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1 Introduction

Background

Human disease and mortality derived from drinking water or food occurs more and more frequently on a global basis, and is a major health concern in Europe. Within the EU it is estimated that 7 million people annually are taken ill from contaminated food, and that 1-2 million of these are directly related to subquality water; of that 7000 people die.

The primary tool available to public health workers and industry for microbial monitoring are the use of indicator microorganisms to determine the presence or absence of pathogens (disease-causing microbes or states). However, all of the available standard indicator tests are retrospective, i.e. all requiring 18 to 48 hours to provide a result. Consequently, the water tested has already been supplied to the consumer, or has already been bottled and distributed to stores. All of the standard tests are also done manually, or with some level of semi-automation, and usually require a rather high set of analytical skills and experience. Clearly there are many improvements that are needed in the industry to get microbial monitoring on a par with advances in physical and chemical testing, which in many cases, can be used for on-line monitoring.

Objectives

The main global objective of the project is to demonstrate in the water industries that sensitive and rapid microbial at-line and laboratory systems are comparable to the relevant reference methods. Also that the new approaches enable "Early Warning" of selected indicators, and provide automation, thereby simplifying the methods. The specific scientific and technical objectives are the demonstration of:

- "Early warning" of indicators: the ability of the system to detect within 3 hours maximum, as an example of coliforms, high levels of contamination in the drinking water industry, and 16 hours maximum, as an example of *P. aeruginosa* in the bottled water or swimming pool sectors, in Presence-Absence formats. The following organisms will be evaluated for demonstration: Coliforms, *E.coli*, *P. aeruginosa*, and/or Enterococci.

- "Rapid Detection": the systems should be sensitive enough to detect the lowest acceptable level of indicators for each given method in 50% of the time needed for the respective reference method, and with a specificity of no less than 90%.

- The ability of the at-line instrument to operate in industrial conditions, especially regarding robustness and integration of the device with the processes being monitored.

- Pre-normative comparability of the instruments and methods with European, International and national standards (CEN, ISO, etc.) for corresponding reference methods.

The project duration was 2 years, beginning 1 December 2001. The following partners and persons were involved in the project:

Ecole Nationale de Nationale de La Santé Publique (ENSP), Rennes France,
Person in charge: Prof. Dr. Jean Lesne

Instituto Superiore di Sanitá (ISS), Roma Italy,
Person in charge: Dr. Lucia Bonadonna

Thames Water Utilities Ltd. (TW), Reading UK,
Persons in charge: Dr. David Holt/ Dr. Annette Prescott.

2 Material and methods

2.1 Introduction

Experimental work was performed at ENSP, ISS, and TW. An overview of applications tested and methods used at the sites are given in Table 1.

Table 1. Overview of applications and methods

Partner	**Instrument** [a]	**Target organism**	**Method *E.coli***	**Method *P. aeruginosa***
ENSP, France	CA-lab	*E.coli* *P. aeruginosa* [c]	ISO 9308-1+ Confirmation + API 20 NE[c]	EN ISO 12780 (only presumptive)
ISS, Italy	CA–lab	*E. coli* *P. aeruginosa*	ISO 9308-1 + Confirmation + API 20 NE[c]	EN ISO 12780 + Confirmation + API 20 NE[c]
TW, UK	CA–lab	Coliforms *E.coli*	Colilert + API 20 NE[c]	Not analysed
	CALM–At-line	Coliforms *E.coli*	Colilert + API 20 NE[c]	Not analysed

[a] Colifast instrument & siting; CA = Colifast Analyser, lab; CALM = Colifast At Line Monitor, in-plant and at-line
[b] BioMerieux
[c] Scarcity of results

2.2 Microbial methods

A brief description of the microbial methods used in this project is given below. The ISO and EN methods used for comparison against the Colifast technology are given below. These methods are hereafter referred to as the reference methods. In Appendix 1 more detailed information regarding the performance and setting of the instruments are given.

2.2.1 Colifast technology

Colifast technology combines selective growth media with automated analysers.

Colifast Media

The Colifast technology is based on an enzymatic reaction between bacteria possessing certain enzymes and complementary substrates amended in a selective media.
Among the constituents in the Colifast growth media are fluorogenic substrates, specifically paired with target organisms or groups. During growth of bacteria enzymes hydrolyse the

fluorogenic substrate, with subsequence increase in fluorescence. The formation of the fluorescent products 4-methylumbelliferone (MU) and 7-amino-4-methylcoumarin (AMC), representing the presence of *E.coli*/coliforms and *Pseudomonas aeruginosa* respectively, are monitored and detected by a fluorometer. In this project *E.coli*, the coliform group (faecal/thermotolerant), and *Ps. aeruginosa* were the target organisms, and detected indirectly by the activity of the enzymes β-D-glucuronidase, β-D-galactosidase, and amino-peptidase respectively.

Colifast analysers

In this project, the Colifast Analyser (CA) and Colifast At Line Monitor (CALM) were demonstrated in laboratory and in industrial conditions. A brief description of the analysers is given in Table 2.

Table 2. Description of the Colifast instrumentation.

Information	**CALM**	**CA**
Location	In-plant/remote	Laboratory
Sampling from water source	Fully automated	Manual (liquid sample or membrane filter)
Subsampling	Fully automated	Fully automated
Quantification software options	Fully automated	Fully automated
Number of samples/run	1-76	1-76
	MPN, P/A, ESQ	MPN, P/A, ESQ
Remote warning	Yes	Yes
Number of different tests /run	1-3	1-3
Data handling	Fully automated	Semi automated
Time to results (hours)	4*-12 * = ESQ	2*-12 * =MUP
Skill requirement	Moderate	Moderate

* Internal Colifast methods, not applied in this project.

In both instruments, enzymatic activity is monitored by measuring the increase in fluorescence due to the formation of MU or AMC. After subtracting background fluorescence and use of a calibrant with known MU or AMC concentration, results are obtained as ppb MU or ppb AMC. A fluorescence threshold value set above the background noise of the instruments and above possible low non target activity present in the water sample is applied to determine the level of CFU in the sample. The time required to reach this threshold value is used as a predictive for estimation of the level of the number of CFU or the present/absence of target organisms. It is well known from the literature that there is a high correlation coefficient between the activity of the β-D-glucuronidase, β-D-galactosidase and plate counts of *E. coli* and coliforms, respectively (Fiksdal et al., 1994; Davies and Apte, 1996; George et al., 2000).

