

Modification of the standard method used in the United Kingdom for counting *Escherichia coli* in live bivalve molluscs

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Summary: *The standard method for counting Escherichia coli in live bivalve molluscs is labour intensive and takes three days to obtain a result. Modifications to the standard method were investigated in a collaborative trial conducted in five centres. No significant difference was found between results based on the presence of acid at 24 hours (h) in first stage tests and those based on the presence of acid and gas after 48 h (standard method). The use of the chromogenic medium BCIG (5-bromo-4-chloro-3-indolyl-β-D glucuronide) agar incubated at 44°C to confirm first stage tests was also found to give equivalent results to conventional confirmation tests. The preferred, modified method removes the presence of gas as a criterion of detection, uses a chromogenic agar medium to confirm the presence of E. coli, and gives results within 48 h. A distribution of simulated samples and selected strains of E. coli to other laboratories using the PHLS external quality assurance scheme for shellfish found no significant difference between results obtained by the standard and modified methods.*

Key words:
chromogenic compounds
Escherichia coli
food microbiology
methods
shellfish

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Introduction

The classification of harvesting areas for bivalve molluscs in the United Kingdom (UK) is based on the level of *Escherichia coli* detected in shellfish flesh, as defined in EU Directive 91/492/EEC¹. The directive states that a five tube, three dilution most probable number (MPN) method should be used for testing, but does not give precise details¹. MPN methods are based on the probability of finding growth after a series of cultures of successive dilutions of a sample in a liquid medium.

A standard method which uses a two stage, five tube, three dilution MPN was adopted in the UK in 1992². In the first stage of the method minerals modified glutamate broth (MMGB) is inoculated with dilutions made from a homogenate of the shellfish flesh, then incubated at 37°C for up to 48 hours (h). Tubes showing the presence of acid and gas are then examined for the presence of *E. coli* by subculture to brilliant green bile broth (BGBB) and tryptone water (TW). These media are incubated at 44°C for 24 h and then examined for the presence of gas and the formation of indole respectively, positive results for both denoting the presence of *E. coli*. An additional option allows for the incorporation of 4-methylumbelliferyl-β-D-glucuronide into tryptone water to enable β-glucuronidase activity, which is reported to be exhibited by 95% to 97% of

E. coli strains^{3,4}, to be detected. Thus, determination of the presence of *E. coli* can be based either on the detection of gas and indole or on a combination of at least two of gas production, indole detection, and β-glucuronidase activity².

This method of counting *E. coli* is labour intensive, requires a substantial quantity of glassware and media, and can take up to three days to obtain a result. A desire to simplify and improve identification procedures led to a five centre collaborative trial to assess amendments to the method. Acid production is usually detected before gas production and therefore the first objective was to determine whether the period of incubation of MMGB tubes in the first stage could be reduced and whether gas production could be omitted as a criterion for forwarding tubes to the second, confirmatory stage. The second objective was to determine whether the use of a chromogenic agar to detect β-glucuronidase positive *E. coli* could replace the existing confirmatory tests. The use of indicators of β-glucuronidase activity for determining levels of *E. coli* has been reported in a wide range of foods⁵⁻⁹, including shellfish and other seafoods¹⁰⁻¹². The agar evaluated contained 5-bromo-4-chloro-3-indolyl-β-D glucuronide (BCIG agar, also referred to as X-GLUC), a chromogenic medium previously used to detect *E. coli* in foods^{9,10}. In the first trial, these modifications were investigated in parallel with the conventional method

by the five laboratories that took part in the study.

In a later trial, laboratories that take part in the PHLS external quality assurance (EQA) scheme for shellfish were asked to test one distribution of samples by the standard method (with traditional second stage tests) in parallel with the use of BCIG agar to confirm the presence of *E. coli* in MMGB tubes that showed acid production after incubation for 24 h. The results of both trials are presented and an adaptation of the standard method is proposed.

