



**Report of the third inter-laboratory study
on the enumeration of *Escherichia coli*
in Live Bivalve Molluscs (PT32) –
2022**

Edited by:

Margherita Montalbano Di Filippo, Arianna Boni, Arnold Knijn, Guendalina Fornari Luswergh, Paola Chiani, Fabio Minelli, Federica Gigliucci, Valeria Michelacci, Gianfranco Brambilla, Stefano Morabito, Rosangela Tozzoli

1. INTRODUCTION AND OBJECTIVES OF THE STUDY

As an effect of Regulation (EU) 222/2018, the European Union Reference Laboratory for *Escherichia coli* (EURL-VTEC) has taken over the activities carried out by the former EU Reference Laboratory for monitoring the bacteriological contamination of bivalve molluscs as regards the analytical tests for *E. coli*. In this specific case, the EURL has the task (i) to assess the performance of the designated National Reference Laboratories (NRLs) for the microbiological contamination of bivalve molluscs in the EU and (ii) to assist the EFTA Member States, EU Candidate Countries and third countries in using the method for the enumeration of *E. coli* in live bivalve molluscan shellfish.

The EU reference method for enumeration of *E. coli* in live bivalve molluscs is represented by ISO 16649-3 **“Microbiology of the food chain - Horizontal method for the enumeration of β -glucuronidase-positive *Escherichia coli* Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide”**.

This scheme is intended to provide proficiency testing (PT) samples for laboratories performing the analysis of live bivalve molluscs, from production areas in accordance with Regulation (EC) N° 854/2004 and from throughout the production chain in accordance with Regulation (EC) N° 2073/2005.

The third round organized by the EURL-VTEC, PT32, was carried out on commercial common live mussels, without any additional contamination. The live bivalve molluscs were obtained from establishment labeled as Area C, with no further treatments for depuration applied.

The main objective of this study was to provide data on the proficiency of the laboratories for ISO 17025 accreditation purposes, in the application of ISO 16649-3.

This document represents the evaluation report of the PT32 study. The study was conducted according to the International Standard ISO/IEC 17043:2010 “Conformity assessment – General requirements for proficiency testing”.

2. PARTICIPANTS

Twenty-five NRLs, representing 23 EU Member States plus 2 non EU (one EFTA country), accepted the invitation to participate.

Each NRL received its own individual laboratory numerical code, which was used to label the laboratories in the result tables.

The Laboratories participating in the study were:

Austria	Austrian Agency for Health and Food Safety, Institute for Food Safety (AGES)
Belgium	Laboratory of Foodborne Pathogens (SCIENSANO)
Bulgaria	National Diagnostic and Research Veterinary Institute (NDRVMI)
Croatia	Croatian Veterinary Institute Laboratory for food and feed microbiology (RIJEKA)
Croatia	Croatian Veterinary Institute HVI - VETERINARSKI ZAVOD SPLIT
Croatia	Laboratory for Food Microbiology, Croatian Veterinary Institute (ZAGREB)
Cyprus	Laboratory for the Control of Food of Animal Origin (LCFAO)
Denmark	Microbiological laboratory Ringsted (FVST)
Germany	NRL <i>E. coli</i> , Bundesinstitut für Risikobewertung (BfR)
Greece	Department of Food Hygiene of Athens (NRL Greece for <i>E.coli</i> in LBM)
Greece	Veterinary Laboratory of Kavala, Ministry of Rural Development & Food, Kavala
Ireland	Shellfish Microbiology Unit, Marine Institute (MARINE)
Italy	Istituto Superiore di Sanità (ISS)
Italy	IZS Umbria e Marche, Sezione di Ancona
Latvia	Institute of Food safety, Animal health and environment - BIOR
Netherlands	National Institute for Public Health and the Environment - RIVM
Netherlands	Wageningen Food Safety Research - WUR
Norway	Institute of Marine Research - HMR
Poland	National Veterinary Research Institute (NVRI)
Romania	Institute for Diagnoses and Animal Health (IDAH)
Slovakia	State veterinary and food institute - SVPU
Slovenia	University of Ljubljana, Veterinary Faculty - Unit for food safety
Spain	Centro Nacional de Alimentación - AESAN
Sweden	Swedish Food Agency, The Biology department
UK	Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS)

3. MATERIALS AND METHODS

3.1. Sample preparation

A single batch of 25 Kg of common mussels (*Mytilus gallusprovincialis*) were collected from a commercial harvesting area class C in Italy, on 17/05/2022. Prior to packing, the shellfish were placed in a large, disinfected container and thoroughly mixed. Samples of approximately 500 g of randomly selected mussels were aliquoted, and the parcels were shipped to the participating laboratories by courier.