TTD (time-to detect)

Time-to-detect (TTD) is defined as the time required to reach a certain fluorescence threshold. This threshold is determined by the slope of the linear increase during growth. The time required to reach this threshold value is determined by the number of target organisms present at start.

2.2.2 The ISO 9308-1 method (Detection and enumeration of *E.coli* and coliform bacteria. Part 1: membrane filtration method)

The method ISO 9308-1 was used to detect *E.coli* in the different water samples. In this method, 100 ml sample is first membrane filtered (0.45 µm). The membranes filters are then placed on a Lactose-TTC agar, and incubated at 36±2°C for 21±2 hours. Colonies that show a yellow colour, due to lactose fermentation are counted as presumptive. For oxidase and indole tests, the yellow colonies are subcultured (36±2°C for 21±2 hours) on nonselective agar, and in tryptophan broth. Colonies, which are capable to produce indole from tryptophan and give negative results on the oxidase test, are counted as *E.coli*. Colonies giving a negative oxidase reaction are counted as coliforms.

2.2.3 The EN 12780 method (Detection and enumeration of *Ps. aeruginosa* by membrane filtration)

The method EN 12780 was used to detect *P. aeruginosa* in the different water samples. In this method, 100-250 ml sample is first membrane filtered (0.45 µm). The membranes filters are then placed on a selective medium (Ps. agar Base/CN Agar), and incubated at 36 ±2°C for 44±4 hours. Table 1 summarises the selection of colonies and confirmation steps required for confirmation of *P. aeruginosa*. Due to the confirmation of fluorescence on King's B medium, 5 days subcultivation may be required.

Table 3. Steps required for the confirmation of colonies growing on CN-agar.

Description of colony on CN- agar	**Ammonia from acetamide**	**Production of oxidase**	**Fluorescence on King's B**	**Confirmed as *P. aeruginosa***
Blue/green	NT	NT	NT	Yes
Fluorescent	+	NT	NT	Yes
Reddish brown	+	+	+	Yes
Other types	NT	NT	NT	Yes

2.2.4 The Colilert® method

In this project, the Colilert-18® method was used to detect coliforms and *E.coli*. The Colilert-18 method is a defined substrate most probable number technique for detecting *E.coli* and coliforms. The Colilert substrate medium is dissolved in 100 ml of sample, which is added to a tray containing 51 wells. The tray is sealed and incubated at 18-22 hours at 37°C. A most probable number is obtained from the number of wells in the pouch that exhibit growth. If coliforms are present, an enzymatic cleavage of ortho-nitrophenyl-β-D galactopyranoside (ONPG) turns the medium yellow. The presence of *E.coli* is observed as fluorescence, due to cleavage of the fluorogenic substrate 4-methylumbelliferyl- β-D-glucuronide (MUGlu).

2.2.5 BioMerieux

The BioMerieux system API 20E where used for species/subspecies identification of *Enterobacteriaceae* and *Ps. aeruginosa*. The product comprises strips that contain 20 miniature biochemical tests. For more information, see www.biomerieux.com.

2.2.6 Specificity and sensitivity

Sensitivity and specificity were calculated.

Sensitivity is defined as the fraction of the total number of positive cultures correctly assigned in the presumptive inspection:

SE = TP/(TP+FN)
Specificity is defined as that fraction of the total number of negative cultures correctively assigned in the presumptive inspection:

SP = TN/(TN+FP)

Where, TP = total positive, FN = false negative, TN = total negative, and FP = false positive.

2.2.7 Statistics

The ISO standard 17994 was applied for determination of the equivalence between the standard reference methods and the Colifast method. In the statistical test, the following formula was used:

$$\chi 2 = \frac{(Na - Nb)^2}{(Na + Nb)}$$

where,
Na = the number of samples where method A is positive and method B negative
Nb = the number of samples where method A is negative and method B positive

and

i) When $\chi 2 < 4$ the methods are not different
ii) When $\chi 2 \geq 4$ the methods are different

3 Results

Results from the project are given below. These results are based on conclusions drawn from the the two project annual Progress Reports, and the final reports from ISS, ENSP, TW, and the independent evaluators. The work performed at Colifast consisted of three parts: i) the design and production of the instruments, ii) the production of the diagnostic media, and iii) the production of software and documentation. The work performed at ISS and ENSP can be divided into three parts: i) determination of sensitivity and specificity of the Colifast methods and the reference methods, ii) determination of the equivalence between the Colifast methods and the reference methods, and iii) the time to detect results based on the concentration of target organisms present. The work performed at TW can be characterised as performance of the Colifast technology under industrial scale-operations.

Since the water sources, confirmation procedures, performance, and presentation of the results at ENSP, ISS, and TW are differentiated, the results are presented from each participant.

3.1 Results from Colifast

Instrumentation

Two types of instruments were developed, prototyped, and delivered to the project partners, which are described below. Six instruments were produced: 4 Colifast Analysers (CA) and 2 Colifast At-line Monitors (CALM); of these 3 CA's were delivered and one retained for developmental work, and 1 CALM was delivered with 1 retained for developmental work. The operators at each site were trained and the instruments were maintained for the duration of the project. Further information on the products developed in this project is available at Colifast homepage, http://www.colifast.no.

Colifast Analyser (CA)

The CA is a semi-automated analyser. Sample and Colifast media have to be manually filled into vials and placed on the incubator block. Sub-sampling is performed automatically at programmed intervals. 76 different samples can be loaded into the incubator block. The CA applications span from raw water, in- process water, to waste water, and pasteurised milk. Results have to be imported into a chart such as Excel® before the number of CFU/100 ml is estimated.

Colifast At-line Monitor (CALM)

The CALM is a fully-automated at line monitor. By connecting the CALM directly to the water source, the instrument performs sampling and sub-sampling at programmed intervals. 76 samples from the water source can be analysed before the incubator blocks have to be refilled with vials containing the Colifast media. The CALM is equipped with a system for remote warning. The CALM applications span from raw water, in- process water, to waste water. Results are obtained directly in number of CFU/100ml, based on MPN and/or ESQ analysis format.