Methods

Examination of samples by the conventional (standard) method

Samples were processed and inoculated into tubes of MMGB according to the prescribed method². This involved preparation of a 1 in 10 homogenate of the shellfish meat and liquor and a further 1 in 100 dilution. An additional dilution of 1 in 1000 was prepared for samples suspected to contain high levels of *E. coli*. Five tubes of double strength MMGB were inoculated with 10 mL of 1 in 10 homogenate, five tubes of single strength MMGB were inoculated with 1 mL of 1 in 10 homogenate, and five further tubes of single strength medium were inoculated with 1 mL of the 1 in 100 dilution. Each tube contained an inverted Durham tube that allowed detection of gas. The tubes were incubated at $37 \pm 1^\circ\text{C}$, and examined after 24 ± 2 h and 48 ± 2 h for the presence of acid and gas. At the time when both acid and gas were detected the tubes were subcultured into brilliant green bile broth (BGBB) containing an inverted Durham tube and 1% tryptone water containing 0.005% MUG (TMUG) to confirm the presence of *E. coli*. These media were incubated at $44 \pm 1^\circ\text{C}$ for 24 ± 2 h. Tubes of BGBB were examined for the presence of gas. Tubes of TMUG were examined first for fluorescence due to β -glucuronidase activity with an ultraviolet lamp (366 nm wavelength). Kovac's reagent was then added to detect indole production, denoted by the formation of a red ring. The results were recorded and the MMGB tubes deemed to contain *E. coli* if at least two out of three tests were positive.

E. coli NCTC 10418 or NCTC 9001 and *Klebsiella aerogenes* NCTC 9528 were used as positive and negative controls, respectively.

Use of chromogenic medium

In addition to the conventional examination, all tubes of MMGB that showed acid production (whether or not they had produced gas) after 24 h or 48 h were subcultured to BGBB and TMUG and on to plates of tryptone bile agar containing 75 mg/L 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (BCIG agar; Oxoid CM 945 TBX agar, Lab M LAB162 tryptone bile glucuronide agar). Each plate was subdivided into a maximum of five sections. Each tube that contained acid was subcultured to duplicate BCIG agar plates; the positive and negative controls described above were also inoculated. One plate was incubated at $37 \pm 1^\circ\text{C}$ and the other at $44 \pm 1^\circ\text{C}$ for 24 ± 2 h. The

growth of blue-green colonies after incubation signified the presence of *E. coli*.

After all second stage tests for *E. coli* confirmation had been completed, the combinations of positive MMGB tubes for the three dilution ranges were recorded for confirmation of *E. coli* by the conventional method and by BCIG agar. The level of *E. coli* per 100 g was then determined from MPN tables².

Biochemical confirmation of *E. coli*

In order to confirm that the blue-green colonies were indeed *E. coli*, a total of 204 blue-green colonies were further characterised by the individual laboratories. These colonies included those isolated when conventional tests in BGBB and TMUG did not indicate the presence of *E. coli*. After subculture to obtain pure growth, the isolates were tested biochemically with the API 20E system (BioMerieux).

Study design

The five laboratories that took part in this trial (in England (Truro, Poole, and Ashford), Wales (Carmarthen), and Scotland (Aberdeen)) routinely participate in shellfish monitoring programmes and examine a range of shellfish types from harvesting sites of categories A, B, and C². Category A samples contain fewer than 230 *E. coli* per 100g, category B 230-4600 *E. coli*/100g, and category C 4600-46 000 *E. coli*/100g. Each laboratory was asked to examine 100 samples, but only one laboratory achieved this number. The total sample size was 360.

Each laboratory recorded the shellfish type and various positive tube combinations (five tubes at three dilutions) for each sample. For the first stage (MMGB), positive tube combinations were recorded according to the following criteria:

- acid (with or without gas) by 24 h
- acid and gas by 24 h
- acid (with or without gas) by 48 h
- acid and gas by 48 h

Tubes positive by (a) or (c) (which include tubes positive by (b) or (d)) were then tested by the three confirmatory methods. Positive tube combinations could then be calculated for the three confirmatory methods based on criteria (a), (b), (c), and (d). Results for each sample were entered on to worksheets and returned to the coordinating centre where the data were entered into an Epi Info¹³ database for analysis.

