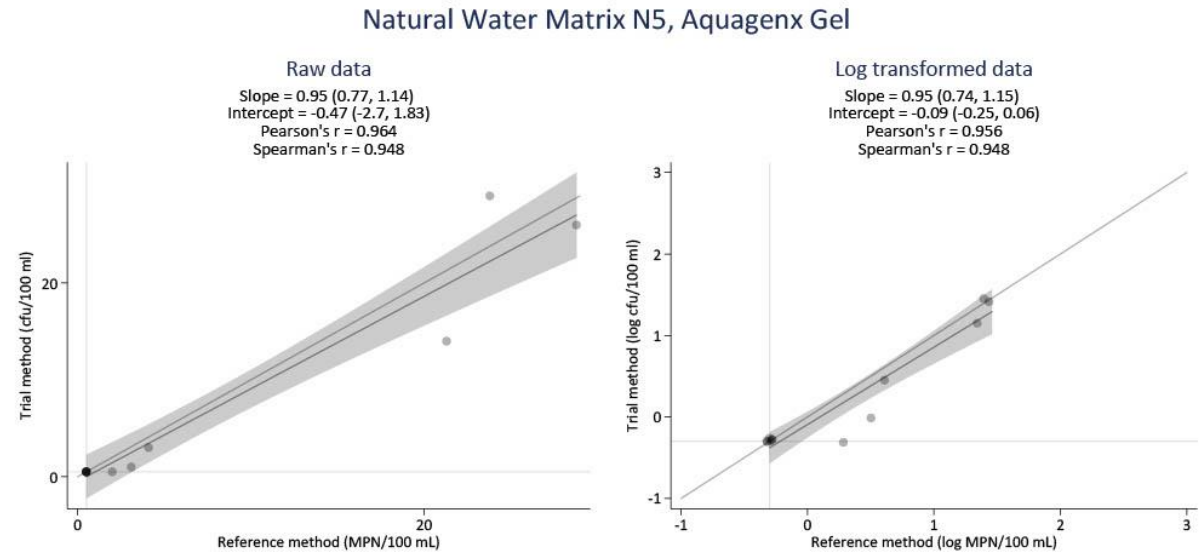
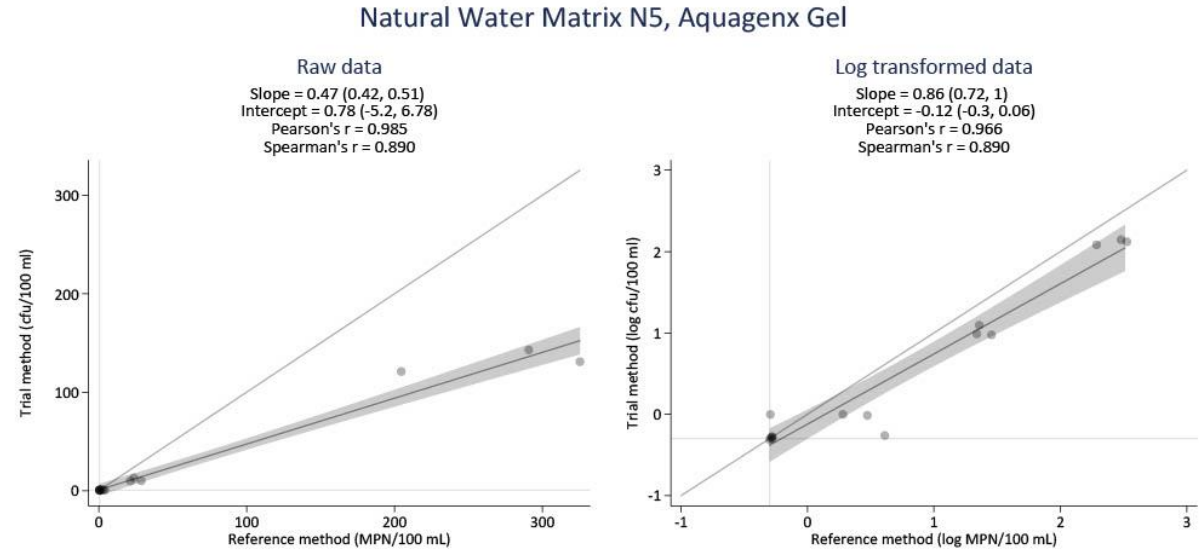


Figure 26: Statistical analysis Natural Matrix N5 after 20 hours at 25°C.



Incubation temperature: 25°C. Incubation duration: 48h. 12 paired samples.

Figure 27: Statistical analysis Natural Matrix N5 after 48 hours at 25°C.



Incubation temperature: 35-37°C. Incubation duration: 20h. 15 paired samples.

