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Equivalence of the Colilert®-18/Quanti-Tray® water potability test for the detection and enumeration of *Escherichia coli* and coliform bacteria in drinking water for human consumption compared to the NF EN ISO 9308-1:2000 reference method

Laboratory expert report

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Solicited request 2010-SA-0323;
DGS n°100019

Laboratory Expert
REPORT

June 2014

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Presentation of participants

PREAMBLE : The external experts, members of the expert committees or working groups, or designated rapporteurs, have all been appointed in a personal capacity, intuitu personae, and do not represent their respective parent organisations

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CONTENT

Presentation of participants **3**
Acronyms and Abbreviations **8**
List of tables **8**
List of figures **8**

1 Context, purpose and procedure for responding to the request 9

1.1 Purpose of the request **9**
1.2 Regulatory and standards background to the request **9**
1.3 Expert appraisal method as regards the request **9**

2 Conditions of the supplementary equivalence study 11

2.1 Lead laboratory and participing laboratories **11**
2.2 Study procedure for participating laboratories **11**
2.3 Selection of samples **12**
2.4 Description of methods ans identification tests to carry out **13**
2.4.1 Comparison of detection methods 13
2.4.2 Number of confirmation tests performed 14
2.4.3 Supplementary tests 14
2.5 Statistical analysis **15**

3 Results 17

3.1 Statistical analysis according to the criteria specified in the NF EN ISO 17994 standard **17**
3.1.1 Coliform bacteria: Statistical analysis taking into account the criteria specified in the NF EN ISO 17994:2004 standard 17
3.1.2 *E. coli* bacteria: Statistical analysis taking into account the criteria specified in the NF EN ISO 17994:2004 standard 19
3.2 Supplementary analyses on *Escherichia coli* bacteria **20**
3.2.1 Statistical analysis taking into account the presumptive enumerations obtained using the Colilert®-18/Quanti-Tray method 20
3.2.2 Statistical analysis taking into account the enumeration results obtained using the NF EN ISO 9308-1 method corrected by additionnal tests confirming β-D-glucuronidase activity in indole-producing *Escherichia coli* (notion of false positive) 21
3.2.3 Analysis of results on phase 2 samples: introduction to the notion of underestimation 23
3.2.4 Statistical analysis taking into account only enumerations >1 UFC or MPN enumeration results confirming the presence of *Escherichia coli* in the samples by both methods 24

4 Conclusions 27

5 References 31

5.1 Standards **31**

5.2 Legislation and Regulations 31

ANNEXES 32

Annex 1 : Request Letter 33

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AFSSA - LERH

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RÉPUBLIQUE FRANÇAISE

Ministère de la Santé et des Sports

Direction générale de la santé

Paris, le 25 MAI 2010

Sous direction *Prévention des risques liés à l'environnement et à l'alimentation*
Bureau *Qualité des eaux*

DGS/EA4 – N° 224

Le Directeur général de la santé

à

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Monsieur le Directeur du Laboratoire d'Etudes et de Recherches en Hydrologie (LERH – AFSSA)
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Objet : Demande d'appui scientifique et technique – Equivalence du test de potabilité des eaux Colilert®-18 / Quanti-tray® pour la recherche et le dénombrement des *Escherichia coli* et des coliformes dans les eaux destinées à la consommation humaine par rapport à la méthode de référence NF EN ISO 9308-1

N/Réf : DGS N° 100019 (*numéro de dossier à rappeler dans toute correspondance*)

La directive 98/83/CE du 3 novembre 1998 relative à la qualité des eaux destinées à la consommation humaine (EDCH) prévoit la recherche de paramètres microbiologiques et fixe, à cette fin, les méthodes d'analyse, dites de référence, à utiliser (annexe III, partie 1) tout en spécifiant que des méthodes alternatives peuvent être utilisées « *à condition qu'il puisse être démontré que les résultats obtenus sont au moins aussi fiables que ceux obtenus par les méthodes spécifiées à l'annexe III, partie 1* ». La directive 98/83/CE ne précisant pas de référentiel normatif ou de guide d'évaluation des méthodes alternatives, la Direction générale de l'Environnement de la Commission Européenne (CE) en charge de l'eau d'alimentation a mandaté en 2007 un groupe consultatif, l'European Microbiology Group (EMG), composé d'experts en microbiologie, afin d'évaluer les dossiers de demande d'équivalence des méthodes alternatives. La direction générale de la santé (DGS) a chargé l'Agence française de sécurité sanitaire des aliments (AFSSA) et votre laboratoire d'y représenter la France.

Pour mémoire, l'EMG s'appuie sur des lignes directrices, basées sur l'unique norme internationale existante définissant une procédure d'évaluation dans le domaine de l'eau, la norme NF EN ISO 17994 : 2004. Il appartient aux industriels souhaitant commercialiser des méthodes alternatives, de fournir les preuves de l'équivalence. Lorsqu'elle est accordée, cette équivalence est reconnue uniquement dans l'Etat-membre qui a porté le dossier auprès de la CE. Parallèlement, l'Agence française de normalisation (AFNOR) a initié en 2004 un projet de validation par tierce partie des méthodes alternatives. Initialement, le groupe de travail mis en place par l'AFNOR-Validation avait décidé de retenir le référentiel NF EN ISO 17994 : 2004 et de le compléter. Mais finalement, le groupe de travail a retenu la norme NF EN ISO 16140, utilisée pour la validation des méthodes microbiologiques alternatives pour les aliments.

Le rapport remis par votre laboratoire à la suite de ma demande d'appui scientifique et technique relatif à l'équivalence des méthodes alternatives par rapport aux méthodes de référence dans le domaine de l'eau d'alimentation (saisine 2007-SA-0192, juin 2007) met en évidence un certain nombre de différences entre le référentiel de l'AFNOR-Validation et celui utilisé par l'EMG, la principale étant l'absence d'essais sur des échantillons représentatifs des eaux distribuées en France dans le référentiel de l'AFNOR-Validation.

Acronyms and Abbreviations

DGS: Direction Générale de la Santé

EMG: European Microbiology Group

LHN: Laboratoire d'Hydrologie de Nancy

MALDI-TOF: Matrix Assisted Laser Desorption Ionization -Time of Flight Mass Spectroscopy

NPP: Nombre le plus probable

ONPG: Ortho-nitrophényl-β-galactoside

STEP: Station d'Epuration des eaux usées

List of tables

Table 1 : Results of comparison tests performed on coliform bacteria enumerated using the Colilert®-18/Quanti-Tray® method and confirmed using standardized tests compared to the enumeration results obtained using the NF EN ISO 9308-1 standard 17

Table 2 : Results of comparison tests performed on E. coli bacteria enumerated using the Colilert®-18/Quanti-Tray® method and confirmed using standardized tests compared to the enumeration results obtained using the NF EN ISO 9308-1 standard 19

Table 3 : Comparison of equivalence test findings for the Colilert®-18/Quanti-Tray® method obtained in the French and Spanish dossiers for *E. coli* enumeration 20

Table 4 : Results of comparison tests performed on presumed E. coli bacteria enumerated using the Colilert®-18/Quanti-Tray® method compared to the enumeration results obtained using the NF EN ISO 9308-1 standard 21

Table 5 : Results of comparison tests performed on *E. coli* bacteria enumerated using the Colilert®-18/Quanti-Tray® method and using NF EN ISO 9308-1 method after corrections of the enumeration values by adding a test for β-D-glucuronidase activity. 22

Table 6 : Results of comparison tests performed on *E. coli* enumerated using the Colilert®-18/Quanti-Tray® method (confirmation of presumed positive wells) compared to the enumeration results using the NF EN ISO 9308-1 standard method. Enumeration sets for which neither method detected any *E. coli* in samples were discarded. 25

Table 7 : Results of comparison tests performed on *E. coli* enumerated using the Colilert®-18/Quanti-Tray® method (confirmation of presumed positive wells) compared to the enumeration results using the NF EN ISO 9308-1 standard method after correction of enumerations by adding a test to detect β-D-glucuronidase activity. Enumeration sets for which neither method detected any *E. coli* in samples were discarded..... 25

List of figures

Figure 1: Operating method and criteria used to select representative sites (data obtained from the national SISE-Eaux database, source: French Ministry of Health). 13

1 Context, purpose and procedure for responding to the request

1.1 Purpose of the request

The French Directorate General for Health (DGS) submitted a request (no. 100019) on May 25, 2010 to the Nancy Laboratory for Hydrology for scientific and technical support regarding the equivalence of the Colilert®-18/Quanti-Tray® water potability test for the detection and enumeration of *Escherichia coli (E. coli)* and coliform bacteria in drinking water for human consumption compared to the NF EN ISO 9308-1:2000 reference method.

ANSES carefully considered this DGS request with regard to its admissibility. To fulfil the request, the specific and unique background of this alternative technique was considered, especially concerning its positioning within several European Union countries, previous efforts of the European Microbiology Expert Group, and the opinion issued by ANSES on methods to establish the equivalence of alternative methods (2007-SA-0192).

Any later requests by an industrial entity must comply with the required standards and, barring exceptional circumstances, should not be systematically submitted to the ANSES Nancy Laboratory for Hydrology for a technical expert appraisal.