Software and Documentation

Software for both instruments was delivered at the outset of the project and revised during the project. Documentation included Users Manuals, Application Manuals, and Quick Guides for both instruments. Other documents included MSDS for the diagnostic media and quality control procedures and certificates for the same.

Diagnostic Media and Reagents

Media and reagents were produced and quality controlled for the duration of the project. Adequate supplies of all consumables for the duration of the study were supplied. These included consumables like batches of each medium, media developer, carrier solution, and acid. In addition, technical supplies like UV-lamps, coils, nuts, and bolts, tubes, luers, and needles were also provided.

3.2 *E.coli*/CA results from ISS

Specificity and sensitivity of CA

Isolates from CA were submitted to confirmation tests (oxidase and indole). Based on these results the specificity and sensitivity of the CA for the detection of E. coli were calculated. These results are shown in Table 4.

Table 4. Values used for calculation of specificity and sensitivity of the CA/E. coli. (n = 522)

	Isolates from vials (presume. and after biochem confirmation of vials)		
CA method	No. pres. Vials	No. of confirmed vials (%)	No. of not confirmed vials (%)
Positive (+)	245	237 (97)	8 (3.2)
Negative (-)	277	272 (98)	5 (1.8)
TOTAL	522	509 (98)	13 (2.4)
Total positive	*242*		
Total negative	*280*		

Based on the numbers in Table 4, the calculated sensitivity and specificity of CA/*E. coli* is 98% and 97%, respectively.

TTD

TTD was calculated on the basis of the different concentration range of target organisms. In Table 5 the TTD for different concentration ranges for confirmed *E.coli* is reported.

Table 5. Average TTD for different concentration ranges of *E.coli* in water sample (CA/*E.coli*).

Concentration range (*E.coli* CFU/100 ml)	**TTD** (h)
0-10	14
11-30	12
31-50	10
51-150	8

Equivalence between CA and ISO 9308-1

Statistical calculation for determination of the equivalence between the CA/ISO 9308-1 is shown in Table 6.

Table 6. Results from single samples of natural waters for *E.coli*. Data were obtained on discordant and concordant pairs between the methods. The values of the χ^2 are based on the Poisson index of dispersion are also reported.

Parameter	**Period**	**No of samples**	**CA+/ ISO+**	**CA-/ ISO-**	**CA-/ ISO+**	**CA+/ ISO-**	**Sum diverg.**	χ^2
E.coli	Trial 1 & 2	143	79	55	3	6	9	1.0
E.coli	Trial 3	65	17	23	16	9	25	2.0

3.3 Ps. *aeruginosa*/CA results from ISS

Specificity and sensitivity of CA

Isolates from CA were submitted to confirmation tests according to the ISO EN 12780. Based on these results the specificity and sensitivity of the CA for the detection of Ps. *aeruginosa* were calculated. These results are shown in Table 7.

Table 7.Values used for calculation of specificity and sensitivity of the CA/Ps. a*eruginosa* on the total number of confirmed isolates (n = 441).

	Isolates from vials (presume. and after biochem confirmation of vials)		
CA method	No. pres. Vials	No. of confirmed vials (%)	No. of not confirmed vials (%)
Positive (#)*	172	122 (71)	50 (29)
Positive (+)	98	5 (5)	93 (95)
Negative (-)	171	170 (99)	1 (0.5)
TOTAL	441	297 (67)	144 (33)
Total positive	*128*		
Total negative	*313*		

*(#) a sample is considered as positive by the CA when a value of 1000 ppb AMC has been reached, and at this level the software has been programmed to stop the analysis. Values < 50 ppb AMC are considered as negative (-) by the instrument, samples > 50 ppb, but < 1000 ppb are reported as (+)

Based on these numbers the calculated sensitivity and specificity of CA/*Ps. aeruginosa* is 99% and 98%, respectively.

TTD

TTD was calculate on the basis of the different concentration range of target organisms. In Table 8 the TTD for different concentration ranges for confirmed CA/Ps. a*eruginosa* is reported.

Table 8.Average TTD for different concentration ranges of Ps. a*eruginosa* in water sample (CA/Ps. a*eruginosa*).

Concentration range (Ps. a*eruginosa* CFU/100 ml)	**TTD** (h:min)
0-10	16:48
11-30	14:42
31-50	14:00

Equivalence between CA and ISO EN 12780

Statistical calculation for determination of the equivalence between the CA and ISO EN 12780 is shown in Table 9.

Table 9. Results from single samples of natural waters for Ps. a*eruginosa*. Data were obtained on discordant and concordant pairs between the methods. The values of the χ^2 are based on the Poisson index of dispersion are also reported.

Parameter	**Period**	**No of samples**	**CA+/ ISO+**	**CA-/ ISO-**	**CA-/ ISO+**	**CA+/ ISO-**	**Sum diverg.**	χ^2
Ps. a*eruginosa*	Trial 1	103	22	71	4	6	10	0.4
Ps. a*eruginosa*	Trial 2	39	16	15	3	5	8	0.5

Ps. a*eruginosa*	Trial 3	75	20	46	5	4	9	0.1

3.4 *E.coli*/CA results from ENSP

Specificity and sensitivity of CA

Isolates from CA were submitted to confirmation tests (oxidase and indole). Based on these results the specificity and sensitivity of the CA for the detection of E. coli were calculated. (Table 10).

Table 10. Values used for calculation of specificity and sensitivity of the CA/E. coli. (n = 245).

Confirmed / Presumptive	**CA (+)**	**CA (-)**	**Total**
CA (+)	62	14	76
CA (-)	2	167	169
Total	64	181	245

Based on the numbers in Table 10, the calculated sensitivity and specificity of CA/E. coli is 97% and 93%, respectively.

TTD

TTD was calculated based on the different concentration range of target organisms. In Table 11 the TTD for different concentration ranges for positive vials confirmed *E.coli* is reported. Threshold value is 100 rfu (relative fluorescence units).

Table 11. Mean TTD for different concentration ranges of *E.coli* in water sample (CA/*E.coli*).