Figure 28: Statistical analysis Natural Matrix N5 after 20 hours at 35-37°C.

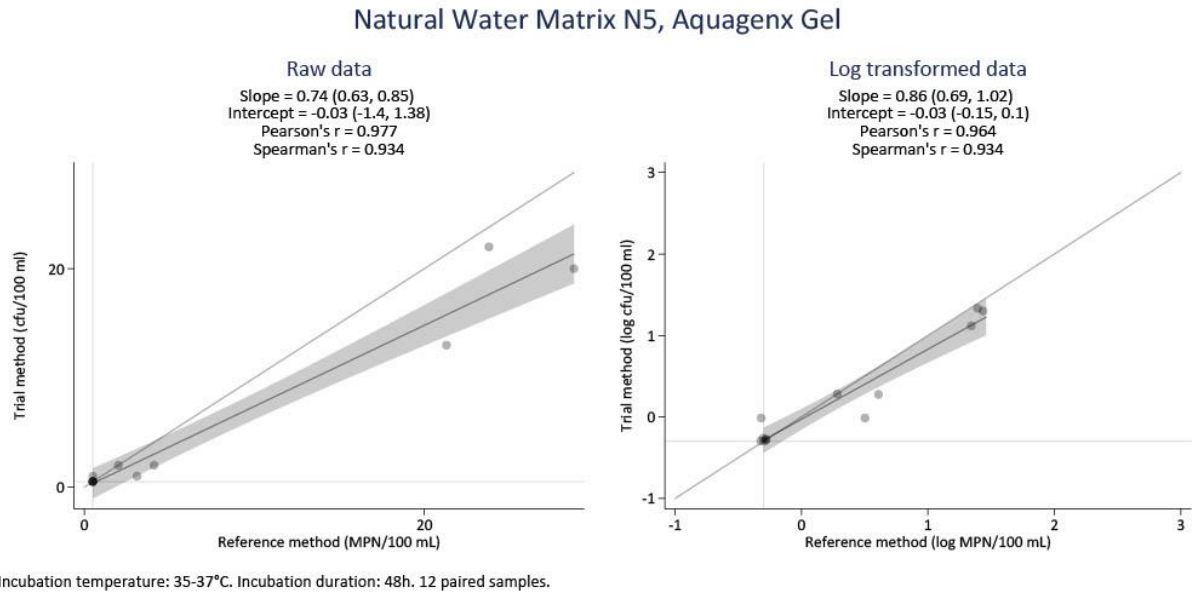


Figure 29: Statistical analysis Natural Matrix N5 after 48 hours at 35-37°C.

Interpretation and overview of results through linear regression on both raw data and log-transformed data is summarised below in Table 17 and Table 18.

Table 17: Overview of the regression analysis of the experiments at 25°C.

Water matrix	Time (h)	Number of paired samples	Maximum value	Slope (raw)	Intercept (raw)	Slope (log)	Intercept (log)	Spearman's r
Lab water	20	39	<1	0.00	0.51	0.00	-0.29	0.00
	48	39	>>	0.21	6.21	0.81	-0.14	0.961
N1	20	15	7	0.01	0.50	0.29	-0.34	0.695
	48	15	131	0.28	3.88	0.86	-0.12	0.951
N2	20	15	12	0.04	0.48	0.38	-0.31	0.702
	48	15	>>	0.62	0.51	0.85	0.01	0.922
N3	20	15	10	0.02	0.92	0.34	-0.31	0.693
	48	15	158	0.44	12.4	0.93	0.03	0.876
N4	20	15	16	0.03	0.34	0.37	-0.35	0.703
	48	15	>>	1.05	0.25	0.95	0.06	0.883
N5	20	15	<1	0.00	0.51	0.00	-0.29	0.000
	48	15	>>	0.95	-0.47	0.95	-0.09	0.948

Table 18: Overview of the regression analysis of the experiments at 35°C.

Water matrix	Time (h)	Number of paired samples	Maximum value	Slope (raw)	Intercept (raw)	Slope (log)	Intercept (log)	Spearman's r
Lab water	20	39	>>	0.26	6.08	0.88	-0.25	0.944
	48	39	>>	0.28	7.84	0.86	-0.16	0.945
N1	20	15	159	0.40	1.79	0.89	-0.19	0.932
	48	15	>>	1.24	-1.30	0.98	-0.12	0.891
N2	20	15	123	0.66	-1.2	0.88	-0.14	0.937
	48	15	>>	0.73	0.24	0.93	-0.08	0.909
N3	20	15	310	0.87	16.6	1.02	0.02	0.820
	48	15	310	0.87	16.7	1.01	0.05	0.820
N4	20	15	>>	0.59	0.28	0.84	-0.06	0.915
	48	15	>>	0.79	0.76	0.94	0.04	0.990
N5	20	15	143	0.47	0.78	0.86	-0.12	0.890
	48	15	>>	0.74	-0.03	0.86	-0.03	0.934