The methods (three confirmatory methods based

TABLE 1 Numbers and types of molluscs tested

Laboratory	Cockles	Mussels	Oysters	Other*	Total
1	3	63	26	8	100
2	36	25	9	–	70
3	26	9	–	–	35
4	3	22	31	2	58
5	4	35	56	2	97
Total	72	154	122	12	360

* scallops, winkles, razor shells

TABLE 2 Distribution of most probable number of counts for samples examined by the standard method

Laboratory	Category A			Category B			Category C	
	<20	20-90	130-230	250-500	600-1750	2100-4600	5400-16000	18000-46000
1	25	27	12	19	8	5	4	–
2	9	27	5	18	7	–	3	1
3	1	2	4	7	2	4	7	8
4	17	14	10	9	6	1	1	–
5	3	9	3	15	27	14	16	10
Total	55	79	34	68	50	24	31	19

on four first-stage interpretations) were then compared using the following criteria:

- (i) the percentage of samples whose tube combinations were identical or differed by just one tube out of the 15 (This statistic is used because a difference of one tube rarely affects sample's classification);
- (ii) the mean number of tubes positive after the first stage and the mean number confirmed;
- (iii) the number/percentage of samples falling into the same classification category and the number of samples whose category changed.

EQA trial

In the second distribution of the PHLS EQA shellfish scheme, 19 laboratories were sent three sample vials containing freeze-dried preparations simulating shellfish, report forms, instruction sheets, and agar powder for the preparation of BCIG agar plates. Participants were asked to examine the samples in triplicate by the standard method, and in addition to subculture MMGB tubes showing the presence of acid after 24 h to BCIG agar, followed by incubation at $44 \pm 1^\circ\text{C}$ for 24 ± 2 h. They were then asked to examine for the presence of blue-green colonies and record the results. Each sample when reconstituted was equivalent to 100 g of shellfish homogenate. The mean target values for two samples were 1.0×10^3 *E. coli* and 1.0×10^3 *Citrobacter freundii* per 100 g of shellfish. The mean target values for the third sample were 4.0×10^3 *E. coli* and 4.0×10^3 *C. freundii*/100 g and 10 *Salmonella haardt*/g. The level of *E. coli* was established by the scheme organisers by tests in triplicate on 15 vials and

the results were used to compare the data generated by the other laboratories.

Results

The number and types of bivalve molluscs examined at each laboratory are shown in table 1, and the distribution of MPN counts for samples examined by the standard method in table 2. Forty-seven per cent of samples were classified as category A, 39% as B, and 14% as C. Scrutiny of the counts and categories by shellfish type revealed similar distributions in each type.

Evaluation of modifications to the first stage

MMGB tests

The effect of using different criteria for forwarding MMGB tubes for confirmation was assessed by examining results from 5400 tubes derived from 360 samples. By the standard method the mean number of tubes forwarded (out of 15) per sample was 8.3. Removing the need for gas detection increased this number to 9.7, cutting the incubation time to 24 h reduced this number to 6.7 (containing acid and gas) and to 7.6 (acid with or without gas).

Forwarding fewer tubes is of importance only if the tubes missed are likely to be positive on confirmation. Table 3 shows that changing the criteria for forwarding tubes from those used in the standard method had little effect on the mean number of tubes confirmed. This was particularly so in the change from acid and gas at 48 h (mean tubes confirmed = 5.89) to acid (with or without gas) at 24 h (mean tubes confirmed = 5.85). Table 3 also gives the percentage of samples for which the use of different criteria for

TABLE 3 Comparison of *E. coli* detection in shellfish samples obtained by the standard method with detection using different criteria for forwarding tubes for confirmation

Criteria for forwarding MMGB tubes	Agreement with standard method in at least 14 out of 15 tubes (after confirmation by standard method)	Mean number of tubes out of 15 forwarded for confirmation	Mean numbers of tubes out of 15 confirmed by standard confirmation method
Acid and gas 48 h	standard (100%)	8.26	5.89
Acid (regardless of gas) 48 h	358/360 (99.4%)	9.71	5.94
Acid and gas 24 h	344/360 (95.6%)	6.69	5.67
Acid (regardless of gas) 24 h	357/360 (99.2%)	7.60	5.85

TABLE 4 Percentage agreement in at least 14 out of 15 tubes between the standard method of confirmation and the use of BCIG agar for determining levels of *E. coli*

First stage procedure	Confirmatory stage	Agreement with standard method in at least 14 out of 15 tubes number/total (%)
Acid and gas 48 h	BCIG at 37°C	329/360 (91.4)
Acid and gas 48 h	BCIG at 44°C	339/360 (94.2)
Acid (regardless of gas) 24 h	BCIG at 37°C	322/360 (89.4)
Acid (regardless of gas) 24 h	BCIG at 44°C	330/360 (91.7)

forwarding tubes resulted in no more than one tube differing at the confirmation stage. Agreement between the standard method and forwarding tubes showing acid (with or without gas) at 24 h was high - 99% of samples agreed in 14 out of 15 tubes.