1.2 Regulatory and standards background to the request

The request seeks to confirm whether the Colilert®-18/Quanti-Tray® method developed by the IDEXX Company is equivalent to the NF EN ISO 11290-1:2000 reference method used in France, in accordance with European Council Directive 98/83/EC on the quality of water intended for human consumption.

To determine the equivalence, the Nancy Laboratory for Hydrology followed the recommendations of the European Microbiology Expert Group (EMEG), which was tasked by the European Commission's Directorate General for the Environment (DG ENV) to apply the NF EN ISO 17994:2004 "Water quality – Criteria for establishing equivalence between microbiological methods" international standard.

1.3 Expert appraisal method as regards the request

In response to the DGS's request, the Nancy Laboratory for Hydrology adopted a two-step process.

It first examined the documentation on equivalence studies submitted by IDEXX to the DGS to support the recognition of equivalence. This initial assessment aimed to verify that the information in the documentation met all the requirements and criteria of the NF EN ISO 17994:2004 standard. ANSES submitted a first report regarding this documentation assessment in October 2010. The findings of this assessment, based on the documents provided to assess the equivalence of the Colilert®-18/Quanti-Tray® method with regard to the NF EN ISO 9308-1:2000 reference method, did not enable a definitive determination of the applicability of the alternative method for water in France according to the principles of the NF EN ISO 17994:2004 standard and EMEG recommendations. The most significant limitation concerned the different types of water tested in

the studies that closely followed the NF EN ISO 17994:2004 standard and the streamlined protocol in line with the EMEG's recommendations.

Given the findings of the documentation assessment and in response to the solicited request, ANSES' Nancy Laboratory for Hydrology conducted a supplementary study of equivalence in accordance with the NF EN ISO 17994:2004 international standard and the EMEG's recommendations. This study was the second step of the request. The supplementary study was designed to determine the equivalence of the Colilert®-18/Quanti-Tray® compared to the NF EN ISO 9308-1:2000 reference method based on the data obtained. The conclusions drawn from this study will not prejudice ANSES's opinion, which will serve as the basis for any modification to the March 17, 2003 ministerial order regarding analysis methods and their performance characteristics as set out in Article 1321-21 of the French Public Health Code.

2 Conditions of the supplementary equivalence study

2.1 Lead laboratory and participing laboratories

This study was conducted based on the principles of independence, impartiality and technical robustness. As a result, IDEXX was not involved in managing the study. The company was asked to provide the necessary products and equipment at no cost to carry out and analyze the tests.

The study was managed by a mirror group composed of several water microbiology experts, a statistical analysis expert and a hydrogeologist, who ensured that the approach applied, was fit for the purpose of assessing the method's equivalence.

The number of participating laboratories was determined according to the number of geographical areas selected for the project. Initially, 12 laboratories were selected based on the geographical zone of interest as well as (i) their accreditation for the NF EN ISO 9308-1:2000 reference method and (ii) their certification for carrying out official water monitoring analyses. Three laboratories dropped out of the study, so only the results of nine laboratories were finally taken into account. A specific agreement outlining study conditions was signed with each laboratory.

2.2 Study procedure for participating laboratories

For the participating laboratories, the study proceeded in three distinct phases as described below

- The first phase (Phase 1) :

She sought to familiarize employees at the participating laboratories with the Colilert®-18/Quanti-Tray® method and ensure they fully understood the equivalence assessment protocol. All the laboratories—even those already using the Colilert®-18/Quanti-Tray® method—were trained on its use under real laboratory conditions by the lead laboratory (ANSES' Nancy Laboratory for Hydrology) during a two-day training workshop. Given the number of laboratories involved, two separate workshops were held so that participants could get hands-on experience using the method on samples at the lead laboratory. This workshop also aimed to clearly explain the comparison protocol being used, which had been previously communicated to the laboratories.

Following these two workshops, the newly trained employees were able to test the equipment, reagents to be assessed and the comparison protocol on their own samples in their respective laboratories to resolve any ambiguities before starting the study. A validation test to ensure full understanding of the alternative method and comparison protocol was carried out by the lead laboratory on water samples that had been artificially contaminated (i.e., spiked) with a calibrated reference material. For the test, the laboratories were asked to apply both methods to the samples under comparison protocol conditions. After analyzing each laboratory's results, a hearing was convened and, depending on the results obtained, the laboratories were authorized to begin the practical study on assigned water samples.

- The second phase (Phase 2) :

She aimed to set up an equivalence study for frequently contaminated samples (assigned natural samples) taken by the laboratories. The DGS made a SISE-Eaux health database request; from these data, the Nancy Laboratory for Hydrology selected sites from which samples are naturally contaminated by coliform and/or *E. coli* bacteria. These samples were analyzed simultaneously using the two methods according to the comparison protocol. It should be noted that given the small number of this type of samples, only five of the nine participating laboratories (Lab A, Lab D, Lab G, Lab H and Lab J) were tasked with analyzing them. Samples were assigned based on the laboratories' geographical proximity to the sampling points.

- The third phase (Phase 3) :

She aimed to carry out an equivalence study on uncontaminated drinking water samples taken by the laboratories that had been "artificially contaminated" by spiking. This phase enabled the comparison of both methods on several targeted bacteria. The samples were contaminated using environmental water containing coliform and *E. coli* bacteria. The laboratories used surface water and wastewater from domestic wastewater treatment plants (DWTP). These two types of water were diluted using chlorinated water to simulate stress on the target bacterial flora in a drinking water system. The solutions were then calibrated and stored at 4°C until the test water samples were spiked. A specific protocol to prepare the spiked water samples was communicated to all participating laboratories.

2.3 Selection of samples

The target number of samples to test was around 250 samples, including water subjected to various disinfection treatments (chlorine, chlorine dioxide, UV light, ozone, etc.). To ensure the sample water types were representative, data were extracted from the SISE-Eaux health database to select water supply units with different water typologies (i.e., having various physical, chemical, microbiological and hydrological properties) covering all of France where possible (both mainland and overseas territories).

The selection of naturally contaminated water samples (Phase 2 samples) was favored to test the two methods under real-world conditions. The equivalence assessment on this type of sample corresponds to what the NF EN ISO 17994 standard considers an "ideal" process. Consequently, analytical data covering the whole of France were extracted from the national SISE-Eaux database to first select sites with potential coliform bacteria contamination.

Given the small number of naturally contaminated samples (Phase 2 samples) selected (50 separate sites, Fig. 1), their locations across France (sites mainly in karst areas) and volumes produced and supplied (from 1 m3/d to 10 m3/d), an additional 150 sites were randomly selected by flow rate and hydrologic unit to test a more representative panel of samples as seen by health monitoring services.

Next, a "hydrogeology" working group modified and completed the number of sites by including 60 additional sites that took into account three criteria: greater coverage and diversity of bodies of water in certain hydrologic units as well as the selection of sites with strong concentrations of E. coli.

These actions resulted in a total of 250 sites in mainland France and its overseas territories being selected for testing, as shown on the map in Fig. 1.

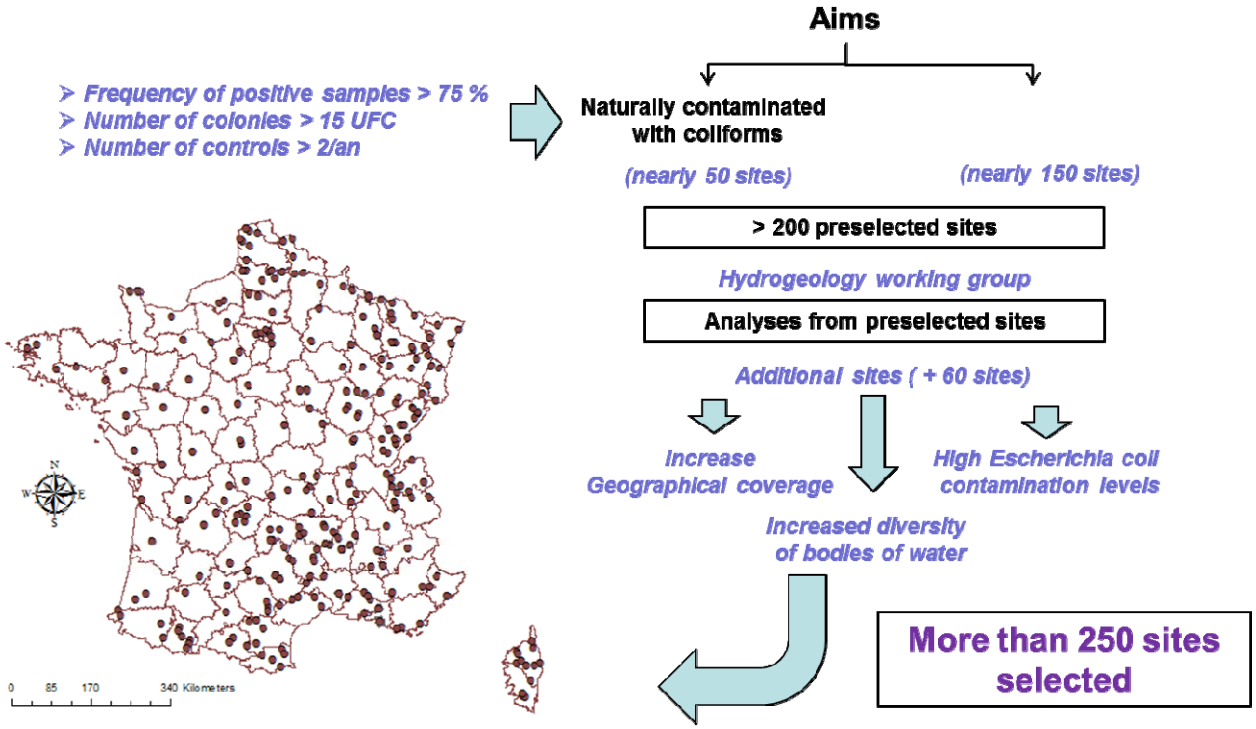

Figure 1: Operating method and criteria used to select representative sites (data obtained from the national SISE-Eaux database, source: French Ministry of Health).