TTD(>100 rfu) / CFU/5-10ml	**4h**	**8h**	**10 h**	**12 h**	**14 h**	**16h**	**Mean**
0-2	1	10	30	8	3	3	10.4
3-10	0	12	19	2	2	0	9.7
11-20	0	3	3	0	0	0	9.0
21-100	-	-	-	-	-	-	-
>100	0	2	0	1	0	0	9.3

Equivalence between CA and ISO 9308-1

Statistical calculation for determination of the equivalence between the CA/ISO 9308-1 is shown in Table 12.

Table 12. Distribution of the four types of pairs of confirmed results.

Parameter	**No of samples**	**CA-/ ISO-**	**CA+/ ISO+**	**CA+/ ISO-**	**CA-/ ISO+**	**Sum divergt**	χ^2
E.coli	228	141	32	30	25	55	0.45

3.5 *Ps. aeruginosa*/CA results from ENSP

It was decided at the progress meeting in Roma 2002, that ENSP should concentrate its task force on *E.coli*, because of lack of suitable water samples containing *Ps. aeruginosa*. Consequently, limited work has been performed. Presented results were also only presumptive. Consequently, neither any discussion nor conclusion can be drawn from these few results.

3.6 *E.coli*/coliforms/CA results from TW

The specificity of CA/coliforms was determined by spiking dechlorinated tap water with a number of environmental isolates. The organisms were spiked in higher and lower range and in duplicates. Results are shown in Table 13.

Table 13. Results obtained with the CA/coliforms for a range of isolates. A total of 266 samples were analysed.

Species or genus	**No. of isolates**	**Colifast (37°C)**	**Colifast (44°C)**	**TTD (h) (37°C)**	**TTD (h) (44°C)**	**Colilert MPN**
E.coli	21	Yes	Yes	6-10	6-9	5-225
E.vulneris	1	Yes	Yes	8	7	11-138
Citrobacter	2	Yes	Yes	9-11	13	23-435
Klebsialla	2	Yes	Yes	7-11	13	5-119
Enterobacter	1	Yes	Yes	9	13	10-166
Aeromonas	1	-	-	-	-	
Pseudomonas	1	-	-	-	-	

The CA/coliforms and Colilert were demonstrated on inlet (ozonated), supernatant, and outlet water from the slow sand filter. The CA/coliforms utilized 10 ml of water sample, while the Colilert 100 ml. Results are presented in Table 14.

Table 14. No of positive samples in the ozone treatet inlet, supernatant, and outlet water from the slow sand filter.

	Method	**No. samples**	**No. + samples Range 0-10**	**No. + samples Range 10-100**	**No. + samples Range >100**
Inlet (ozone)	Colilert coliforms	36	17	19	0
	Colifast 37°C	36	0	2	0
	Colilert E.coli	36	9	0	0
	Colifast 44°C	36	0	0	0
Supernatant	Colilert coliforms	36	0	0	36
	Colifast 37°C	36	0	0	32
	Colilert E.coli	36	9	15	12
	Colifast 44°C	36	5	9	12
Outlet	Colilert coliforms	32	0	16	16
	Colifast 37C	32	0	9	16
	Colilert E.coli	32	16	3	13
	Colifast 44C	32	7	3	13

In an attempt to increase the sensitivity of the CA/coliforms, a membrane filtration step was added. 100 ml of sample was filtered through a 0.45 µm cellulose nitrate filter, and inserted into the vial containing the Colifast medium. In Figure 1 samples containing a range of coliforms numbers and TTD is shown.

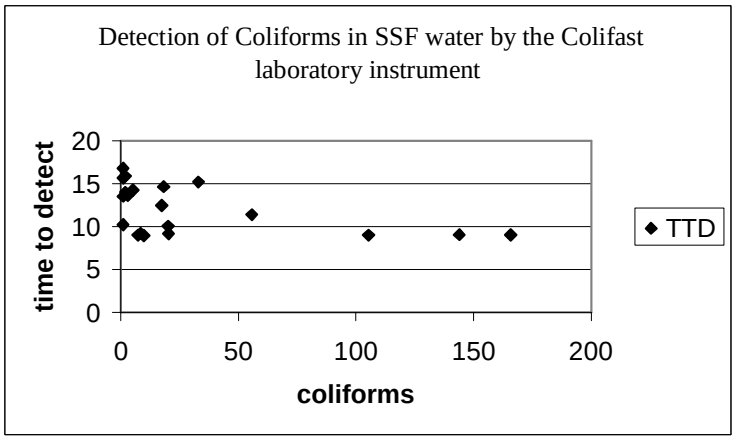

Figure 1. The relation between levels of coliforms/100 ml and TTD.

As shown in Figure 1, TTD increased in samples containing low numbers of coliforms (1-5/100 ml). The time required to detect these organisms ranged from 8-16 hours, which was longer than the previously 8-11 hours.

In order to increase the performance and sensitivity of CA/coliforms, 100 ml samples were split into 25 x 4 ml, and compared to Colilert. Out of the 121 samples analysed , 93.6% agreed on presence/absence basis. 6.4% of the CA/coliforms samples produced a positive reaction in at least one of four vials when the Colilert MPN results were < 1. Figure 2 shows the TTD for samples containing more that 200cfu/100 ml with 100% of samples were detected within 10 hours.

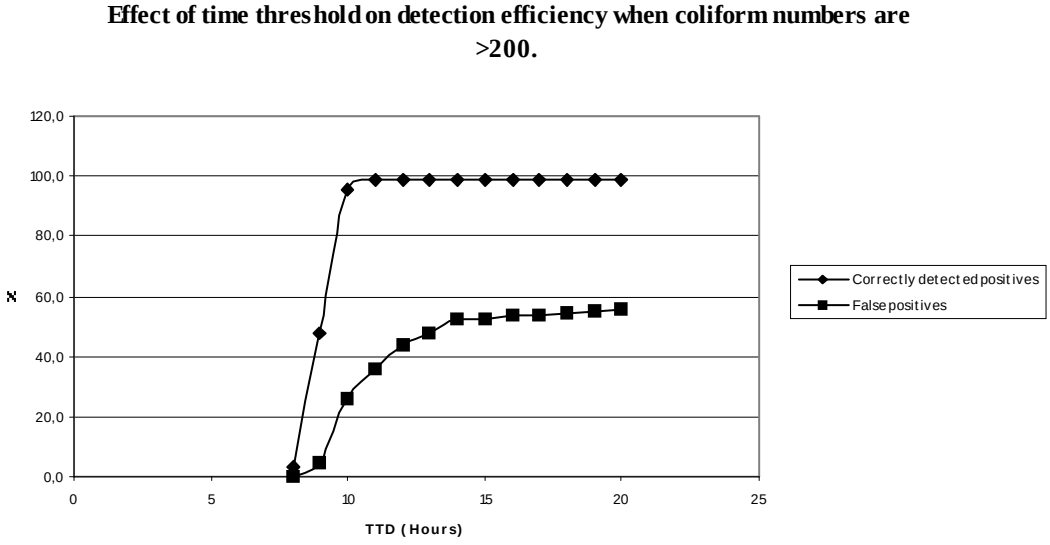

Figure 2. Effect oftime threshold on detection efficiency when coliforms numbers are > 200.