Use of chromogenic media in the confirmatory stage

All tubes forwarded for confirmation were subcultured on to BCIG plates, which were incubated at 37°C and 44°C. Table 4 shows close agreement between the standard method and the use of BCIG agar in confirming the presence of *E. coli*. BCIG plates incubated at 44°C were easier to interpret than those incubated at 37°C due to reduction of background flora and greater colour intensity. The results were not influenced by the shellfish type or the extent of contamination with *E. coli*.

Identification of β -glucuronidase positive colonies

A total of 204 isolates producing blue-green colonies were characterised by the *API 20E* system; all isolates were identified as *E. coli*. One profile predominated (5114572) but at least 26 different profiles were obtained indicating that various strains were recovered.

Effect of laboratory, shellfish type, and category on the agreement between methods

Statistical methods were used to examine whether agreement between the results from the standard method and the modified method (subculturing MMGB tubes showing acid (with or without gas) at 24 h to BCIG agar incubated at 44°C) varied with laboratory, shellfish, or category of the sample by the standard method. Chi squared tests were used to compare the proportions of samples agreeing in 14 out

of 15 tubes. No significant differences were found between laboratories ($\chi^2 = 6.1$, 4 df, $p = 0.19$), shellfish types ($\chi^2 = 2.2$, 3 df, $p = 0.54$), or categories ($\chi^2 = 0.48$, 2 df, $p = 0.78$). Similarly there were no significant differences with regard to the proportion of samples whose category changed. Results by laboratory are shown in table 5. Changes in category (table 6) occurred mostly in samples for which the standard method gave tube combinations on the borderline between categories. The poorer classification obtained in nine samples was offset by a better classification in eight others.

Use of the modified method in the EQA trial

Results obtained in the EQA trial by subculturing MMGB tubes showing acid (with or without gas) at 24 h to BCIG agar incubated at 44°C showed 89.3% agreement in at least 14 out of 15 tubes with results from the standard method (table 7). When tube combinations differed, the differences in *E. coli* counts were small. Furthermore, the BCIG results correlated better with target results and eliminated some variation within and between samples. No significant difference was found between mean MPNs obtained by the standard and revised methods.

Discussion

The results of this study showed that the standard *E. coli* MPN method for the classification of shellfish harvesting areas in the UK may be modified to give a simpler, quicker test that yields essentially equivalent results.

Investigation of the recovery stage in MMGB has shown that comparable numbers of tubes subsequently yielding confirmed *E. coli* are obtained whether tubes containing acid are subcultured at 24 h or tubes containing acid and gas are subcultured at 48 h (as in the standard method). The modified method yields

TABLE 5 Effect of using the modified method on classification

Laboratory	Number (%) of samples showing agreement between revised and standard methods in 14 out of 15 tubes	Number (%) of samples showing no category change with use of revised method
1	97/100 (97.0)	99/100 (99.0)
2	62/70 (88.6)	66/70 (94.3)
3	31/35 (88.6)	32/35 (91.4)
4	51/58 (87.9)	54/58 (93.1)
5	89/97 (91.8)	92/97 (94.8)
Total	330/360 (91.7)	343/360 (95.3)

TABLE 6 Number of samples showing category changes with use of modified method (subculture of MMGB tubes showing acid at 24 h to BCIG agar incubated at 44°C)

Category		No (%) of samples*
Standard method	Modified method	
A	B	7 (1.9)
B	A	4 (1.1)
B	C	2 (0.6)
C	B	4 (1.1)
Total		17 (4.7)

* total number of samples tested=360

fewer false positives in this first stage of the test (requiring fewer confirmations to be performed), eliminates the need for Durham's tubes to detect gas production, and shortens the first stage of the procedure by 24 h.