All samples (Phase 2 + Phase 3) were assigned to the laboratories according to their geographical location so that each participating laboratory could follow the standards for sampling, transport and analysis timelines. Accordingly, depending on the participating laboratories' locations with respect to the selected sites, the number of samples allocated to each laboratory varied.

2.4 Description of methods ans identification tests to carry out

2.4.1 Comparison of detection methods

- NF EN ISO 9308-1:2000 method

For this study, the NF EN ISO 9308-1:2000 standard served as the reference method. As a reminder, the principle of this method consists in filtering 100 ml of drinking water for human consumption through a membrane filter with a porosity of 0.45 µm, incubating the membrane in a selective agar medium and confirming the presence of bacteria through appropriate tests. According to this reference standard, coliforms are defined as aerobic and oxidase-negative bacteria that can form colonies within (21 ± 3) h at (36 ± 2)°C in a lactose-based selective growth medium. For the standard assay, the ISO standard allows the incubation period to be extended by 24 hours (refer to standard note 1) to increase the method's sensitivity if no colonies are detected within 24 hours. This provision is included in the regulatory text on drinking water for human consumption (ministerial order of September 17, 2003 regarding water sample analysis methods and their performance characteristics). This incubation time was employed for these assays, i.e., with enumeration after (44±4) h (2 readings: (21±3) h and (44±4) h). For *Escherichia coli*, the production of indole from tryptophan within (21 ± 3) h at (44 ± 0.5)°C completed the process described above. A second membrane was systematically prepared for incubation at (44 ± 0.5)°C. A tolerance of ± 1°C at 44°C was accepted for the study.

- Colilert®-18/Quanti-Tray method

This study was designed to assess the Colilert®-18/Quanti-Tray® method. This is an MPN-type method in which 100 ml of drinking water is mixed with a lyophilized reagent. The mixture is then distributed across 51 wells with the same volume. The coliform detection principle is based on the bacteria's ability to cleave ortho-nitrophenyl-β-galactoside (ONPG) via the β-D-galactosidase enzyme after 18 h of incubation at (36 ± 2)°C, thereby causing the wells—which are initially clear—to gradually turn yellow. To detect *E. coli*, the method reveals the presence of another enzyme, β-D-glucuronidase, over the same incubation period. The yellow wells will appear fluorescent if *E. coli* is present in the samples. Although no additional tests are required with the Colilert®-18/Quanti-Tray® method to confirm the accuracy of the information provided by the colored wells, for the purposes of the comparison test to check equivalence, it was necessary to verify and confirm the presence of coliforms and *E. coli*. Confirmation was obtained with the same tests as those for the reference standard. This additional requirement resulted in the participating laboratories carrying out confirmation by duplicate analysis on each sample. To obtain confirmation, some of the liquid medium was removed from each coliform- or *E. coli*-positive well (those presumed to contain the bacteria) and placed on MacConkey agar to determine the positive presence of lactose from the bacteria after incubation at (36 ± 2)°C for (21 ± 3) h. The oxidase and indole tests were systematically performed on positive lactose colonies obtained on the MacConkey agar subcultured from wells indicating the presence of coliforms and *E. coli*. It should be noted that if several lactose-positive colony types were identified on the MacConkey agar, the laboratories were instructed to subculture the different observed types for confirmation. To report the final results, if several colony types were obtained from the MacConkey agar, the wells were considered to be confirmed as containing coliforms or *E.coli* if at least one of the subcultured colonies presented the characteristics outlined in the reference standard. To obtain the definitive enumeration results, the MPN tables provided by the test kit producer were used according to the instructions for use. An additional 2 h for reagent incubation time was tolerated at participating laboratories. All the laboratories involved in this study were supplied with the specific equipment required to carry out the Colilert®-18/Quanti-Tray® assays (Quanti-Tray® sealer to seal the plates, 365 nm UV lamp, Quanti-Tray® comparator, and MPN plate s). IDEXX supplied this equipment to the lead laboratory, which then distributed it to each participating laboratory.

2.4.2 Number of confirmation tests performed

It is important to confirm the colonies or positive wells during a comparison test. Ideally, all the presumptive colonies obtained on the selective medium should be confirmed using the reference standard or the wells from the Colilert®-18/Quanti-Tray® method. However, for cost-related reasons, it was not feasible to ask all the participating laboratories to confirm all colonies, especially during the analyses of spiked (i.e., "artificially contaminated") Phase 3 samples. As a result, the laboratories were asked to confirm all the colonies obtained from **Phase 2** "naturally contaminated samples" using the standard and all positive wells from the alternative method. During **Phase 3** of the study, the laboratories analyzing spiked samples adopted the approach applied to drinking water samples during official monitoring controls, i.e., if there are more than ten presumptive colonies or positive wells from the alternative method, ten samples are chosen for confirmation, E. coli-positive samples being favored.

2.4.3 Supplementary tests

All participating laboratories were asked to conduct supplementary tests on the coliform and *E. coli* bacteria that were isolated and confirmed using NF EN ISO 9308-1 reference standard tests. The Nancy Laboratory for Hydrology sent all the laboratories a chromogenic medium with which they were to test the presence of β-D-galactosidase (coliform) and β-D-glucuronidase (*E. coli*)) enzyme activity on at least three isolated and confirmed colonies of coliform bacteria and at least three isolated and confirmed colonies of *E. Coli*. Testing for β-D-glucuronidase was recommended as part of a technical corrigendum issued by the ISO/TC147 Technical Committee in 2007. The aim is

to avoid false positives, especially those due to the presence of *Klebsiella oxytoca* strains, which can give positive indole results under the same incubation conditions.

Additionally, the laboratories were also instructed to freeze all samples analyzed using each method in cryobeads at -20°C to send at least three confirmed colonies each of coliform and *E. coli* strains to the Nancy Laboratory for Hydrology. The bacteria obtained from analyzed samples were identified mainly using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry as well as by biochemical identification tests. The test results of each method and the identification data obtained were integrated in a specially created database.

2.5 Statistical analysis

Statistical analysis was performed on the enumeration results obtained using the NF EN ISO 9308-1 reference method and the Colilert®-18/Quanti-Tray® method, taking into account the guidelines of the NF EN ISO 17994 international standard. This method involves determining the relative difference, expressed as a percentage, between each confirmed count pair obtained with the two methods using the formula:

$x = [\ln(a) - \ln(b)]$ x 100 % where a and b are the enumeration values obtained for each method

Two methods are considered as quantitatively equivalent (“no difference”) if the mean relative difference measured for the confirmed enumeration pairs is close to zero and if the calculated expanded uncertainty does not exceed the maximum accepted deviation set for each sample type. This accepted deviation “D” for the water samples was set at 10%. For this study, the decision was made to accept the Colilert®-18/Quanti-Tray® method as equivalent whenever its average performance was quantitatively equivalent or better than the reference method. For each analysis performed, we have indicated the relative difference obtained as well as the standard deviation, standard error, expanded uncertainty “U” and the overall trend of the test, which can be expressed as follows depending on the individual test results:

- Overall positive trend (+) = the Colilert®-18/Quanti-Tray® method’s detection performance is higher than that of the NF EN ISO 9308-1 method
- Overall negative trend (–) = the Colilert®-18/Quanti-Tray® method’s detection performance is lower than that of the NF EN ISO 9308-1
- Overall inconclusive trend (o) = results do not permit a conclusion to be made due to a lack of data
- Overall trend with no difference (MND) = equivalent method with no difference

Before carrying out statistical tests, a critical analysis of the enumeration results was performed. For example, the enumeration sets for each tested sample with zero deviation between the two methods were eliminated from the analysis. Enumeration outliers were also sought in order to exclude them from the statistical analysis. The NF EN ISO 17994:2004 standard does not indicate official tests to detect outlier laboratories, inappropriate sample types or isolated outlier test results. It is the statistical analyst’s decision to identify outlier results or not. To address this issue, each laboratory’s results were analyzed. Based on these results, the enumerations from all laboratories were then grouped together for a comprehensive statistical analysis. Given the number of results ($n > 25$ and < 500), the decision was made to perform a Rosner’s test to eliminate outliers.