The trial method was also assessed using the semi-quantitative risk classes defined in Table 5. An analysis was considered to correctly match the risk class if stock 1 yielded a result above 100 CFU/100 mL, if stock 2 yielded a result of at least 11 and no more than 100 CFU/100 mL, if stock 3 yielded a result of at least 1 and no more than 10 CFU/100 mL, and if stock 4 had either no detectable *E. coli* or a maximum of 1 CFU/100 mL. Detailed tables for each natural water matrix are shown in Table 19 -Table 22.

Table 19: Results matching expected risk class after 20 hours at 25°C. (% results in risk class)

		Water Matrix					
Test Water	Risk Class	N1	N2	N3	N4	N5	Average
S1	>100 CFU/100 mL (very high risk)	0%	0%	0%	0%	0%	**0%**
S2	11-100 CFU/100 mL (high risk)	0%	0%	0%	0%	0%	**0%**
S3	1-10 CFU/100 mL (medium risk)	0%	0%	0%	0%	0%	**0%**
S4	<=1 CFU/100 mL* (low risk)	100%	100%	100%	100%	100%	**100%**
Average	n/a	**25%**	**25%**	**25%**	**25%**	**25%**	**25%**

Table 20: Results matching expected risk class after 48 hours at 25°C. (% results in risk class)

		Water Matrix					
Test Water	Risk Class	N1	N2	N3	N4	N5	Average
S1	>100 CFU/100 mL (very high risk)	67%	100%	100%	100%	100%	**93%**
S2	11-100 CFU/100 mL (high risk)	100%	100%	100%	100%	100%	**100%**
S3	1-10 CFU/100 mL (medium risk)	67%	100%	100%	100%	67%	**87%**
S4	<=1 CFU/100 mL* (low risk)	100%	100%	100%	100%	100%	**100%**
Average	n/a	**83%**	**100%**	**100%**	**100%**	**92%**	**95%**

Table 21: Results matching expected risk class after 20 hours at 35-37°C. (% results in risk class)

		Water Matrix					
Test Water	Risk Class	N1	N2	N3	N4	N5	Average
S1	>100 CFU/100 mL (very high risk)	100%	67%	100%	100%	100%	**93%**
S2	11-100 CFU/100 mL (high risk)	100%	0%	100%	100%	33%	**67%**
S3	1-10 CFU/100 mL (medium risk)	67%	67%	67%	100%	67%	**73%**
S4	<=1 CFU/100 mL* (low risk)	100%	100%	100%	100%	100%	**100%**
Average	n/a	**92%**	**58%**	**92%**	**100%**	**75%**	**83%**

Table 22: Results matching expected risk class after 48 hours at 35-37°C. (% results in risk class)

		Water Matrix					
Test Water	Risk Class	N1	N2	N3	N4	N5	Average
S1	>100 CFU/100 mL (very high risk)	100%	100%	100%	100%	100%	**100%**
S2	11-100 CFU/100 mL (high risk)	100%	100%	100%	100%	100%	**100%**
S3	1-10 CFU/100 mL (medium risk)	67%	67%	67%	100%	100%	**80%**
S4	<=1 CFU/100 mL* (low risk)	100%	100%	100%	100%	100%	**100%**
Average	n/a	**92%**	**92%**	**92%**	**100%**	**100%**	**95%**

Finally, the utility of the test to produce dichotomous presence/absence results was assessed at different thresholds. (see Table 23 - Table 26).

Table 23: Summary of presence/absence results after 20 hours at 25°C.

	Presence/absence cut-off		
Water matrix	1 CFU/100 mL	10 CFU/100 mL	100 CFU/100 mL
N1	50%	50%	75%
N2	50%	58%	75%
N3	50%	50%	75%
N4	50%	58%	75%
N5	25%	50%	75%
All	**45%**	**53%**	**75%**

Table 24: Summary of presence/absence results after 48 hours at 25°C.

	Presence/absence cut-off		
Water matrix	1 CFU/100 mL	10 CFU/100 mL	100 CFU/100 mL
N1	92%	100%	92%
N2	100%	100%	100%
N3	100%	100%	100%
N4	100%	100%	100%
N5	92%	100%	100%
All	**97%**	**100%**	**98%**

Table 25: Summary of presence/absence results after 20 hours at 35-37°C.