Concomitant investigation of an alternative confirmation procedure based on subculture to a chromogenic medium for the detection of β -glucuronidase activity of *E. coli* also yielded results equivalent to those of the standard method. The alternative method is simpler to perform and removes the use of tubed or bottled media from the confirmation step. Although β -glucuronidase negative strains of *E. coli* will not be detected by this method, most strains isolated from food and the environment are positive for β -glucuronidase, and the detection of indole negative and anaerogenic or weak gas-producing strains^{3,4,14} (not currently detected) will offset any disadvantage. The use of a chromogenic substrate to detect β -glucuronidase activity in a second stage test overcomes the problem of false positive reactions that have been obtained with shellfish and fish samples when using a fluorogenic substrate in a direct test for *E. coli*^{11,15}.

Combining these two modifications yields results equivalent to the standard method, with the advantages of reduction in test time and complexity and a decrease in the need for bottled/tubed media. Changes in potential classification status due to use of the revised method will be rare and will tend to affect only those samples that yield results near the edges of categories. The effects of such variations with individual samples will be ameliorated by the fact that classifications for individual areas are based on multiple results over time and often from more than one sampling point.

In summary, the authors recommend that the standard method for the detection of *E. coli* in shellfish

is modified as follows: base the first stage on examination of the MMGB tubes for acid only after 24 h at 37°C and confirm the presence of *E. coli* on BCIG agar incubated at 44°C for 24 h. The appendix describes the amended standard method incorporating these modifications, together with methods for the isolation of *Salmonella* spp. and *S. typhi*, in order to present the complete procedure for the bacteriological examination of bivalve molluscs.

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TABLE 7 Comparison of results obtained by standard and revised methods in the EQA trial

Sample	Number (%) agreed in at least 14 out of 15 tubes	Mean MPN/100 g		p-value for difference between means
		Standard method	Revised method	
1	49/57 (86.0)	1045	1156	0.32
2	46/54 (85.2)	2716	2520	0.46
3	55/57 (96.5)	968	996	0.78
Total	150/168 (89.3)			

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APPENDIX

1. Examination of shellfish for *Escherichia coli*

The numbers of *Escherichia coli* in live bivalve molluscs are estimated as a marker of faecal contamination in shellfish flesh. This allows harvesting areas to be classified and is used to monitor the quality of the marketed product.

1.1 Sample size

Oysters and clams	10 - 15
Mussels	15 - 30
Cockles	30 - 50

1.2 Sample transport

Deliver samples to the laboratory as soon as possible. Use a cool box with freezer packs to keep temperature near 4°C, but do not allow direct contact between samples and freezer packs. No more than 24 h should elapse between sample collection and start of tests. Store samples at 4°C during this time; do not freeze.

1.3 Sample preparation

- (i) Wear gloves during sample preparation; use a separate pair for each sample.
- (ii) Discard any gaping shellfish and those with obvious signs of damage.
- (iii) Select at least ten oysters or clams, 15 mussels, or 30 cockles.
- (iv) Scrub shellfish clean under cold, running tap water of potable quality.
- (v) Dry with clean paper towels.
- (vi) Open shellfish with a sterile shucking knife (flamed and cooled), as follows.

1.3.1 Oysters/clams

Insert the knife between the two shells towards the hinge end of the shellfish. Push the knife further into the shellfish and prise open the upper shell, allowing any liquor to drain into a sterile weighed bag or beaker. Push the blade through the shellfish and sever the muscle attachments by slicing across. Remove the upper shell and scrape the contents of the lower shell into the bag or beaker. Repeat for ten oysters/clams and collect in the same bag or beaker.

1.3.2 Mussels/cockles

Insert the knife between the shells through the byssal opening of the shellfish and separate the shells by twisting the knife. Collect the liquor in a weighed sterile bag or beaker. Cut the muscle between the two shells and scrape the contents into the sterile bag or beaker. Repeat for at least 15 mussels or 30 cockles and collect in the same bag or beaker.

1.4 Homogenisation and dilution of sample

1.4.1 Dilution fluid

0.1% peptone in water, pH 7.2 \pm 0.2

1.4.2 Homogenisation in stomacher

Place the bag containing shellfish flesh and liquor inside two more bags; this will prevent small pieces of shell from puncturing the bag. Remove excess air and place in the stomacher. Operate stomacher for 2-3 minutes (min).