It should be noted that for the enumeration sets for samples with a zero deviation result using one of the methods but not both, the data were retained for statistical analysis. It is virtually impossible to avoid obtaining this type of combination (a, 0) and (0, b). To avoid omitting these samples, the relative differences were calculated by applying the formulas below:

- When the result was (a, 0), the relative difference was calculated using: $x = \ln(a + 1)$ X 100%

- When the result was (0, *b*), the relative difference was calculated using: $x = -\ln(b+1)$ x 100%

The main statistical analysis consisted in comparing the confirmed enumerations obtained using the NF EN ISO 9308-1 reference method to those obtained using the Colilert®-18/Quanti-Tray® method after confirmation of the presumed bacteria in the yellow wells (coliforms) and fluorescent yellow wells (*E. coli*) using the tests described in the reference method. This analysis corresponds to that performed in most assessment dossiers and complies with EMEG guidelines. The coliform test results are discussed in Section 6.1.1 while *E. coli* results are discussed in Section 6.1.2. To offer greater details on the findings of certain tests, additional statistical analyses taking into account the guidelines of the NF EN ISO 17994 standard were also performed, namely by directly using the enumeration results for the presumed positive wells from the Colilert®-18/Quanti-Tray® method or the corrected enumeration results obtained from the reference standard after confirmation of β-D-glucuronidase enzyme activity in isolated and confirmed colonies (indole-producing *E. coli*).

3 Results

3.1 Statistical analysis according to the criteria specified in the NF EN ISO 17994 standard

3.1.1 Coliform bacteria: Statistical analysis taking into account the criteria specified in the NF EN ISO 17994:2004 standard

The first step of the statistical analysis was to determine the equivalence of the Colilert®-18/Quanti-Tray® method compared to the NF EN ISO 9308-1 reference method by examining the confirmed results obtained after verification of the lactose-positive and oxidase-negative traits of the bacteria isolated from the presumed positive wells (yellow wells: β-galactosidase activity). The statistical analysis included 220 samples, of which 45 were naturally contaminated (Phase 2) and 175 were artificially contaminated (Phase 3). The results of the statistical tests performed on each sample type (Phase 2 or Phase 3) are provided in Table 1 with all results broken down by laboratory and phase grouping.

Table 1 : Results of comparison tests performed on coliform bacteria enumerated using the Colilert®-18/Quanti-Tray® method and confirmed using standardized tests compared to the enumeration results obtained using the NF EN ISO 9308-1 standard

Laboratories & grouping	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
Lab A	14	77.05	111.3	29.75	59.49	+
Lab C	12	74.91	75.85	21.90	43.79	+
Lab D	37	60.94	100.22	16.48	32.95	+
Lab E	9	5.58	37.18	12.39	24.78	o
Lab F	13	45.89	34.01	9.43	18.87	+
Lab G	11	-0.62	78.44	23.65	47.3	o
Lab H	12	47.1	116.62	33.67	67.33	o
Lab J	84	36.37	124.81	13.62	27.24	+
Lab K	28	19.03	46.07	8.71	17.41	+
Phase 2 + 3	220	41.02	101.7	6.86	13.71	+
phase 2	45	35.93	147.39	21.97	43.94	MSD
phase 3	175	42.34	86.7	6.55	13.11	+

+ : Colilert®-18/Quanti-Tray® method with a higher detection performance
- : Colilert®-18/Quanti-Tray® method with a lower detection performance
O : Results not permitting a conclusion due to a lack of data
MSD : Method with No Difference

In this study, six laboratories obtained a positive conclusion on their assigned samples (Labs A, C, D, F, J and K), indicating that the Colilert®-18/Quanti-Tray® method obtained higher enumerations

than those obtained using the NF EN ISO 9308-1 standard method. Relative differences ranging from 19.03% to 77.05% were observed depending on the laboratory, with a variability between 34.01% and 124.81%. The measurement data for the various tested samples at three of the participating laboratories (Labs E, G and H) did not permit a definitive conclusion. For these laboratories, it would have been necessary to increase the number of samples tested to obtain a trend.

The grouping of all enumerations from Phases 2 and 3 confirms that the alternative method offers a higher detection performance (41.02%) for coliform bacteria numbers in water compared to the NF EN ISO 9308-1 reference method.

The statistical analysis performed separately on samples from Phase 2 and Phase 3, respectively, also concluded that the Colilert®-18/Quanti-Tray® method was equivalent. However, the conclusion is much less clear cut for Phase 3 samples than for Phase 2 samples (MND trend), which could be explained by a greater variability in the latter samples (147.39% versus 86.7% for Phase 3 samples).

This study's findings, based on over 200 samples, demonstrate that the Colilert®-18/Quanti-Tray® method is equivalent to the NF EN ISO 9308-1 standard with regard to enumeration of coliform bacteria in water samples. Overall, this conclusion is consistent with those drawn in different equivalence studies carried out in other EU countries on water samples representative of distributed water. It is difficult to say precisely why there is such a large difference in favor of the Colilert®-18/Quanti-Tray® method. Various authors have theorized that the target bacteria grow better in the liquid chromogenic medium than in the solid Tergitol with TTC agar. For others, the difference could be explained by the presence of interfering flora in water samples that might alter the standard method's counts. Incidentally, the negative impact of interfering flora on counts using the NF EN ISO 9308-1 method is indicated in its scope.

It is interesting to note that the equivalence results based on the enumerations obtained for samples from Phase 2 and Phase 3 differ. When Phase 3 samples sourced from surface water and DWTP wastewater were spiked with coliform bacteria, a significant enumeration difference was observed. However, this difference disappeared in the naturally contaminated Phase 2 samples. Based on the tested samples, there is little difference between the two methods due to the considerable variability among enumerations. Again, it is difficult to determine the reasons for this difference, aside from the fact that in these types of water, coliform bacteria are most certainly very different physiologically than those artificially added to the samples, despite the treatment applied to simulate chlorine stress. Few other published studies have examined the use of naturally contaminated samples to assess the equivalence of the two methods.

In conclusion, this study's findings demonstrate that the Colilert®-18/Quanti-Tray® method is equivalent to the NF EN ISO 9308-1 method in enumerating coliforms in the tested water samples.

3.1.2 *E. coli* bacteria: Statistical analysis taking into account the criteria specified in the NF EN ISO 17994:2004 standard

For *E. coli*, the mean relative difference was determined from the enumerations obtained using the reference method and the Colilert®-18/Quanti-Tray® method by taking into account the confirmed results of bacteria from positive fluorescent yellow wells (presumed *E. coli* bacteria) with the following traits: lactose positive (+), oxidase negative (–) and indole positive (+). The equivalence assessment was estimated on 202 samples, of which 38 were from naturally contaminated samples (Phase 2) and 164 artificially contaminated samples (Phase 3). The results are shown in Table 2.

Table 2 : Results of comparison tests performed on E. coli bacteria enumerated using the Colilert®-18/Quanti-Tray® method and confirmed using standardized tests compared to the enumeration results obtained using the NF EN ISO 9308-1 standard

Laboratories & grouping	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
Lab A	12	-74.59	117.74	33.99	67.98	-
Lab C	11	-80.15	109.54	33.03	66.05	-
Lab D	32	-38.57	130.67	23.10	46.2	o
Lab E	9	-113.06	64.51	21.50	43.01	-
Lab F	13	-20.75	71.47	19.82	39.64	o
Lab G	11	-53.66	105.32	31.76	63.51	o
Lab H	10	28.36	78.23	24.74	49.48	o
Lab J	76	-31.97	105.28	12.08	24.15	-
Lab K	28	-33.54	94.74	17.90	35.81	o
Phase 2 + 3	202	-39.47	106.41	7.49	14.97	-
Phase 2	38	-74.35	117.15	19.00	38.01	-
Phase 3	164	-31.39	102.46	8.00	16.00	-

+ : Colilert®-18/Quanti-Tray® method with a higher detection performance
- : Colilert®-18/Quanti-Tray® method with a lower detection performance
O : Results not permitting a conclusion due to a lack of data
MSD : Method with No Difference

Of the nine participating laboratories, only four obtained lower *E. coli* enumeration results from their assigned samples with the Colilert®-18/Quanti-Tray® method than with the NF EN ISO 9308-1 standard method. The relative differences measured varied between -31.97% and -113.06%.

The Phase 2 plus Phase 3 grouping confirmed the trend observed by these three laboratories. The mean relative difference measured varied between -31.39% for Phase 3 spiked samples and -74.35% for Phase 2 naturally contaminated samples. It should be noted that the variations observed are relatively high as they are close to 100%.

Negative findings for the Colilert®-18/Quanti-Tray® method are not widely reported in published European equivalence dossiers. In most cases, higher enumerations demonstrating the equivalence of the Colilert®-18/Quanti-Tray® method compared to the reference standard are observed. Only the findings presented in the Spanish equivalence dossier are consistent with

those obtained in the present study. Indeed, the nine Spanish laboratories that compared the Colilert®-18/Quanti-Tray® method with the NF EN ISO 9308-1 standard using 275 artificially spiked samples found similar values related to the overall results—namely the standard deviation, standard error and expanded uncertainty "U"—to those measured in the present study (Table 3).

Table 3 : Comparison of equivalence test findings for the Colilert®-18/Quanti-Tray® method obtained in the French and Spanish dossiers for *E. coli* enumeration

Samples	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
French	202	-39.47	106.41	7.49	14.97	-
Spanish*	275	-38.16	103.18	6.22	12.44	-

*** results in percentages**

These findings, which are relatively similar to the enumerations of the samples tested in the Spanish dossier, reinforce the validity of the comparison methodology used for this study on water samples that are representative of French water sources, including naturally contaminated samples. On this sample type (Phase 2) and for E. coli, a much higher negative mean relative difference (-74.35 versus -31.39%) was observed than that for Phase 3 samples, which could be indicative of an influence linked to the type of samples tested (Phase 2 samples versus Phase 3 samples).