These studies indicate that the CA/coliforms is more sensitive than looking at 10 ml samples. This was further confirmed as shown in Figure 3.

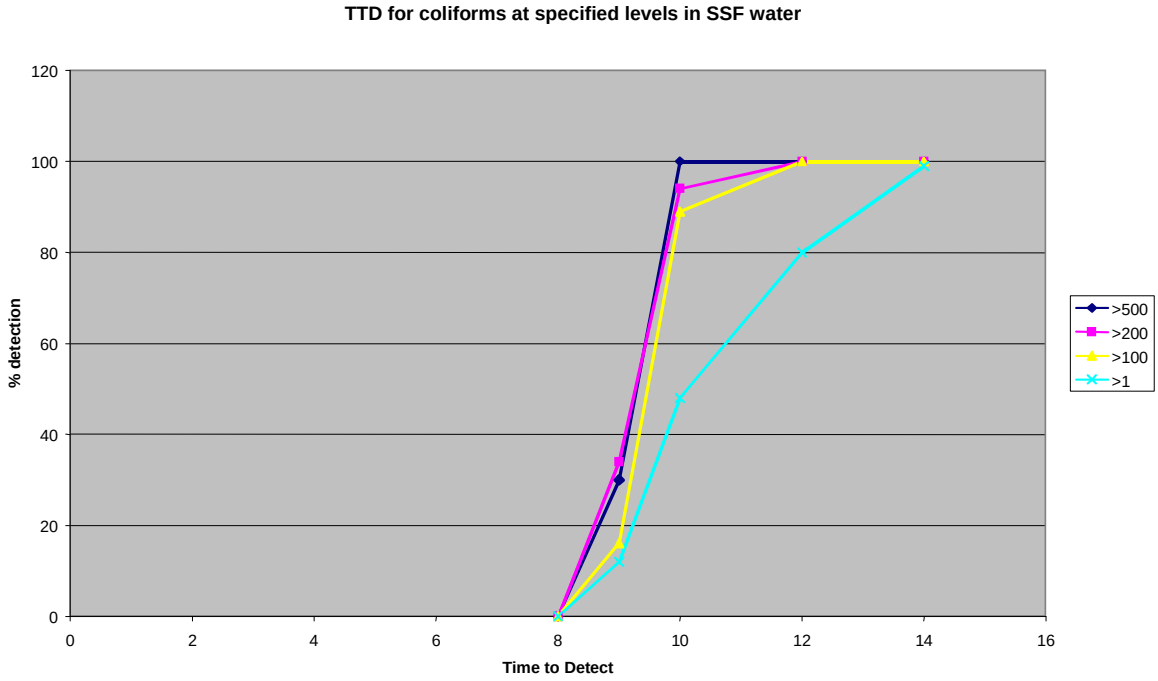

Figure 3. The time to detect different concentration of coliforms in slow sand filter.

As shown here, the CA/coliforms were able to detect 1 coliform in 100 ml sample within 13-14 hours.

3.7 *E.coli*/coliforms/CALM results from TW

The results from the CA experiments at TW above were incorporatede into the use of the CALM. The CALM/coliforms were used to monitor the performance of a slow sand filter bed. Samples were taken twice daily in duplicate and at the same time for the anlysis by Colilert to obtain most probable number (Figure 4).

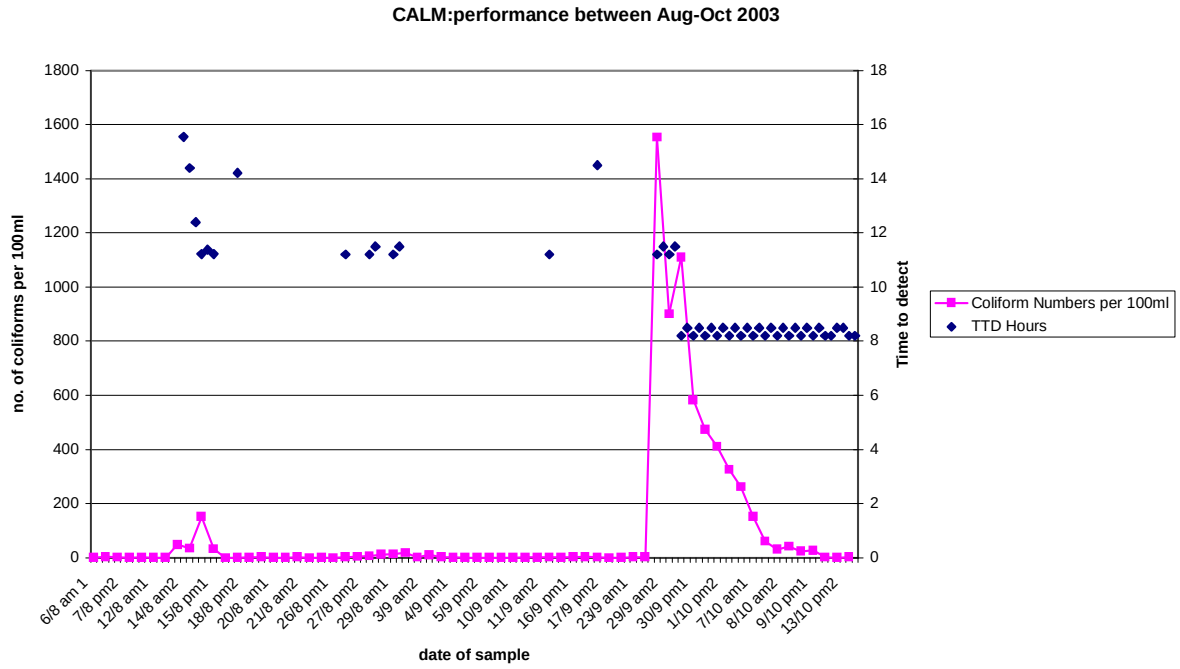

Figure 4. The performance of CALM at slow sand filter bed outlet. The continuous line is the coliforms number per 100 ml as determined by Colilert. The diamonds represent the time to detect. If a diamond is absent the time to detect is > 18 hours or the event has remained undetected. 10 ml sample was anlysed with the CALM/coliforms.