Remove 50g* of homogenate to another stomacher bag. From a measured 450 mL volume

* Another 25 g of homogenate may be removed at this stage for detection of salmonella.

TABLE A1 Most probable number (MPN) of organisms: tables for multiple tube methods using 5 × 1 g, 5 × 0.1 g, 5 × 0.01 g¹⁶

1 g	0.1 g	0.01 g	MPN/100g	
0	0	0	<20	
0	0	1	20	
0	1	0	20	
1	0	0	20	
1	0	1	40	
1	1	0	40	
1	2	0	50	
2	0	0	40	
2	0	1	50	
2	1	0	50	
2	1	1	70	
2	2	0	70	
2	3	0	110	
3	0	0	70	
3	0	1	90	
3	1	0	90	
3	1	1	130	
3	2	0	130	
3	2	1	160	
3	3	0	160	
4	0	0	110	
4	0	1	140	
4	1	0	160	
4	1	1	200	
4	2	0	200	Category A
5	0	0	220	(<230 <i>E. coli</i>)
4	2	1	250	Category B
4	3	0	250	(>230 <i>E. coli</i>)
4	3	1	310	(<4600 <i>E. coli</i>)
4	4	0	320	
4	4	1	380	
5	0	1	290	
5	0	2	410	
5	1	0	310	
5	1	1	430	
5	1	2	600	
5	1	3	850	
5	2	0	500	
5	2	1	700	
5	2	2	950	
5	2	3	1200	
5	3	0	750	
5	3	1	1100	
5	3	2	1400	
5	3	3	1750	
5	3	4	2100	
5	4	0	1300	
5	4	1	1700	
5	4	2	2200	
5	4	3	2800	
5	4	4	3450	
5	5	0	2400	Category B
5	5	1	3500	(<4600 <i>E. coli</i>)
5	5	2	5400	Category C
5	5	3	9100	(>4600 <i>E. coli</i>)
5	5	4	16000	(<46000 <i>E. coli</i>)
5	5	5	>18000*	

* needs further dilutions to clarify classification

of sterile dilution fluid, add about 100 mL to the 50 g sample. Homogenise in the stomacher for 2-3 min. Then add the remainder of the dilution fluid and mix well. This forms the master 1 in 10 dilution.

Prepare a 1 in 100 dilution by adding 10 mL of the 1 in 10 dilution to 90 mL dilution fluid. If levels of *E. coli* are expected to be high, - e.g., for category C and prohibited harvesting areas - prepare 1 in 1000 and 1 in 10000 dilutions in the same way.

TABLE A2 Most probable number (MPN) of organisms: tables for multiple tube methods using 5 × 0.1 g, 3 × 0.01 g, 5 × 0.001 g¹⁶

0.1 g	0.01 g	0.001 g	MPN/100g	
0	0	1	200	
0	1	0	200	Category A
1	0	0	200	(<230 <i>E. coli</i>)
1	0	1	400	Category B
1	1	0	400	(>230 <i>E. coli</i>)
1	2	0	500	(<4600 <i>E. coli</i>)
2	0	0	400	
2	0	1	500	
2	1	0	500	
2	1	1	700	
2	2	0	700	
2	3	0	1100	
3	0	0	700	
3	0	1	900	
3	1	0	900	
3	1	1	1300	
3	2	0	1300	
3	2	1	1600	
3	3	0	1600	
4	0	0	1100	
4	0	1	1400	
4	1	0	1600	
4	1	1	2000	
4	2	0	2000	
4	2	1	2500	
4	3	0	2500	
4	3	1	3100	
4	4	0	3200	
4	4	1	3800	
5	0	0	2200	
5	0	1	2900	
5	0	2	4100	
5	1	0	3100	
5	1	1	4300	
5	1	2	6000	Category C
5	1	3	8500	(>4600 <i>E. coli</i>)
5	2	0	5000	(<46000 <i>E. coli</i>)
5	2	1	7000	
5	2	2	9500	
5	2	3	12000	
5	3	0	7500	
5	3	1	11000	
5	3	2	14000	
5	3	3	17500	
5	3	4	21000	
5	4	0	13000	
5	4	1	17000	
5	4	2	22000	
5	4	3	28000	
5	4	4	34500	
5	5	0	24000	
5	5	1	35000	
5	5	2	54000	Prohibited
5	5	3	91000	(>46000 <i>E. coli</i>)
5	5	4	160000	
5	5	5	>180000	

Store remainder of the sample homogenate at 1-4°C until testing is completed.