	Presence/absence cut-off		
Water matrix	1 CFU/100 mL	10 CFU/100 mL	100 CFU/100 mL
N1	92%	100%	100%
N2	92%	75%	92%
N3	92%	100%	100%
N4	100%	100%	100%
N5	92%	83%	100%
All	**93%**	**92%**	**98%**

Table 10: Summary of presence/absence results after 48 hours at 35-37°C.

	Presence/absence cut-off		
Water matrix	1 CFU/100 mL	10 CFU/100 mL	100 CFU/100 mL
N1	92%	100%	100%
N2	92%	100%	100%
N3	92%	100%	100%
N4	100%	100%	100%
N5	100%	100%	100%
All	**95%**	**100%**	**100%**

5.3 Qualitative results

Lastly, a qualitative assessment of the AQUAGENX GEL test kits was made with reference to categories ranging from the ease of use to the safety of the user and environment. Summary of these results can be found below in Table 28.

Table 28: Scoring of the user friendliness of the Aquagenx Gel test kits.

Subjects		Assessment	Explanation
User manual		Clear	
Execution test		Easy	For use in a laboratory, with many samples, it is labour intensive
Interpretation results		Easy	Explanation of the results is easier because the user can also form an image. If a lot of *E.coli* grows in the medium, you will also have a lot of blue green dots.
Contamination risk to:	Sample User	Medium Medium	Sample is transferred from one plastic bag to another plastic bag. This could potentially cause contamination to the sample and the user. At high concentrations, *E.coli* can form gas. Due to this gas formation, the gel bag can open and the gel can run out.
Dispose of materials with a high concentration of *E. coli*		Is described	Questions: is this disinfection procedure sufficient to kill all the *E.coli* at high concentrations?

6 Appendix

6.1 Risk class matching

The following tables show, for each combination of natural water matrix, incubation temperature and duration, the number of tests with results that fell into the expected risk class. Note that values with no detected *E. coli* in a 100 mL sample, or with a maximum of 1 CFU/100 mL, were considered as matching the low risk class.

Table29: Risk class matching expected risk class, Natural Matrix N1 after 20 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	7	-2	0%	0%
		7	-2	0%	
		2	-2	0%	
S2	11-100 CFU/100 mL (high risk)	<1	-2	0%	0%
		<1	-2	0%	
		<1	-2	0%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	0%
		<1	-1	0%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**1.25**	**25%**	
Presence/Absence (1 CFU cut-off)				50%	
Presence/Absence (10 CFU cut-off)				50%	
Presence/Absence (100 CFU cut-off)				75%	

Table 30: Risk class matching expected risk class, Natural Matrix N1 after 48 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	131	0	100%	67%
		72	-1	0%	
		110	0	100%	
S2	11-100 CFU/100 mL (high risk)	19	0	100%	100%
		19	0	100%	
		25	0	100%	
S3	1-10 CFU/100 mL (medium risk)	1	0	100%	67%
		<1	-1	0%	
		4	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.17**	**83%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				92%	

Table 31: Risk class matching expected risk class, Natural Matrix N1 after 20 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	139	0	100%	100%
		159	0	100%	
		142	0	100%	
S2	11-100 CFU/100 mL (high risk)	15	0	100%	100%
		12	0	100%	
		17	0	100%	
S3	1-10 CFU/100 mL (medium risk)	1	0	100%	67%
		1	0	100%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.08**	**92%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 32: Risk class matching expected risk class, Natural Matrix N1 after 48 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	35	0	100%	100%
		21	0	100%	
		25	0	100%	
S3	1-10 CFU/100 mL (medium risk)	2	0	100%	67%
		2	0	100%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.08**	**92%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 33: Risk class matching expected risk class, Natural Matrix N2 after 20 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	5	-2	0%	0%
		7	-2	0%	
		12	-1	0%	
S2	11-100 CFU/100 mL (high risk)	<1	-2	0%	0%
		<1	-2	0%	
		<1	-2	0%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	0%
		<1	-1	0%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**1.17**	**25%**	
Presence/Absence (1 CFU cut-off)				25%	
Presence/Absence (10 CFU cut-off)				58%	
Presence/Absence (100 CFU cut-off)				75%	

Table 34: Risk class matching expected risk class, Natural Matrix N2 after 48 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	12	0	100%	100%
		16	0	100%	
		12	0	100%	
S3	1-10 CFU/100 mL (medium risk)	2	0	100%	100%
		2	0	100%	
		4	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.00**	**100%**	
Presence/Absence (1 CFU cut-off)				100%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 35: Risk class matching expected risk class, Natural Matrix N2 after 20 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	123	0	100%	67%
		106	0	100%	
		90	-1	0%	
S2	11-100 CFU/100 mL (high risk)	10	-1	0%	0%
		7	-1	0%	
		3	-1	0%	
S3	1-10 CFU/100 mL (medium risk)	3	0	100%	67%
		<1	-1	0%	
		1	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.42**	**58%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				75%	
Presence/Absence (100 CFU cut-off)				92%	