As shown in Figure 4, two peaks in coliforms number were observed, each representing times when the filter bed was out of operation immediately after cleaning. The first increase in mid August, although below the 200 coliform treshold was detecetd by the CALM/coliforms with a TTD between 11-12 hours. The large increase in coliforms number at the beginning of October was also detected within the same time treshold. From the small increase in mid August, a treshold dtection limit of the instrument is at 30 coliforms with a TTD of 13 hours.

4 Discussion & Conclusions

4.1 General conclusion from independent evaluators

E.coli

The ISO 9308-1 method relies on the ability of E.coli to ferment lactose and produce indole from tryptophan. The Colifast technology is based on another principle, which is the detection of the β-D-glucuronidase activity of E.coli strains. This divergence of principle will produce divergence in results for strains of E.coli which are lactose negative and β-D-glucuronidase positive and strains of E.coli which are lactose/indole positive and β-D-glucuronidase negative. Fortunately, most strains of E.coli are both lactose/indole and β-D-glucuronidase positive.

The objective of the work performed at ISS and ENSP was to compare a liquid CA/E.coli presence/absence (P/A) method to the ISO 9308-1 method, which include membrane filtration on TTC. This method is a quantitative method, giving number of CFU/100 ml. In order to perform the comparison, the ISO method was transformed to a P/A format, which represents a"down-grading" adaptation of the ISO method. In addition, the fields of appliaction of the ISO 9308-1 is water intended for human consumption, including treated drinking waters and

other types of potable waters with low levels of bacteria. Since the TCC is a growth media with low selectivity, background contamination will also be detected. Waters like rivers and swallow wells are not a field of application for ISO 9308-1. This also means that collecting samples for ISO 9308-1 is a difficult task, because natural waters contaminated with E.coli are scarce. In order to overcome this problem, it is allowed to use prepared samples by dilution, spiking, and mixing of different kinds of waters.
Application of ISO17994 (for *E.coli*) requires a high amount of samples with divergent results : for example, for two P/A methods, the required number of samples for detecting an average relative recovery greater then 20% is 380 samples, 1540 samples for detecting a relative recovery greater then 10%. That means that the smaller the relative difference between the 2 methods, the higher the number of samples needed to detect this difference. Among ISS natural samples (analysed in quintuplets), 78 samples are negative and 96 samples are both positive by CA and REF method. Only 34 samples give divergent results (CA+ and REF- or CA- and REF+). Among ENSP samples, 141 samples are negative and 32 samples are both positive by CA and REF method. Only few samples (approximately 35 samples – data not available in the ENSP report) give divergent results (CA+ and REF- or CA- and REF+). According to ISO 17994 at least 5 times more divergent samples would be needed for for comparing equivalence of two P/A methods.
In the study, CA method has a low rate of false positive and low rate of false negative. CA method can also give results within a reasonable time (within 10h) (ideal would be real time) and can be used *in situ*. These 3 aspects are obviously an advantage for process monitoring of water drinking treatment plants, for which the method could be recommended.

Ps. aeruginosa

The principle of CA method is based on the detection of amino-peptidase activity, which is considered as specific for *Ps. aeruginosa*. It is a P/A method. The principle of the ISO EN 12780 method is based on the ability of *Ps. aeruginosa* to grow on a specific culture medium followed by confirmation (it is a quantitative method). This divergence of principle will produce divergence in results, which relative proportion is to be assessed.
The objective of ISS and ENSP studies was to compare liquid CA presence/absence (P/A) method in 100ml (Colifast P. aeruginosa) with -an adaptation of- the standard filtration quantitative ISO EN 12780 method in 100ml (filtration on selective agar). In order to make comparisons possible, quantitative results cfu in 100ml from ISO EN 12780 needed to be transformed in P/A results in 100ml, which represent a “down-grading” adaptation of this method in terms of expression of results and do not represent standard counting results from this method.

The field of application of the ISO EN 12780 standard includes filterable waters and in particular bottled and swimming pools waters. It is a quantitative selective method, which is suitable for water samples collected in the ISS and ENSP studies. Bottled waters (majority of ISS samples) and swimming pools samples (majority of ENSP) used in this comparison study (reference vs alternative) are representative from types of waters covered by the reference method.

Among ISS samples, most samples gave negative results (absence of contamination). 58 samples were positive both CA and REF methods. Only few samples were divergent (n=27).
Among ENSP 38 samples, only 2 samples were positive (with discordant pairs of results); 36 samples were unfortunately negative by both methods, which is not appropriate for

comparison. In total (ISS + ENSP), a total of 29 divergent results on 217 samples were observed. ISO17994 requires a much higher amount of samples : for example, for two P/A methods, the required number of samples for detecting an average relative recovery greater then 30% is 170 samples, 1540 samples for detecting a relative recovery greater then 10%. That means that the smaller the relative difference between the 2 methods, the higher the number of samples needed to detect this difference. According to ISO 17994, many more samples are needed (at least 20 times more) for comparing equivalence of these two P/A methods. According to data results achieved during the DEMOWATER project (ISS and ENSP), it can not be demonstrated that *Ps. Aeruginosa* ISO 17994 and CA methods are equivalent. Further investigation is needed.