In conclusion for *E. coli* bacteria, this study's findings demonstrate that the enumeration performance of the Colilert®-18/Quanti-Tray® method is lower than that of the reference standard for both naturally contaminated or artificially spiked water samples when applying EN ISO 17994 criteria. At this point of the study, the equivalence of this method compared to the NF EN ISO 9308-1 method cannot be admitted for the detection of *E. coli* bacteria in the tested water samples.

3.2 Supplementary analyses on *Escherichia coli* bacteria

Given the *E. coli* bacteria results, additional work was carried out for this study to understand the observed trend for this type of bacteria. The supplementary tests performed below are not recommended by either the NF EN ISO 17994 standard or EMEG guidelines. However, some of them were carried out in several equivalence dossiers, including the Spanish dossier, and the findings of these supplementary data were used to validate the equivalence of the Colilert®-18/Quanti-Tray® method compared to the NF EN ISO 9308-1 method.

3.2.1 Statistical analysis taking into account the presumptive enumerations obtained using the Colilert®-18/Quanti-Tray method

In the equivalence studies carried out based on the NF EN ISO 17994 standard, *E. coli* bacteria detected using the Colilert®-18/Quanti-Tray® method must be confirmed by the tests described in the reference method. To do this, tests are performed to detect indole production in *E. coli* bacteria drawn from fluorescent wells (β-D-glucuronidase activity). If indole production is detected, these results validate the presumed positive wells, which either confirms the presumptive enumeration or leads to a new enumeration based on the number of confirmed wells.

To assess the impact of the indole production confirmation step on the final result of the statistical test, a new test was performed which took into account the enumeration results obtained using the Colilert®-18/Quanti-Tray® method based on the unconfirmed presumed positive wells. The results obtained for samples from Phase 2, Phase 3 and the Phase 2 plus Phase 3 grouping are shown in Table 4.

Table 4 : Results of comparison tests performed on presumed E. coli bacteria enumerated using the Colilert®-18/Quanti-Tray® method compared to the enumeration results obtained using the NF EN ISO 9308-1 standard

Laboratories & grouping	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
Phase 2 + 3	203	-29.95	103.58	7.27	14.54	-
Phase 2	39	-66.66	115.09	18.43	36.86	-
Phase 3	164	-21.22	99.04	7.73	15.47	-

+ : Colilert®-18/Quanti-Tray® method with a higher detection performance
- : Colilert®-18/Quanti-Tray® method with a lower detection performance
O : Results not permitting a conclusion due to a lack of data
MSD : Method with No Difference

The statistical analysis was performed on 203 different samples, i.e., one sample more than when the wells from the Colilert®-18/Quanti-Tray® were confirmed with NF EN ISO 9308-1 method tests (Table 3). The findings of the statistical analysis of the data from the Phase 2 and Phase 3 samples show that the lower detection counts using the Colilert®-18/Quanti-Tray® method vary between -66.66% and -21.22% respectively. The sample grouping led to a relative difference of -29.95% which disadvantages the Colilert®-18/Quanti-Tray® method.

In conclusion, the confirmation of presumed wells through an additional indole test had little effect on the observed enumeration findings, and therefore on the trend and the conclusions of the statistical tests performed. The indole test confirmation step does not explain the differences in enumeration between the two methods.

3.2.2 Statistical analysis taking into account the enumeration results obtained using the NF EN ISO 9308-1 method corrected by additionnal tests confirming β-D-glucuronidase activity in indole-producing *Escherichia coli* (notion of false positive)

Several equivalence dossiers noted that the characterization of *E. coli* bacteria based only on indole production is not specific enough to confirm the presence of *E. coli* in a water sample. Indeed, for certain authors, bacteria other than *E. coli*, such as *Klebsiella oxytoca*, may also be detected by these confirmation tests, thereby generating false positives. This argument was indicated in technical corrigendum 1 of the ISO 9308-1 standard issued in 2007 by the ISO/TC 147 Technical Committee (water quality, Sub Committee 4). In this context, the actual number of *E. coli* bacteria in a water sample will be overestimated when the NF EN ISO 9308-1 standard is used, unfavorably biasing the statistical analysis against the Colilert®-18/Quanti-Tray® method.

In this study, we wanted to first estimate the proportion of false positive results obtained by both methods. This assessment was performed on a pool of bacteria isolated from the fluorescent yellow wells (*E. coli*) from the Colilert®-18/Quanti-Tray® method as well as bacteria isolated using the NF EN ISO 9308-1 method from Tergitol agar and revealed as being oxidase negative (–), lactose positive (+), with indole production for *E. coli* bacteria and taking into account the identification of species using MALDI-TOF mass spectrometry or biochemical tests. The assessment was carried out on bacterial strains in the Phase 2 and Phase 3 water samples. By considering the results of the various confirmation tests according to the particular method, speciation can determine the percentage of false positives obtained by both methods.

As regards the bacteria identified as *E. coli* using the **NF EN ISO 9308-1** method, out of 267 strains identified (77 strains from 31 different Phase 2 samples and 190 strains from 93 different Phase 3 samples), 182 bacteria (68.1%) were found to actually be *E. coli*. This means the number of false positives was 31.8% (85 bacteria) for the standard method.

With the **Colilert®-18/Quanti-Tray®** method, out of 241 identified strains, isolated from fluorescent yellow wells (48 strains from 23 different Phase 2 samples and 193 strains from 86 different Phase 3 samples), 190 bacteria were found to actually be *E. coli* (78.8%). This means the number of false positives was 21.1% (51 bacteria) for this method.

These findings clearly show that *E. coli* bacteria in water samples are overestimated regardless of the testing method, with a higher number of false positives resulting from the NF EN ISO 9308-1 standard.

To take into account the impact of overestimated results obtained using the NF EN ISO 9308-1 method on the statistical analysis findings, the decision was made to correct the enumeration results obtained with this method by considering the results of non-standard supplementary tests used to characterize β-D-glucuronidase production activity in indole-producing *E. coli*. This was possible as all participating laboratories were instructed to look for β-D-glucuronidase activity on at least three *E. coli* colonies identified using the NF EN ISO 9308-1 method on every tested sample. The findings of these supplementary tests enabled the enumeration results to be corrected by eliminating the positive results from the indole test if no β-D-glucuronidase activity was found in the tested bacteria.

Based on these new corrected enumeration data from the NF EN ISO 9308-1 method, a statistical analysis was performed as set out in the NF EN ISO 17994 standard. These new results were compared with the enumerations obtained using the Colilert®-18/Quanti-Tray® method (confirmation of the positive indole activity of bacteria in fluorescent yellow wells). The results obtained on the Phase 2, Phase 3 and Phase 2 plus Phase 3 sample grouping are shown in Table 5.

Table 5 : Results of comparison tests performed on *E. coli* bacteria enumerated using the Colilert®-18/Quanti-Tray® method and using NF EN ISO 9308-1 method after corrections of the enumeration values by adding a test for β-D-glucuronidase activity.

Laboratories & grouping	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
Phase 2 + 3	193	-13.37	110.71	7.97	15.94	o
Phase 2	34	-48.71	108.36	18.58	37.17	-
Phase 3	159	-5.81	110.07	8.73	17.46	o

+ : Colilert®-18/Quanti-Tray® method with a higher detection performance
- : Colilert®-18/Quanti-Tray® method with a lower detection performance
O : Results not permitting a conclusion due to a lack of data
MSD : Method with No Difference

The use of supplementary tests to show the β-D-glucuronidase activity of *E. coli* confirmed using the standard and the resulting corrected enumerations alters the overall conclusion of the statistical test when the values from the comparison study of the samples from Phases 2 and 3 are grouped together. When these supplementary tests are taken into account, and although negative mean relative differences are obtained, it is no longer possible to draw a conclusion or indicate an overall trend for the Colilert®-18/Quanti-Tray® method. These findings are the result of reduced count deviations between the two methods. The number of samples tested and included in the analysis was lower (193 versus 202 initially). To draw a definitive conclusion, the statistical test indicates that at least 500 additional samples are necessary.

It is interesting to note that the overall test trend is different depending on the type of samples analyzed. While it is impossible to draw a conclusion concerning Phase 3 samples or when Phase 2 and 3 samples are grouped together, this is not the case when naturally contaminated samples (Phase 2) are taken on their own for the statistical analysis. For these latter samples, the negative trend against the Colilert®-18/Quanti-Tray® remains true even when an additional step to test for

β-D-glucuronidase is added. A lower count of -48.71% was obtained from the 34 samples tested using the Colilert®-18/Quanti-Tray® method. However, it should be noted that for Phase 2 samples, this new confirmation configuration for *E. coli* bacteria shows that 34 samples were declared non-compliant for *E. coli* instead of the 38 initially declared non-compliant.