Table 36: Risk class matching expected risk class, Natural Matrix N2 after 48 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	141	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	20	0	100%	100%
		16	0	100%	
		12	0	100%	
S3	1-10 CFU/100 mL (medium risk)	3	0	100%	67%
		<1	-1	0%	
		2	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.08**	**92%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 37: Risk class matching expected risk class, Natural Matrix N3 after 20 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	6	-2	0%	0%
		4	-2	0%	
		10	-2	0%	
S2	11-100 CFU/100 mL (high risk)	<1	-2	0%	0%
		<1	-2	0%	
		<1	-2	0%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	0%
		<1	-1	0%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**1.25**	**25%**	
Presence/Absence (1 CFU cut-off)				50%	
Presence/Absence (10 CFU cut-off)				50%	
Presence/Absence (100 CFU cut-off)				75%	

Table 38: Risk class matching expected risk class, Natural Matrix N3 after 48 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	144	0	100%	100%
		158	0	100%	
		149	0	100%	
S2	11-100 CFU/100 mL (high risk)	34	0	100%	100%
		27	0	100%	
		26	0	100%	
S3	1-10 CFU/100 mL (medium risk)	2	0	100%	100%
		2	0	100%	
		2	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.00**	**100%**	
Presence/Absence (1 CFU cut-off)				100%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 39: Risk class matching expected risk class, Natural Matrix N3 after 20 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	258	0	100%	100%
		310	0	100%	
		297	0	100%	
S2	11-100 CFU/100 mL (high risk)	28	0	100%	100%
		28	0	100%	
		33	0	100%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	67%
		4	0	100%	
		3	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.08**	**92%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 37: Risk class matching expected risk class, Natural Matrix N3 after 48 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	258	0	100%	100%
		310	0	100%	
		297	0	100%	
S2	11-100 CFU/100 mL (high risk)	28	0	100%	100%
		28	0	100%	
		33	0	100%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	67%
		5	0	100%	
		4	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.08**	**92%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 38: Risk class matching expected risk class, Natural Matrix N4 after 20 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	16	-1	0%	0%
		10	-2	0%	
		10	-2	0%	
S2	11-100 CFU/100 mL (high risk)	<1	-2	0%	0%
		<1	-2	0%	
		<1	-2	0%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	0%
		<1	-1	0%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**1.17**	**25%**	
Presence/Absence (1 CFU cut-off)				50%	
Presence/Absence (10 CFU cut-off)				58%	
Presence/Absence (100 CFU cut-off)				75%	

Table 39: Risk class matching expected risk class, Natural Matrix N4 after 48 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	48	0	100%	100%
		44	0	100%	
		50	0	100%	
S3	1-10 CFU/100 mL (medium risk)	5	0	100%	100%
		6	0	100%	
		5	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.00**	**100%**	
Presence/Absence (1 CFU cut-off)				100%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 11: Risk class matching expected risk class, Natural Matrix N4 after 20 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	30	0	100%	100%
		26	0	100%	
		24	0	100%	
S3	1-10 CFU/100 mL (medium risk)	3	0	100%	100%
		1	0	100%	
		5	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.00**	**100%**	
Presence/Absence (1 CFU cut-off)				100%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 12: Risk class matching expected risk class, Natural Matrix N4 after 48 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	38	0	100%	100%
		33	0	100%	
		37	0	100%	
S3	1-10 CFU/100 mL (medium risk)	3	0	100%	100%
		6	0	100%	
		8	0	100%	
S4	<=1 CFU/100 mL (low risk)	1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.00**	**100%**	
Presence/Absence (1 CFU cut-off)				100%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 13: Risk class matching expected risk class, Natural Matrix N5 after 20 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	<1	-3	0%	0%
		<1	-3	0%	
		<1	-3	0%	
S2	11-100 CFU/100 mL (high risk)	<1	-2	0%	0%
		<1	-2	0%	
		<1	-2	0%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	0%
		<1	-1	0%	
		<1	-1	0%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**1.50**	**25%**	
Presence/Absence (1 CFU cut-off)				25%	
Presence/Absence (10 CFU cut-off)				50%	
Presence/Absence (100 CFU cut-off)				75%	

Table 14: Risk class matching expected risk class, Natural Matrix N5 after 48 hours at 25°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	26	0	100%	100%
		29	0	100%	
		14	0	100%	
S3	1-10 CFU/100 mL (medium risk)	3	0	100%	67%
		<1	-1	0%	
		1	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		<1	0	100%	
Average			**0.08**	**92%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

Table 15: Risk class matching expected risk class, Natural Matrix N5 after 20 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	121	0	100%	100%
		143	0	100%	
		131	0	100%	
S2	11-100 CFU/100 mL (high risk)	10	-1	0%	33%
		13	0	100%	
		10	-1	0%	
S3	1-10 CFU/100 mL (medium risk)	<1	-1	0%	67%
		1	0	100%	
		1	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.25**	**75%**	
Presence/Absence (1 CFU cut-off)				92%	
Presence/Absence (10 CFU cut-off)				83%	
Presence/Absence (100 CFU cut-off)				100%	

Table 16: Risk class matching expected risk class, Natural Matrix N5 after 48 hours at 35-37°C.