4.2 Conclusion ISS

At the present, with the use of conventional cultural methods, the assessment of the hygienic quality of drinking water is only available after a minimum of 24 hours. Much time is necessary for the final results when confirmation of colonies has to be done. In that respect the results obtained by the CA Technology are satisfactory. The speed of detection depends upon the level of contamination within the sample. In water samples with low contamination as those analyzed for water for human consumption, detection times ranged from 8 to 14 hours for *E. coli* and from 10 to 16 hours for samples containing 1-10 CFU/100 ml *Pseudomonas aeruginosa.*

High sensitivity and specificity for the target microrganisms were observed. Based on the numbers from table 4, the calculated sensitivity and specificity of CA/*E. coli* was 98% and 97%, respectively. Calculated sensitivity and specificity of CA/Ps. *aeruginosa* was 99% and 77%, respectively (Table 7).

Although a strict adherence to the ISO 17994 principles was not applied because of the scarcity of number of divergent samples, it is possible to hypothesize a good equivalence of the CA methods respect to the standard methods. In table 14 final results of the equivalence tests in the ISS comparative trials are reported.

Table 14. Results and conclusions from statistical tests of the comparisons of methods

Comparison	**Evaluation of the difference (χ^2) between methods**
CA/ISO 9308-1 (*E. coli*)	No difference (< 4)
CA/ISO EN 12780 (*Ps. aeruginosa*)	No difference (< 4)

4.3 Conclusion ENSP

At the present, with the use of conventional cultural methods, the assessment of the hygienic quality of drinking water is only available after a minimum of 24 hours. Much time is necessary for the final results when confirmation of colonies has to be done. Real-time analysis would be ideal for the management and control of microbial water quality and the safeguard of public health. In that respect the results obtained by the CA Technology are satisfactory. Colifast Technology can be an efficient and valid alternative to the cultural

methods for *Escherichia coli* detection, especially when high numbers of samples per day have to be analyzed. In water samples with low contamination as those analyzed for drinking water, detection times ranged from 9 to 10 hours for *E. coli* (1-20 CFU/ tested volume), and the low cost per sample and low complexity of the procedure makes the cultural techniques universally applicable to laboratories.

Higher selectivity for the target microorganism was observed with the CA method. Furthermore overgrowth of background organisms on agar media was a frequent occurrence resulting in difficulty in reading the membranes and in the selection of the colonies to be confirmed, whereas CA method was able to give a result. False positive results were obtained in both the types of methods, even if higher numbers of false positive were observed with the ISO method. On the other side, some rare false negative colonies were isolated and identified from the CA method (*E. coli* β-D-glucuronidase negative) and from the ISO method (*E. coli* indol negative). It is known that no single method is able to recover all the biotypes of a particular organism or group of organisms. But the problem is accentuated with the membrane filtration method currently in use because identification relies only on the ability of *E. coli* to ferment lactose and produce indol from tryptophan. Generally, the occurrence of strains of *E. coli* which are negative for one or more of these traits is higher than the occurrence of strains which are negative for β-D-glucuronidase.

Although a strict adherence to the ISO 17994 principles was not applied because of the scarcity of samples giving divergent results, it is possible to hypothesize a good equivalence between the CA method and the standard method for *Escherichia coli* detection. In that respect the results are satisfactory. As a preliminary conclusion, it can be stated that, when rigorously confirmed by the identification of the bacteria grown on the two different culture media, the two methods are not significantly different. Further testing is just needed for full compliance to the ISO statistical standard of comparison of bacteriological methods.

4.4 Conclusion TW

The Colifast laboratory instrument can be used to accurately detect coliforms in operational slow sand filtered waters at a level of 200 coliforms/100ml. Although combining membrane filtration with the colifast methodology can increase sensitivity, there is considerable variation in the time to detect low levels in these environmental samples. Use of 4X 25ml samples also increases the sensitivity of the method but the cost of performing these tests are considerably higher. Good correlation between Colifast and Colilert was observed and in some instances when Colifast contained <1 organism a positive result was observed in at least one of four vials used in the Colifast method.

The CALM instrument has provided an opportunity to provide on-line microbial analysis for operational proposes. The CALM machine was able to accurate detect coliforms at a level of 200 coliforms/100ml. This level of contamination was observed immediately after filter bed cleaning when the water from the outlet of the bed is out of supply. Further research in this area is necessary to determine whether this technology can be used as an on-line monitor for slow sand filter bed operation.

5 Exploitation and dissemination of results

Colifast will actively participate at conferences and exhibitions, both national and international. In these conferences and exhibitions, sales and marketing will be the focus.

Colifast will give oral presentations and publications in journals when this is possible. Two brochures have been produced from the project, one at the beginning and one summarising the conclusions from the project. These brochures have been given to the distributors and customers.

Below is a list of publications and presentations related to this project given by the participants:

ISS:
Bonadonna L. Rapid analysis of microbial contamination of water. p. 161-182 in Rapid and on-line instrumentation for food quality assurance. Woodhead Publishing in Food Science and Technology. I.E. Tothill. 406 pp.

Bonadonna L., C. Cataldo, M. Semproni. *Pseudomonas aeruginosa* in water: an early warning system for its determination (submitted to J Microbiol Method)

Bonadonna L., M. Semproni, C. Cataldo. A rapid method for the analysis of *Escherichia coli* in water (submitted to J Appl Microbiol)

Bonadonna L., M. Semproni, C. Cataldo. Metodi rapidi per l'analisi delle acque: la determinazione di *Escherichia coli* (submitted to Igiene e Sanità Pubblica)

TW:
Annette M. Prescott and David Holt. 2003. Demonstration of a Rapid Microbial Monitor for Water Quality Monitoring. IWA 2003 South Africa Sustainability Conference.

Annette M. Prescott, Jaime Massanet Nicolau, and David Holt. 2003 Demonstration of a Rapid Microbial Monitor for Water Quality Monitoring. Water Quality Technology Conference Proceedings, Philadelphia.

6 Policy related benefits

During the project the two monitoring devices for novel early warning of microbial contamination were demonstrated. This was performed in industry scale and at laboratory sites. Parameters measured were sensitivity, specificity, and time to result. Colifast intends to further commercialese and distribute the system in order to provide European water companies benefits of a cost effective (less that 2 euro/test), rapid and easily implemental solution.

The laboratory methods in the second year were subject to comparison based upon the ISO Standard 17994. Hundreds of water samples from various sources such as bottled water, well water, and swimming pools were analysed, yielding thousands of paired results for analyses. A key statistical parameter in the analyses is the number of discordant results. Although there were over 1000 results, too few of the discordant pairs were found, to make any firm conclusion as to method equivalency. This made it difficult to elaborate with standardisations organisations in Europe.