3.2. Sample distribution and examination

Each individual sample was packed in a polystyrene refrigerate box containing one plastic pocket with a stomacher bag containing common mussels and a disposable data-logger for checking the shipment temperature. A total of 25 boxes were shipped and 25 samples were despatched on 17/05/2022 to the participants. Participants were requested to analyse the samples as soon as possible after receipt and within 24 hours of arrivals, following the Part 3 of the method ISO 16649.

3.3. Collection and Elaboration of the NRLs Results

The results were submitted through a website pre-arranged by the EURL for *E. coli*. The instruction on how to report the results was sent by E-mail to all the participants' laboratories. In particular, the participants were requested to fill in both (i) the Evaluation form (arrival date/time/condition/temperature, storage temperature, analysis' start date/time and notes field in order to specify any problem with the samples delivery/packaging) and (ii) the Sample Results section.

3.4. Analysis of the NRLs' results

3.4.1 Parameters used for the assignment of the scores

A scoring system has been used to assess the participants' performance. *E. coli* MPN scores allocated to participants are detailed in the Table 1.

Table 1: *E. coli* MPN scores - dark green boxes represent the maximum value' score (12), the dark red ones the minimum (0) (graduation colour bar)



Results	Returning of results	Score allocated		Score
		Replicate 1	Replicate 2	
Both replicates MPN results are within the expected range*	2	5	5	12
One replicate MPN result reported is outside the expected range and falls between the median \pm 3 SD and the median \pm 5 SD value	2	5	2	9
Both replicates MPN results reported are outside the expected range and fall between the median \pm 3SD and the median \pm 5 SD value	2	2	2	6
One replicate MPN result reported is outside the median \pm 5 SD value	2	5	0	7
Both replicates MPN results reported are outside the median \pm 5 SD value	2	0	0	2
Single MPN result reported only **	2	5	0	7
Tube combination inconsistent with MPN reported (only one replicate)	2	7		9
Tube combination inconsistent with MPN reported (both replicates)	2	5		7
Sample not examined or results returned late, or no explanation received	0	0	0	0
High censored result (i.e. MPN => 18000 per 100g)		Score not assigned		

***expected range:** Participants' Median \pm 3SD – SD stands for Theoretical Standard Deviation = 0,24
 The expected range values are reported in detail in **Table 3** (Results Section).

**** Single result report only in the expected range.**

4. RESULTS

4.1. Reference results

Ten randomly selected samples from the shipment lot consisting in approximately 100 g of mussels each, were analyzed in duplicate on 18/05/2022 following the ISO 16649-3 method. Sample homogeneity was assessed according with the requirements of ISO 17043:2010. The sample material distributed was considered sufficiently homogenous. The reference results are reported in Table 2.

Table 2: *E. coli* MPN/100 g reference results.

Sample Number - Type	Range (<i>E.coli</i> MPN/100g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample 1 - Mussels	0	78	32,5	1,71E+02	6,19	5,15E+02	2,05

SDT stands for Theoretical Standard Deviation = 0,24

Note: 1,71E+02 stands for $1,71 \times 10^2$ which is 1,71 times 10 (E) to the 2nd power (+02)

4.2. Participants' results

Performance assessment was carried out according to the scoring parameters reported in Table 1 – Section Materials and Methods. Participants' results and scores are shown in Tables 3, 4, 5 and Figure 1.

The samples used in this PT displayed a very low level of natural contamination with *E. coli*. The reference results were also low and a further reduction was expected due to the shipment conditions of the animals. Accordingly, the majority of the participants reported very low *E. coli* MPN values (<18 MPN/100g) (results in Table 5). As a consequence, with many reported results equaling 0, the participants' median and upper and lower limits (± 3 SD and ± 5 SD) were not calculated (Table 4) and the analysis was carried out using the references' median, upper, and lower limits only (± 3 SD and ± 5 SD).

Considering how above, the proficiency of the participants reporting <18 MPN could not be assessed and the scoring applied has to be considered as indicative. A satisfactory proficiency was instead assigned to all the laboratories reporting a MPN>20.

Table 3: Summary statistics of participants' results (total results received 25 laboratories).