Test water	Risk class	CFU/100 mL	Risk class difference	Correct risk class	
				Single test	Triplicates
S1	>100 CFU/100 mL (very high risk)	>>	0	100%	100%
		>>	0	100%	
		>>	0	100%	
S2	11-100 CFU/100 mL (high risk)	20	0	100%	100%
		22	0	100%	
		13	0	100%	
S3	1-10 CFU/100 mL (medium risk)	2	0	100%	100%
		2	0	100%	
		1	0	100%	
S4	<=1 CFU/100 mL (low risk)	<1	0	100%	100%
		<1	0	100%	
		1	0	100%	
Average			**0.00**	**100%**	
Presence/Absence (1 CFU cut-off)				100%	
Presence/Absence (10 CFU cut-off)				100%	
Presence/Absence (100 CFU cut-off)				100%	

6.2 Traffic light assessment scheme.

In order to assist with the interpretation of the Phase 2 results, the following 'traffic light' assessment scheme is used, in which results are considered to be 'green' if the results meet the statements listed in the kit's manual, 'yellow' if there is some disparity between results and the expected results, or there is a potential risk of infection to the user, and 'red' if the results deviate significantly from the expected results. The detailed assessment scheme is described below.

Results do not meet the guidelines listed in the kit's manual.

False positives:

Two or more tests are positive

False negatives:

Two or more tests are negative

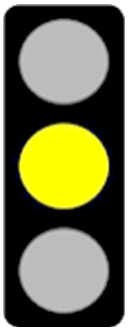
Incubation temperature:

A score is given to each temperature and the score deviates by a factor of more than 2.

Natural waters:

The results match the expected risk class less than 50% of the time in at least one natural water matrices, or less than 80% of the time in at least three natural water matrices

Disparity between results and the kit's guidelines compared to the potential risk to the user.



False positives:

If only one test is positive. Risk of infection to the user is minimal.

False negatives:

One test is negative

Incubation temperature:

A score is given to each temperature. If the score does not deviate by a factor of more than 2, the results stay in the same risk class.

Natural waters:

The results match the expected risk class at least 50% of the time in all five natural water matrices, and at least 80% of the time in at least three natural water matrices.

Results meet the guidelines listed in the kit's manual.

False positives:

None of the tests are positive.

False negative:

All the tests are positive.

Incubation temperature:

Incubation results matches the temperature range in the kit's manual.

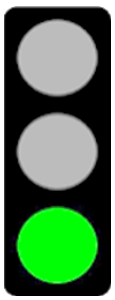
Natural waters:

The results match the expected risk class at least 80% of the time in all five natural water matrices, and at least 90% of the time in at least three natural water matrices.

6.2.1 False positive due to non-target bacteria.

Table 17: Results of the false positives test at 25°C.

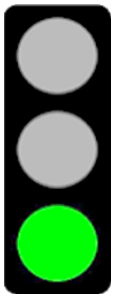
Non-target bacteria ($1*10^8$ CFU/100 mL)	Quantitative test results (CFU/100 mL) 20h	48h
Aeromonas	-	-
Citrobacter	-	-
Enterobacter	-	-
Klebsiella	-	-
Pseudomonas	-	-
E. coli *	2	>>



* *E. coli* has been analysed as a positive control to ensure growth conditions.

Table 18: Results of the false positives test at 35-37°C.

Non-target bacteria ($1*10^8$ CFU/100 mL)	Quantitative test results (CFU/100 mL) 20h	48h
Aeromonas	-	-
Citrobacter	-	-
Enterobacter	-	-
Klebsiella	-	-
Pseudomonas	-	-
E. coli *	>>	>>



* *E. coli* has been analysed as a positive control to ensure growth conditions.

6.2.2 False negatives due to competition

Table 19: Results of the false negatives test at 25°C.

Non-target bacteria (30,000 CFU/100 mL)	Target bacteria (30 CFU/100 mL)	Quantitative test results (CFU/100 mL) 20h	48h
Aeromonas	*E. coli*	0	33
Citrobacter	*E. coli*	0	3
Enterobacter	*E. coli*	0	19
Klebsiella	*E. coli*	0	17
Pseudomonas	*E. coli*	0	26
	E. coli	0	19

Table 20: Results of the false negatives test at 35-37°C.