The rapid innovative detection system will detect contamination earlier, then will rationalize use of natural resources, and then enhance environment. The Colifasts technology embodies

multiple, automated sub-sampling. This enables a time to result which is proportional to the level of contamination. i.e. a serious contamination event with a higher number of cells can be detected in few hours. Thus the plant operator can be taken corrective action even sooner when its matter. Technically the handling of samples will be reduced, reducing time-consumption for the operators and the analysis will be automatically processed inside the instruments. Since time to detect results will decline using the Colifast technique, the bottled water will be released earlier to the market. Detection of contaminated water will be possible at an earlier stage compared to the reference method. This will reduce the risk of producing high volumes of contaminated bottled water.

Results from the project may to some extent push the technical limit and better address the European priority. The project may be considered as a common contribution to the awareness that the European Standards in the field of microbial detection in the water industry need improvements. The partners will by their experience from this project contribute to implement and improve EU standards of microbial contamination.

Colifast intends to continue to commercialize and distribute the technology. Colifast has actively been dissemination scientific and technical information to customers and distributors. This involves publication in journals, presentation at conferences, and exhibitions. The project has been announced on Colifast website, with periodic status and partnership. Scientific papers have been published and are recently submitted from the participants. Colifast has been participated at international congresses and distributors inside and outside Europe. Colifast has gained new distributors in Europe and outside Europe. The next generation technology has partly been realized. At the moment 4 at-line monitors and 4 lab-version instruments have been sold.

7 Literature

Davies, C.M. and Apte, S.C. 1996. Rapid enzymatic detection of faecal pollution. Wat. Sci. Tech. 32: 169-171

Fiksdal, L. Pommepuy, M., Caprais, M. P. and Midttun I. 1994. Monitoring of faecal pollution in costal waters by use of rapid enzymatic techniques. Appl. Environ. Microbiol. 60:1581-1584.

George, I, Petit, M. and Servais P. 2000.Use of enzymatic methods for rapid enumeration of coliforms in freshwater. J. Appl. Microbiol. 88:404-13.

Appendix 1.

Analyses performed at ISS

Instrument settings for CA/*E.coli*:
Warm up time: 4 hours
Time between sub-samples: 2 hours
Threshold: 100 ppb
Range of time to sub-sampling: 4-16 hours
Confirmation tests: oxidase and indole. Selected colonies were determined at species level by biochemical identification (API 20 NE, Biomeruex)
Number of samples analysed: see Chapter 3 Results
Sources: drinking water, bottle water and well water
No of trials: 1 & 2 (July 2002 to May 2003) and 3 (June 2003 to November 2003)
Analysis format: Present/Absent (P/A)

Instrument settings for CA/Ps. *aeruginosa*:
Warm up time: 10 hours
Time between sub-samples: 2 hours
Threshold: 200 ppb
Range of time to sub-sampling: 6-22 hours
Confirmation tests: according to ISO EN 12780. Selected colonies were determined at species level by biochemical identification (API 20 NE, Biomeruex).
Number of samples analysed: see Chapter 3 Results
Sources: natural samples of water for human consumption and swimming pools
Trials: 1 & 2 (July 2002 to May 2003) and 3 (June 2003 to November 2003)
Analysis format: Present/Absent (P/A)

Analyses performed at ENSP
Instrument settings for CA/*E.coli*:
Warm up time: 4 hours
Time between sub-samples: 2 hours
Threshold: 100 ppb
Range of time to sub-sampling: 4-16 hours
Volume water: 100 ml membrane filtered or 5 ml sample
Confirmation tests: only lactose positive colonies were selected for further confirmation with indole and oxidase. Selected colonies were determined at species level by biochemical identification (API 20 NE, Biomeruex)
Number of samples analysed: see Chapter 3 Results
Sources: spiked water (piped drinking water and river water), raw water, ground water, tap water, crushed ice, and well water.
Trials: 1 (September 2002 to December 2002), 2 (February 2003 to May 2003), and 3 (September 2003 to January 2004)
Analysis format: Present/Absent (P/A)

Instrument settings for CA/Ps. *aeruginosa*:
Warm up time: 10 hours
Time between sub-samples: 2 hours
Threshold: 200 ppb
Range of time to sub-sampling: 6-22 hours

Confirmation tests: according to ISO EN 12780, but only presumptive colonies are consdered
Number of samples analysed: see Chapter 3 Results
Sources: natural drinking water, ground water, bottled water, and swimming-pool water.
Trials: (September 2002 to December 2002), 2 (February 2003 to December 2003), and 3 (September 2003)
Analysis format: Present/Absent (P/A)

Analyses performed at TW

Instrument settings for CA/faecal coliforms/ total coliforms
Warm up time: 3-5 hours
Sample time: 7.5-12 hours
Experiment time: 12-15 hours
Subsamples: 4 -10 samples
Incubation temperature: 37 and 44°C.
Range of time to sub-sampling: 4-16 hours
Volume sample: 4 x 25 ml, 10 ml, 100 ml filtered (0.45 µm)
Confirmation tests: An selected no. of *E.coli* and coliforms were isolated and purified, and analysed by Colilert, purification on nutrient agar and McConkey ager, and further selection of colonies for API 20E tests.
Other analyses performed: Colilert
Sources: Samples from inlet, outlet, and supernatant of slow sand filters (SSF); beds of sand and granulated activated carbon. Dechlorinated water spiked with environmental isolates.
Analysis format: Present/Absent (P/A)

Instrument settings for CALM/Coliforms:
Warm up time: 4 hours
Time between sub-samples: 2 hours
Threshold: 100 ppb
Range of time to sub-sampling: 4-16 hours
Volume water: 100 ml membrane filtered or 5 ml sample
Confirmation tests: only lactose positive colonies were selected for further confirmation with indole and oxidase. Selected colonies were determined at species level by biochemical identification (API 20 NE, Biomeruex)
Number of samples analysed: see Chapter 3 Results
Sources: Samples from inlet, outlet, and supernatant of slow sand filters (SSF); beds of sand and granulated activated carbon. Dechlorinated water spiked with environmental isolates.
Analysis format: Present/Absent (P/A)

Appendix 2. Final brochure from project.