In conclusion, this study's findings demonstrate that the number of *E. coli* bacteria is overestimated by both methods when enumerating *E. coli* bacteria in water samples. The proportion of overestimated bacteria is higher with the NF EN ISO 9308-1 method than the Colilert®-18/Quanti-Tray® method. Based on the enumeration data characterizing *E. coli* bacteria for indole production and β-D-glucuronidase activity, it is no longer possible to conclude that the Colilert®-18/Quanti-Tray® method is equivalent to the NF EN ISO 9308-1 method. The addition of supplementary tests to reveal β-D-glucuronidase activity by indole-producing bacteria helps mitigate overestimation and, as indicated in the technical corrigendum from 2007 not yet in force in Europe, appears to be beneficial for reducing the number of false positives. However, as regards the naturally contaminated Phase 2 samples, correcting enumerations obtained using the NF EN ISO 9308-1 standard by adding supplementary tests maintains the negative trend of the statistical test against the Colilert®-18/Quanti-Tray® method.

3.2.3 Analysis of results on phase 2 samples: introduction to the notion of underestimation

Following on from this assessment, it appeared essential to determine the reasons for the negative trend against the Colilert®-18/Quanti-Tray® method when testing the Phase 2 samples. The detailed analysis of the enumeration results of the naturally contaminated samples showed a higher underestimation of *E. coli* bacteria in water samples using the Colilert®-18/Quanti-Tray® method than when using the NF EN ISO 9308-1 standard method, even when supplementary tests (to confirm β-D-glucuronidase activity of *E. coli* bacteria detected using the standard method) were carried out.

Of the 34 Phase 2 samples, *E. coli* was detected by both methods in just 23 samples (67.7%). The Colilert®-18/Quanti-Tray® method did not detect *E. coli* in ten samples (29.4%) even when the bacteria were detected using the NF EN ISO 9308-1 method. Conversely, the standard method did not detect *E. coli* in one of 34 samples (2.9%) revealed positive by the Colilert®-18/Quanti-Tray® method. Given these results, it would appear that the detection of *E. coli* in Phase 2 samples was underestimated using the Colilert®-18/Quanti-Tray® method. However, this trend must be qualified given the low number of samples tested. To date, there are few available data from European studies on this type of natural sample because assessments are generally carried out on artificially contaminated samples.

By comparison, each method underestimated counts in pooled Phase 3 samples in an equal proportion (around 10%). The enumeration confirmation for the NF EN ISO 9308-1 method using tests designed to confirm β-D-glucuronidase activity reverses the trend, disadvantaging the NF EN ISO 9308-1 standard, which underestimates *E. coli* bacteria in 18.2% of the Phase 3 samples versus 8.1% for the Colilert®-18/Quanti-Tray® method.

The risk of underestimation (false negative results) is linked to the presence of *E. coli* bacteria not presenting the trait that testing is designed to detect. For the NF EN ISO 9308-1 standard method, the false negatives are due to *E. coli* not producing indole. For the Colilert®-18/Quanti-Tray® method, the false negatives are due to *E. coli* not expressing β-D-glucuronidase activity. In both cases, the bacteria are counted as coliforms. In the natural environment, there is a variable proportion of *E. coli* that does not produce indole or express β-D-glucuronidase activity. The most representative example of this phenomenon is cited in the new ISO 9308-2:2012 standard (Water quality – Enumeration of *Escherichia coli* and coliform bacteria – Part 2: Most probable number method), which takes the example of enterohemorrhagic strains of *E. coli* (e.g., O157:H7), which

are Risk Group 3 bacterial pathogens than can contaminate people and do not express β-D-glucuronidase activity.

Using the database created for the present study and taking into account the results of the various tests performed using both methods as well as the results of speciation using MALDI-TOF mass spectrometry or biochemical tests, we estimated the proportion of the two types of *E. coli* bacteria (indole-negative and β-D-glucuronidase-negative strains) in the analyzed samples.

With the reference standard, the proportion of *E. coli* bacteria from the isolated bacteria pool not expressing indole production was determined from bacteria having been subcultured on Tergitol agar with lactose-positive and oxidase-negative results (bacteria counted as coliforms). Of 308 strains not producing indole identified by MALDI-TOF mass spectrometry or biochemical tests (76 strains from 35 different Phase 2 samples and 232 strains from 101 Phase 3 samples), 14 strains (4.5%) proved to be *E. coli* counted by the NF EN ISO 9308-1 method as coliforms and not *E. coli.*

With regard to the Colilert®-18/Quanti-Tray® method, we were able to determine the proportion of *E. coli* bacteria not expressing β-D-glucuronidase activity among the bacteria that the method had counted as coliforms. Of 415 strains fully identified by MALDI-TOF mass spectrometry or biochemical tests, isolated from non-fluorescent yellow wells (176 strains from 45 Phase 2 samples and 239 strains from 101 different Phase 3 samples), 37 strains (8.9%) proved to be *E. coli* counted by the method as coliforms.

These results show that both methods are likely to underestimate *E. coli* bacteria in samples, with a slightly greater trend for underestimation when the Colilert®-18/Quanti-Tray® method is used. At this point of the assessment, the proportions indicated above should be considered with a certain amount of caution. As mentioned previously, these findings were obtained from tests performed using each method (detection of indole production or fluorescence in the Colilert®-18/Quanti-Tray® wells) and not following proof of a biochemical or molecular mechanism indicating the presence of these two metabolic pathways. There may be estimation biases possibly related to the methodological procedure followed. For example, for the Colilert®-18/Quanti-Tray® method, β-D-glucuronidase activity is detected after an incubation period of 18 h, which means that if this activity is only revealed later, the *E. coli* bacteria pool not detected after the 18-hour period will not be counted as *E. coli* but as coliform bacteria instead, thereby biasing the previously determined proportion.

In conclusion, this study has demonstrated that both methods are likely to underestimate an *E. coli* bacteria pool, though the Colilert®-18/Quanti-Tray® method is slightly more likely to do so. The negative mean relative difference disadvantaging the Colilert®-18/Quanti-Tray® method for Phase 2 samples can be explained by its failure to detect *E. coli* bacteria in 30% of the samples tested using this method.

3.2.4 Statistical analysis taking into account only enumerations >1 UFC or MPN enumeration results confirming the presence of *Escherichia coli* in the samples by both methods

We have observed that the use in statistical analysis of enumeration sets for which one of the results obtained using one of the methods leads to a count of zero could be problematic for later analysis. While the NF EN ISO 17994:2004 standard notes that this type of data is inevitable, the inclusion of too many unbalanced enumeration sets should be avoided when making recommendations.

As regards assessment using *E. coli* bacteria, it is virtually impossible to avoid low enumerations, a situation that may lead to one of the methods giving a zero result, especially for naturally contaminated environment samples. The proportion of *E. coli* bacteria among total coliforms in natural or spiked water is unbalanced, fewer *E. coli* being found than coliforms (*E. coli* being a

mere subpopulation of this vast group). The results relating to *E. coli* enumerations are closely linked to the target value set for coliform bacteria. Therefore, if a value for coliforms is established according to the NF EN ISO 17994 standard recommendations, the counts for *E. coli*, which are enumerated during the same analytical sequence, will be below this target. To obtain an equivalent target value, it would be necessary to increase the proportion of spiked water samples to test with the contaminated solution, which would lead to increased numbers of both coliforms and interfering flora in the samples. An excessive number of interfering flora could obviously bias the assessment by decreasing the detection performance of the standard method (and even the method's scope).

In the various statistical analyses performed thus far, only the sets with zero counts obtained using both methods were eliminated from analysis. To reduce the impact of unbalanced enumeration sets, the decision was made to carry out a new statistical analysis on positive enumeration sets (≥1 CFU or 1 MPN) obtained using both methods. This statistical analysis was performed first by taking into account the enumeration results obtained using the Colilert®-18/Quanti-Tray® method after confirmation of presumed positive wells using standardized tests from the NF EN ISO 9308-1 prescriptive method. The results are shown in Table 6. Next, to limit overestimation of final enumerations with the NF EN ISO 9308-1 method, these results were corrected by taking into account the results of supplementary tests used to characterize β-D-glucuronidase production in isolated bacteria. The results are shown in Table 7.

Table 6 : Results of comparison tests performed on *E. coli* enumerated using the Colilert®-18/Quanti-Tray® method (confirmation of presumed positive wells) compared to the enumeration results using the NF EN ISO 9308-1 standard method. Enumeration sets for which neither method detected any *E. coli* in samples were discarded.

Laboratories & grouping	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
Phase 2 + 3	152	-36.41	102.71	8.33	16.66	-
phase 2	24	-33.08	118.86	24.26	48.53	o
phase 3	128	-37.03	99.91	8.83	17.66	-

+ : Colilert®-18/Quanti-Tray® method with a higher detection performance
- : Colilert®-18/Quanti-Tray® method with a lower detection performance
O : Results not permitting a conclusion due to a lack of data
MSD : Method with No Difference

Table 7 : Results of comparison tests performed on *E. coli* enumerated using the Colilert®-18/Quanti-Tray® method (confirmation of presumed positive wells) compared to the enumeration results using the NF EN ISO 9308-1 standard method after correction of enumerations by adding a test to detect β-D-glucuronidase activity. Enumeration sets for which neither method detected any *E. coli* in samples were discarded.