<i>E. coli</i> MPN – summary statistics'	Sample 1
Participants reporting duplicate results for <i>E. coli</i> MPN	25
Participants reporting a single MPN result	0
Participants reporting both replicate MPN results within expected range*	4/25
Participants reporting both replicate MPN results outside expected range	16/25
Participants reporting one replicate MPN result outside expected range	5/25
Participants reporting one replicate MPN results as censored results	0
Participants reporting both replicate MPN results as censored results	0
Participants reporting tube combination and/or MPN results inconsistent with ISO 7218**	0

*expected range: **note without Participants' Median $\pm 3SD$, the expected range is Reference Median $\pm 3SD$.**

**points deducted from participants returning results with incorrect tube combinations and/or inconsistent with ISO 7218.

Table 4: *E. coli* MPN/100 g participants' results

Sample Number - Type	Range (<i>E.coli</i> MPN/100g)		Median	Median $\pm 3SDT^*$		Median $\pm 5SDT^*$	
	Minimum Value	Maximum Value					
Sample 1 - Mussels	0	6,8E+01	0	-	-	-	-

Note: The median and upper and lower limits ($\pm 3 SD$ and $\pm 5 SD$) were calculated from participants' results. SDT calculations were based on the inherent variability of the 5 x 3 MPN method ($0.24 \log_{10}$).

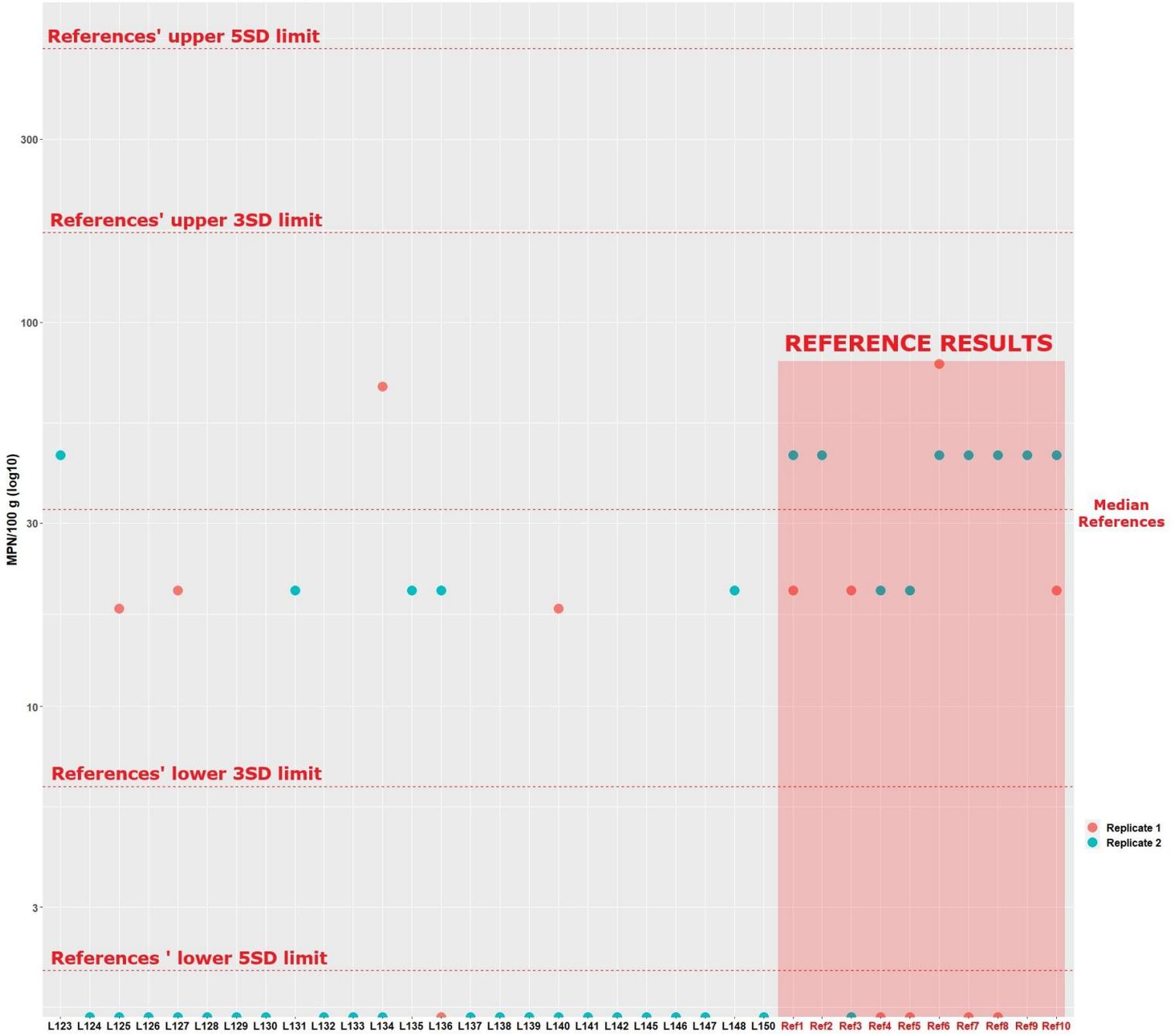
Reference values were excluded from the calculation of the participants' median.