Non-target bacteria (30,000 CFU/100 mL)	Target bacteria (30 CFU/100 mL)	Quantitative test results (CFU/100 mL) 20h	48h
Aeromonas	*E. coli*	25	38
Citrobacter	*E. coli*	29	>>
Enterobacter	*E. coli*	22	25
Klebsiella	*E. coli*	30	31
Pseudomonas	*E. coli*	27	28
	E. coli	31	33

6.2.3 Natural waters

Table 21: Results matching expected risk class, by water matrix after 20 hours at 25°C.

	Water Matrix				
Test Water	N1	N2	N3	N4	N5
S1	0%	0%	0%	0%	0%
S2	0%	0%	0%	0%	0%
S3	0%	0%	0%	0%	0%
S4	100%	100%	100%	100%	100%
Average	**25%**	**25%**	**25%**	**25%**	**25%**
Grand Average			**25%**		

Table 22: Results matching expected risk class, by water matrix after 48 hours at 25°C.

	Water Matrix				
Test Water	N1	N2	N3	N4	N5
S1	67%	100%	100%	100%	100%
S2	100%	100%	100%	100%	100%
S3	67%	100%	100%	100%	67%
S4	100%	100%	100%	100%	100%
Average	**83%**	**100%**	**100%**	**100%**	**92%**
Grand Average			**95%**		

Table 23: Results matching expected risk class, by water matrix 20 hours at 35-37°C.

	Water Matrix				
Test Water	N1	N2	N3	N4	N5
S1	100%	67%	100%	100%	100%
S2	100%	0%	100%	100%	33%
S3	67%	67%	67%	100%	67%
S4	100%	100%	100%	100%	100%
Average	**92%**	**58%**	**92%**	**100%**	**75%**
Grand Average			**83%**		

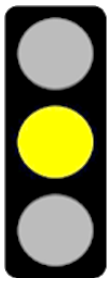
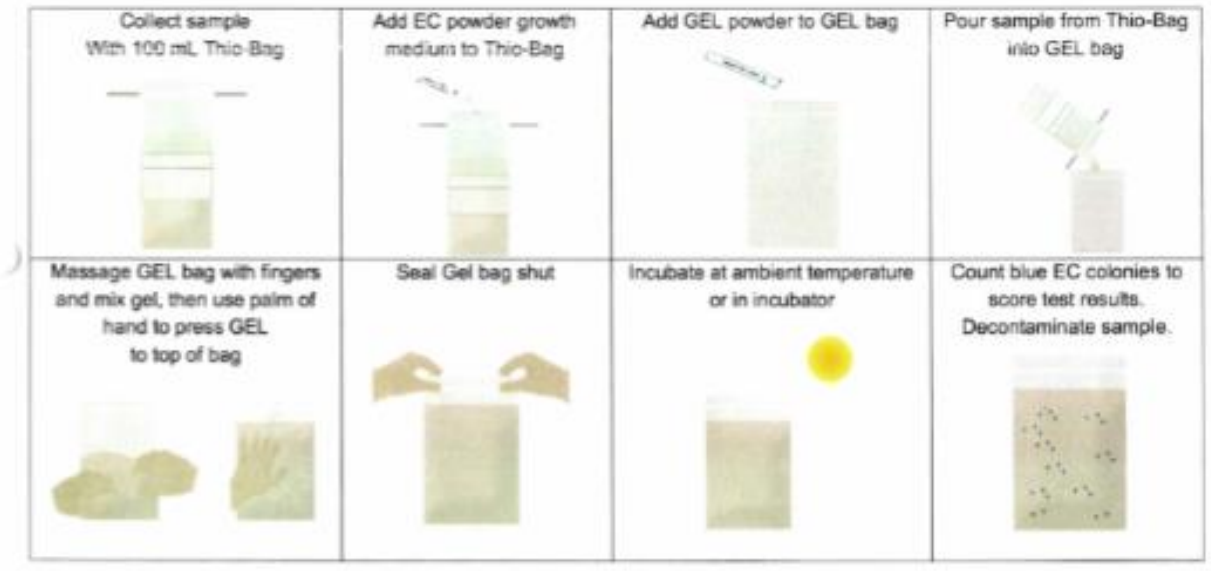


Table 24: Results matching expected risk class, by water matrix 48 hours at 35-37°C.