Laboratories & grouping	Number of samples	Mean relative difference (%)	Standard deviation (%)	Standard error (%)	U (%)	Overall test conclusion
Phase 2 + 3	139	-22.48	102.61	8.70	17.41	-
Phase 2	23	-16.72	104.11	21.71	43.42	o
Phase 3	116	-23.62	102.73	9.54	19.08	-

+ : Colilert®-18/Quanti-Tray® method with a higher detection performance
- : Colilert®-18/Quanti-Tray® method with a lower detection performance
O : Results not permitting a conclusion due to a lack of data
MSD : Method with No Difference

Depending on the selected operating method, 139 to 152 samples of which approximately 15% were from Phase 2 were included in the various statistical analyses. The number of samples per laboratory ranged between 2 and 41.

Of the grouped enumerations, an overall negative trend disadvantaging the Colilert®-18/Quanti-Tray® method was observed, whether or not a supplementary test using the EN NF ISO 9308-1 standard to detect β-D-glucuronidase activity in *E. coli* bacteria was performed. The additional identification step (test detecting β-D-glucuronidase activity) and the correction of enumerations reduced the deviation between the mean relative differences from -36.41% to -22.48% when supplementary tests were performed.

Different trends were observed for the samples grouped by phase. For Phase 2 samples and contrary to Phase 3 samples, a conclusion can no longer be drawn due to an insufficient number of samples and low enumeration results.

In conclusion, the comparison of enumeration results excluding unbalanced sets confirms the initial overall trend observed in Section 3.1.2, namely that the two methods are not equivalent for the detection of *Escherichia coli* in water samples. For *Escherichia coli*, the detection performance of the Colilert®-18/Quanti-Tray® method is lower than that of the NF EN ISO 9308-1 standard.

4 Conclusions

The purpose of this study was to determine the equivalence of the Colilert®-18/Quanti-Tray® method to the NF EN ISO 9308-1 method on water samples that were representative of water treated and distributed in France. The approach consisted in following the NF EN ISO 17994 method recommended by the EMEG. This standard has been used in various equivalence studies carried out by other European Union member states. Equivalence was assessed using more than 200 samples, a quarter of which were from sites naturally contaminated with coliform and *E. coli* bacteria and three-quarters of which were artificially contaminated by surface water or DWTP wastewater spiked with the target bacterial population.

Samples were selected based on various criteria (type of water, treatment, plant size, national coverage, etc.) through the SISE-Eaux national database to ensure that samples were representative of water produced across France. Nine laboratories around France participated in the study after specific training on the method with a view to testing the method's robustness. Samples from naturally contaminated sites were chosen to test both methods under conditions similar to those during official monitoring controls. These samples were used to check that the method's scope suited the type of samples to be tested. It should be noted that there are few assessment results to date regarding this type of sample in equivalence dossiers submitted to the European Commission's Directorate General for the Environment (DG ENV). This type of sample is rare, thereby making selection complex. Spiked water samples (artificially contaminated using environmental water containing the target bacteria) are typically used. Before spiking, these micro-organisms are generally treated with chlorine to simulate real-world situations. Several authors have noted that final test results can be impacted by the source of the water and whether an additional step to simulate stress on a population has been taken. To demonstrate any effect of sample preparation, this study systematically presents the results of the comparative statistical analysis performed on either the enumeration results from grouped samples (naturally contaminated Phase 2 samples + artificially spiked Phase 3 samples) or by taking into account the enumeration data for each sample type (Phase 2 or Phase 3 samples).

The results clearly show that the Colilert®-18/Quanti-Tray® method provides much higher coliform counts than the NF EN ISO 9308-1 method when all the samples are grouped together. Detection performance was increased by more than 41% for this bacteria group. Overall, the results obtained in this study were consistent with the trends shown in various European studies. It is difficult to precisely explain the reasons for this trend. As mentioned in Section 3.1.1, it could be explained by better bacterial growth in the liquid chromogenic medium or by the presence of interfering flora that worsens results from the NF EN ISO 9308-1 method. There was greater variability in the results concerning samples from contaminated sites (Phase 2), thus attenuating the final test conclusion. For these samples, the equivalence between the two methods was confirmed and there is no difference between them. These different results enable us to conclude that the Colilert®-18/Quanti-Tray® method is equivalent to the NF EN ISO 9308-1 method for the representative water types tested for coliform bacteria.

For *E. coli* bacteria, the assessment results are not as clear cut and depend on the type of samples used for statistical analysis and the test methodology applied to confirm their presence in water samples.

The initial findings of the equivalence study on Phase 2 and Phase 3 samples using the NF EN ISO 17994:2004 method showed that the Colilert®-18/Quanti-Tray® method was not equivalent to the NF EN ISO 9308-1 standard due to lower bacteria counts than those obtained using the reference method. Overall, these results are not consistent with those obtained in several European dossiers, which have demonstrated that the methods are equivalent. However, they are

consistent with the results published in the Spanish dossier, which is the most complete to date in this field.

The negative trend measured was shown to be linked to an overestimation of *E. coli* bacteria in samples tested with the NF EN ISO 9308-1 standard. Adding supplementary tests to identify β-D-glucuronidase activity in indole-positive bacteria and therefore correct the counts resulted in no conclusion being able to be drawn with regard to the statistical test trend established for all the samples. The notion of overestimation of *E. coli* by the NF EN ISO 9308-1 standard is described in a technical corrigendum issued by the ISO/TC147 Technical Committee in 2007. It could be due to the presence of *Klebsiella oxytoca* strains, which are thermotolerant and can produce positive results during an indole test. To minimize false positives, the technical committee recommends carrying out supplementary tests to detect β-D-glucuronidase activity. The use of these tests is not recommended by the EMEG. The possibility of overestimation was reported in various equivalence dossiers submitted to DG ENV. In the Spanish dossier, the results from these tests (based on confirmation of β-D-glucuronidase activity) on a pool of colonies used to phenotype the bacteria isolated using biochemical tests provided the justification to prove that the Colilert®-18/Quanti-Tray® method was equivalent to the NF EN ISO 9308-1 method despite a negative initial conclusion. In this study, participating laboratories systematically carried out supplementary tests on all samples analyzed using the NF EN ISO 9308-1 standard and on at least three detected *E. coli* colonies, thereby enabling the correction of all available enumeration results and statistical tests to be performed. Due to the small number of *E. coli* colonies tested, it is possible that the enumerations provided by the NF EN ISO 9308-1 standard method could have led to imprecise results.

Based on data obtained using mass spectrometry or biochemical tests to identify isolated bacteria and only on the results of tests for each method (indole-positive bacteria for the reference standard and β-D-glucuronidase activity for the Colilert®-18/Quanti-Tray® method), we observed that both methods were likely to overestimate a non-negligible number of *E. coli* bacteria, with a greater trend for overestimation by the standard method (21.1% versus 31.8% for the Colilert®-18/Quanti-Tray® and NF EN ISO 9308-1 methods, respectively).

It should be noted that while supplementary tests to detect β-D-glucuronidase activity provide a second confirmation step to limit overestimation of bacteria isolated using the reference standard, these tests could also lead to incorrectly invalidating the presence of *E. coli* bacteria detected using the standard but not expressing β-D-glucuronidase activity. In the natural environment, certain *E. coli* phenotypes do not express β-D-glucuronidase activity, such as enterohemorrhagic strains of shiga toxin-producing *E. coli*, which are present in environmental water. These bacteria may then be counted as coliforms in test results, which could lead to a very real underestimation of risk if they are present in water. The notion of underestimation of these bacteria is clearly addressed in the scope of the new ISO 9308-2 standard, whose principle is based on the method assessed in this dossier.

The notion of underestimation of the number of *E. coli* bacteria, which can in turn lead to an underestimation of health risks, has received little attention in European dossiers. In reality, based on identification data for bacteria isolated by both methods and taking into account the results of the various tests conducted, it has been shown that both methods are likely to underestimate a pool of *E. coli* bacteria in samples, with a slightly higher likelihood for the Colilert®-18/Quanti-Tray® method (8.9% versus 4.5% for the NF EN ISO 9308-1 method). The percentages given above should be considered with caution as they take into account identification data and results from different tests performed using each methodology. Little is known about the proportion of *E. coli* bacteria in the environment whose phenotypic traits cause them to be underestimated by the tests implemented by both methodologies; indeed, this is a topic of controversy within the scientific community.

With regard to the samples from contaminated sites (Phase 2), this study demonstrated that even after correction of the enumeration data obtained using the NF EN ISO 9308-1 standard through additional testing to detect β-D-glucuronidase activity, the trend revealed by the statistical test remained unchanged. When applied to these samples, the Colilert®-18/Quanti-Tray® method results in lower enumerations. This study has demonstrated that this trend is linked to an underestimation of the number of *E. coli* bacteria detected in this type of sample without being able to explain why. Conversely, for the Phase 3 samples that were artificially spiked with stressed target bacteria solutions from surface water or DWTP wastewater, the proportion of samples with underestimated counts is equivalent when the enumeration values obtained using the reference standard are not corrected, with an inversion disadvantaging the standard after correction. These inconsistent results depending on the sample type doubtless reflect the presence of bacterial populations with different phenotypes.