Table 5. Details of the analysis performed by the Laboratories and scores obtained; dark green boxes represent the maximum value' score (12), the dark red ones the minimum (0) (graduation colour bar).



Lcode	Shipment Date	Analysis Start Date and Sample' Temperature (°C)	<i>E.coli</i> MPN/100g				Score
			Replicate 1	Rarity Category	Replicate 2	Rarity Category	
L123	17/05/2022	18/05/2022 - 4°C	45	1	45	1	12
L124	17/05/2022	19/05/2022 - 13°C	0	1	0	1	2
L125	17/05/2022	18/05/2022 - 12°C	18	1	0	0,092	7
L126	17/05/2022	19/05/2022 - 23°C	0	1	0	1	2
L127	17/05/2022	18/05/2022 - 14°C	20	1	0	1	7
L128	17/05/2022	18/05/2022 - 13°C	0	1	0	1	2
L129	17/05/2022	18/05/2022 - 5°C	0	1	0	1	2
L130	17/05/2022	18/05/2022 - 12°C	0	1	0	1	2
L131	17/05/2022	18/05/2022 - 16°C	20	1	20	1	12
L132	17/05/2022	19/05/2022 - 14°C	0	1	0	1	2
L133	17/05/2022	19/05/2022 - 22°C	0	1	0	1	2
L134	17/05/2022	18/05/2022 - 13°C	68	0,354	0	1	7
L135	17/05/2022	18/05/2022 - 13°C	20	1	20	1	12
L136	17/05/2022	18/05/2022 - 9°C	0	1	20	1	7
L137	17/05/2022	20/05/2022 - 21°C	0	1	0	1	2
L138	17/05/2022	19/05/2022 - 16°C	0	1	0	1	2
L139	17/05/2022	19/05/2022 - 7°C	0	1	0	1	2
L140	17/05/2022	18/05/2022 - 7°C	18	1	0	1	7
L141	17/05/2022	18/05/2022 - 6°C	0	1	0	1	2
L142	17/05/2022	19/05/2022 - 13°C	0	1	0	1	2
L145	17/05/2022	18/05/2022 - 9°C	0	1	0	1	2
L146	17/05/2022	18/05/2022 - 7°C	0	1	0	1	2
L147	17/05/2022	18/05/2022 - 12°C	0	1	0	1	2
L148	17/05/2022	20/05/2022 - 22°C	20	1	20	1	12
L150	17/05/2022	18/05/2022 - 14°C	0	1	0	1	2

Figure 1: Results' Dot Graph - Sample 1 Mussels



Note: The median and upper and lower limits (± 3 SD and ± 5 SD) were calculated only from references' results. Sample replicates (Replicate 1 and Replicate 2) are represented by red and blue dots.

5. CONCLUDING REMARKS

1. Twenty-five laboratories participated in the study and all of them returned the results.
2. The contamination of the molluscs with *E. coli* was that naturally present in the animals and was very low; in addition, a further reduction could be observed probably due to the shipment conditions, although the analysis of the shipment temperatures failed to demonstrate an effect on the reported results (Table 5).
3. The low contamination level was the main feature affecting the analysis of the participants' proficiency, which could not be calculated for those reporting MNP values <18.
4. Nine of the 25 participants (36%) reported an MPN>20 and all obtained the highest score. The remaining 16 laboratories (64%) reported results that did not allow to assess their performance.
6. The observed randomness in the contamination levels of animals collected in the wild and the frailty of the samples made up by live molluscs suggests using frozen or lyophilized materials for the next PTs as test matrix..