	Water Matrix				
Test Water	N1	N2	N3	N4	N5
S1	100%	100%	100%	100%	100%
S2	100%	100%	100%	100%	100%
S3	67%	67%	67%	100%	100%
S4	100%	100%	100%	100%	100%
Average	**92%**	**92%**	**92%**	**100%**	**100%**
Grand Average			**95%**		

6.2.4 Manual

Summary of test Procedures for Gel EC Kit



World Health Organization (WHO) Guidelines for Drinking Water Quality, Table 5.4, Fourth Edition, 2017

Procedural Notes

1. Prepare work area

- Sanitize work area with disinfectant cleaning solution, paper towels or wipes.

2. Collect 100 mL water sample with Whirl-Pak™ Thio-Bag™

- White tablet in Whirl-Pak Thio-Bag is sodium thiosulfate, which neutralizes residual chlorine in sample. Do not remove.
- Wearing disposable, thin plastic gloves is recommended. If you don't have gloves, avoid touching inside of Thio-Bag with bare hands.
- Fill Thio-Bag to 100 mL fill mark. Record sample details.

3. Add Aquagenx ECgrowth medium to sample in Whirl-Pak Thio-Bag

- Open medium packet with scissors and pour powder growth medium into Thio-Bag. Growth medium should not be added to sample before you are ready to proceed with the entire testing procedure.
- Do not touch growth medium with bare fingers or hands.
- Roll down Whirl-Pak seal and close Thio-Bag shut.
- Dissolve medium in sample. Gently swirl the bag until the medium is completely dissolved. You can squeeze any clumps of powder to help them dissolve more quickly.

4. Add Aquagenx GEL to larger GEL bag

- Label larger GEL bag or attach barcode asset tag to bag.
- Wearing disposable, thin plastic gloves is recommended. If you don't have gloves, avoid touching inside of GEL bag below the reclosable seal with bare hands.
- Open GEL packet with scissors and pour GEL powder into bag.

5. Pour sample with dissolved EC medium from Thio-Bag into Aquagenx GEL bag

- Pour entire sample from Thio-Bag into GEL bag.
- Use fingers to massage the GEL bag and mix to dissolve the powder. Squeeze and press clumps of powder to dissolve. Examine both sides of the GEL bag and continue dissolving the powder. Squeeze and massage any remaining clumps of powder until dissolved.
- The mixture will become thick and gelatinous after 1-2 minutes.
- After GEL is dissolved and is becoming gelatinous, lie GEL bag on a flat, horizontal surface. Beginning at bottom of bag, gently press bag with your hand to move any air pockets out of the bag. Some small remaining air bubbles are fine.
- Use the palm of your hand to spread the gel evenly in a uniform thickness to nearly the top of the bag and just below the reclosable seal.

Aquagenx, LLC | PO Box 17181, Chapel Hill, North Carolina, 27516 USA | www.aquagenx.com

- Seal GEL bag shut, and once again spread the gel evenly throughout the sealed bag.

6. Incubation Period and Temperatures

- During the incubation period, GEL tests can develop an odor. To control odor, place GEL tests in another sealed plastic bag or container during the incubation period.
- Ambient temperature incubation works at any temperatures between 25°- 44.5°C for detection of *E. coli*.
- Because the GEL test works at variable temperatures, constant temperature control in an incubator is not required. However, at cooler temperatures, constant temperature incubation is recommended, if available.
- For regulatory compliance purposes, samples must be incubated at 35±0.5°C for 20-24 hours to detect and quantify *E. coli*.
- The GEL test also can be used to detect and quantify thermotolerant (or fecal) coliforms if the GEL samples are incubated at a temperature of 44.5°C (between 44-45 °C) throughout an incubation period of 20-24 hours. Strict temperature control is required for this procedure.

Recommended Incubation Periods at Ambient Temperature Conditions:

35-37°C: Incubate 20 hours
31-34°C: Incubate 24-30 hours
25-30°C: Incubate 40-48 hours

Recommended Incubation Period Using an Incubator

35±0.5°C: Incubate 18 hours

7. Score and record CFU test results

- After appropriate incubation period, count the number of colonies in GEL bag:
  - *E. coli* are blue/blue-purple colonies
  - Upper detection limit of *E. coli* is is 200-300 CFU/100 mL

	WHO Health Risk Category Drinking Water
Number of colonies (CFU): *E. coli*	
<1	Low risk: no action required
1 - 10	Intermediate risk: low action priority
11 - 100	High risk: higher action priority
>100	Very high risk: urgent action required

8. Decontaminate sample

- Add 4 mL of liquid bleach (NaOCl) or sufficient chlorine tablets (calcium hypochlorite or sodium dichloroisocyanurate) to GEL bag to provide about 200 milligrams of free chlorine.
- After 30 minutes, pour contents into a sink, toilet or hole in ground and safely dispose the GEL bag.