Through this study, we observed that unbalanced enumeration datasets (zero counts obtained by one or other of the two methods), regardless of the reference method, could be problematic when drawing conclusions based on the statistical test. While these data qualify the robustness of each method assessed in operating conditions similar to real-world conditions, this type of unbalanced set does not permit a conclusion to be drawn on the extent of the relative difference measured for target populations in each tested sample, and therefore to assess each method's performance. The proportion of samples resulting in uneven enumerations is not indicated in current European dossiers. The raw data have not been systematically reported in the published studies; only the mean relative differences are provided. As mentioned previously, it is virtually inevitable that such datasets will be obtained for *E. coli* bacteria subpopulations in such a vast group of coliforms. For these bacteria, obtaining a predefined target value is complex because it depends on the spiking proportion (volume/volume) with the selected environmental water. To obtain a high target value, it would have been necessary to increase the spiked proportion. However, doing so comes with a risk of increasing interfering bacterial flora, thereby impacting the results of one of the two methods and biasing the assessment study. The use of pure solutions with *E. coli* bacteria could resolve this issue. However, the use of pure solutions should preferably never be used in equivalence assessment studies or only used as a last resort, as they are not representative of bacteria found naturally in the environment.

To ensure a "strict" comparative assessment approach by using samples containing the target bacteria detected using both methods, the decision was made to voluntarily exclude unbalanced enumeration sets from Phase 2 and Phase 3 samples. Depending on the methodology, 139 to 152 samples were shown to contain E. coli detected by both methods, of which 15% were from Phase 2. Of these samples, the initial negative trend against the Colilert®-18/Quanti-Tray® method was confirmed, both with and without supplementary tests to confirm β-D-glucuronidase activity in indole-producing bacteria isolated using the reference standard. The significant variations of almost 100% obtained during this study can be explained by the considerable proportion of samples with low bacterial counts (<10 colonies) obtained by one of the methods. To reduce these variations, new statistical analyses were performed taking into account the >5 CFU or MPN enumeration values (obtained by both methods) and confirmed the negative trend that disadvantages the Colilert®-18/Quanti-Tray® method (variation reduced by around half).

It is difficult to say exactly why lower counts were obtained for *E. coli* bacteria in the tested samples with the Colilert®-18/Quanti-Tray® method. It is not possible to indicate whether these results are exclusively linked to a methodological problem with the Colilert®-18/Quanti-Tray® method (insufficient incubation time, unsuitable growth medium, etc.) or the *E. coli* phenotype proportions (percentage of indole-positive or -negative *E. coli*, β-D-glucuronidase-positive or -negative *E. coli*) found in French water that are different from those found in water tested in other European countries.

Consequently, given these different results and based on the knowledge acquired through this comparative study carried out on representative samples from naturally contaminated sites and artificially contaminated samples, we do not recommend the use of the Colilert®-18/Quanti-Tray®

method under the conditions and according to the operating method described by the supplier to detect *E. coli* bacteria in water samples for official monitoring.

Date of validation of the report: 9 February 2017.

5 References

5.1 Standards

NF EN ISO 9308-1 (september 2000) Water quality - Enumeration of Escherichia coli and coliform bacteria - Part 1 : membrane filtration method for waters with low bacterial background flora

NF EN ISO 17994 (april 2014) Water quality - Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods

5.2 Legislation and Regulations

MINISTERE DE LA SANTE, DES FAMILLES ET DES PERSONNES HANDICAPEES. Arrêté du 17 septembre 2003 relatif aux méthodes d'analyse des échantillons d'eau et à leurs caractéristiques de performance. Journal officiel de la République Française, n°258, page 19027, texte n° 20, du 7 novembre 2003. En ligne <https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000247611&fastPos=1&fastReqId=1449584004&categorieLien=id&oldAction=rechTexte>

Council of the European Union. Council Directive 98/83/EC of 3 november 1998 on the quality of water intended for human consumption. Official Journal of the European Communities, L330, page 32, of the 5 december 1998. En ligne <http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1508143657404&uri=CELEX:31998L0083>

ANNEXES

Annex 1 : Request Letter

Reçu par
28 MAI 2010
AFSSA - LERH

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

Ministère de la Santé et des Sports

Direction générale de la santé

Paris, le 25 MAI 2010

Sous direction *Prévention des risques liés à l'environnement et à l'alimentation*
Bureau *Qualité des eaux*

DGS/EA4 – N° 224

Le Directeur général de la santé

à

Monsieur le Directeur du Laboratoire d'Etudes et de Recherches en Hydrologie (LERH – AFSSA)
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Objet : Demande d'appui scientifique et technique – Equivalence du test de potabilité des eaux Colilert®-18 / Quanti-tray® pour la recherche et le dénombrement des *Escherichia coli* et des coliformes dans les eaux destinées à la consommation humaine par rapport à la méthode de référence NF EN ISO 9308-1

N/Réf : DGS N° 100019 (*numéro de dossier à rappeler dans toute correspondance*)

La directive 98/83/CE du 3 novembre 1998 relative à la qualité des eaux destinées à la consommation humaine (EDCH) prévoit la recherche de paramètres microbiologiques et fixe, à cette fin, les méthodes d'analyse, dites de référence, à utiliser (annexe III, partie 1) tout en spécifiant que des méthodes alternatives peuvent être utilisées « *à condition qu'il puisse être démontré que les résultats obtenus sont au moins aussi fiables que ceux obtenus par les méthodes spécifiées à l'annexe III, partie 1* ». La directive 98/83/CE ne précisant pas de référentiel normatif ou de guide d'évaluation des méthodes alternatives, la Direction générale de l'Environnement de la Commission Européenne (CE) en charge de l'eau d'alimentation a mandaté en 2007 un groupe consultatif, l'European Microbiology Group (EMG), composé d'experts en microbiologie, afin d'évaluer les dossiers de demande d'équivalence des méthodes alternatives. La direction générale de la santé (DGS) a chargé l'Agence française de sécurité sanitaire des aliments (AFSSA) et votre laboratoire d'y représenter la France.

Pour mémoire, l'EMG s'appuie sur des lignes directrices, basées sur l'unique norme internationale existante définissant une procédure d'évaluation dans le domaine de l'eau, la norme NF EN ISO 17994 : 2004. Il appartient aux industriels souhaitant commercialiser des méthodes alternatives, de fournir les preuves de l'équivalence. Lorsqu'elle est accordée, cette équivalence est reconnue uniquement dans l'Etat-membre qui a porté le dossier auprès de la CE. Parallèlement, l'Agence française de normalisation (AFNOR) a initié en 2004 un projet de validation par tierce partie des méthodes alternatives. Initialement, le groupe de travail mis en place par l'AFNOR-Validation avait décidé de retenir le référentiel NF EN ISO 17994 : 2004 et de le compléter. Mais finalement, le groupe de travail a retenu la norme NF EN ISO 16140, utilisée pour la validation des méthodes microbiologiques alternatives pour les aliments.

Le rapport remis par votre laboratoire à la suite de ma demande d'appui scientifique et technique relatif à l'équivalence des méthodes alternatives par rapport aux méthodes de référence dans le domaine de l'eau d'alimentation (saisine 2007-SA-0192, juin 2007) met en évidence un certain nombre de différences entre le référentiel de l'AFNOR-Validation et celui utilisé par l'EMG, la principale étant l'absence d'essais sur des échantillons représentatifs des eaux distribuées en France dans le référentiel de l'AFNOR-Validation.

1/2

Bien que la DGS ait régulièrement alerté l'AFNOR-Validation et la société Idexx Laboratories sur ces divergences et le risque de non reconnaissance par la DG Environnement de dossier ne suivant pas le référentiel de l'EMG, celle-ci a déposé fin 2008 une demande de validation de la méthode Colilert®-18 / Quanti-Tray® pour l'eau de consommation humaine auprès de l'AFNOR-Validation. L'AFNOR-Validation a validé cette méthode début 2010 selon son propre référentiel.

Lors de la réunion du 10 mars 2010 avec le cabinet de Mme la Ministre, il a été convenu de la nécessité de tester la méthode sur différentes eaux françaises (EDCH, eaux brutes d'origine superficielle ou souterraine utilisées pour la production d'EDCH). Le choix des types d'eaux sera déterminé à partir de l'examen des résultats des essais menés par la Société Idexx sur différentes eaux européennes.

Aussi, je sollicite votre appui scientifique et technique pour réaliser cette étude complémentaire en mettant en œuvre les moyens que vous jugerez nécessaires. La Société Idexx fournira les tests de potabilité des eaux Colilert®-18 / Quanti-tray®. Un rapport intermédiaire de cet appui scientifique et technique est attendu pour le mois de septembre 2010. Votre avis est d'autant plus important qu'il n'est pas envisagé de consulter le groupe EMG.

Je vous précise, enfin, que ce dossier est enregistré à la Direction générale de la santé sous l'intitulé suivant :

DEMANDE D'APPUI SCIENTIFIQUE ET TECHNIQUE SUR L'EQUIVALENCE DU TEST DE POTABILITE DES EAUX COLILERT®-18 / QUANTI-TRAY® POUR LA RECHERCHE ET LE DENOMBREMENT DES *E. COLI* ET DES COLIFORMES DANS LES EAUX DESTINEES A LA CONSOMMATION HUMAINE PAR RAPPORT A LA METHODE DE REFERENCE NF EN ISO 9308-1

et porte le numéro : DGS N° 100019.

Jocelyne BOUDOT
Sous-directrice de la prévention des risques liés à l'environnement et à l'alimentation

2/2

Notes

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