1.4.3 Homogenisation in blender (homogeniser)

Weigh beaker containing shellfish flesh and liquor and subtract the weight of the beaker to obtain the weight of sample. Add two parts by weight of sterile dilution fluid. Transfer to a sterile blender, replace lid, and homogenise at high speed (about 12 000 rev/min) for a total of 60 seconds (s) (four sessions of 15 s blending

TABLE A3 Most probable number (MPN) of organisms: tables for multiple tube methods using 5 × 0.01 g, 3 × 0.001 g, 5 × 0.0001 g¹⁶

0.01 g	0.001 g	0.0001 g	MPN/100g	
0	0	1	2000	
0	1	0	2000	
1	0	0	2000	
1	0	1	4000	Category B
1	1	0	4000	(>230 <i>E. coli</i>)
2	0	0	4000	(<4600 <i>E. coli</i>)
1	2	0	5000	Category C
2	0	1	5000	(>4600 <i>E. coli</i>)
2	1	0	5000	(<46000 <i>E. coli</i>)
2	1	1	7000	
2	2	0	7000	
2	3	0	11000	
3	0	0	7000	
3	0	1	9000	
3	1	0	9000	
3	1	1	13000	
3	2	0	13000	
3	2	1	16000	
3	3	0	16000	
4	0	0	11000	
4	0	1	14000	
4	1	0	16000	
4	1	1	20000	
4	2	0	20000	
4	2	1	25000	
4	3	0	25000	
4	3	1	31000	
4	4	0	32000	
4	4	1	38000	
5	0	0	22000	
5	0	1	29000	
5	0	2	41000	Category C
5	1	0	31000	(>4600 <i>E. coli</i>)
5	1	1	43000	(<46000 <i>E. coli</i>)
5	1	2	60000	Prohibited
5	1	3	85000	(>46000 <i>E. coli</i>)
5	2	0	50000	
5	2	1	70000	
5	2	2	95000	
5	2	3	120000	
5	3	0	75000	
5	3	1	110000	
5	3	2	140000	
5	3	3	175000	
5	3	4	210000	
5	4	0	130000	
5	4	1	170000	
5	4	2	220000	
5	4	3	280000	
5	4	4	345000	
5	5	0	240000	
5	5	1	350000	
5	5	2	540000	
5	5	3	910000	
5	5	4	1600000	

with 15 s intervals). Stand for 30 s, swirl briefly, and transfer 30 mL of homogenate to 70 mL of sterile dilution fluid. This forms the master 1 in 10 dilution. Prepare further decimal dilutions as described in 1.4.1.

1.5 Examination

1.5.1 Media

Minerals modified glutamate broth (MMGB), e.g. Oxoid CM607 (base) and L124 sodium glutamate. Equivalent formulations from other suppliers may be used.

This medium is prepared as single and double strength and dispensed in quantities of 10 mL in bottles or tubes.

Double strength: Dissolve 5 g ammonium chloride in 1 L of distilled water. Add 22.7 g minerals modified medium base and 12.7 g sodium glutamate.

Single strength: Dissolve 2.5 g ammonium chloride in 1 L of distilled water. Add 11.4 g minerals modified medium base and 6.4 g sodium glutamate.

Mix to dissolve completely. Adjust pH to 6.7 after autoclaving. Dispense in 10 mL. Sterilise in the autoclave for 10 min at 116°C.

5-bromo-4-chloro-3-indolyl-β-D-glucuronide agar (BCIG) tryptone bile agar containing 75 mg/l 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, e.g. Oxoid CM 945 (TBX agar), Lab M LAB 162 (tryptone bile glucuronide agar).

1.5.2 First stage tests

1.5.2.1 Inoculation

- Inoculate 10 mL of 1 in 10 homogenate to each of five tubes containing 10 mL of double strength MMGB. Each tube contains the equivalent of 1 g of tissue.
- Inoculate 1 mL of the 1 in 10 homogenate to each of five tubes containing 10 mL of single strength MMGB. Each tube contains the equivalent of 0.1 g of tissue.
- Inoculate 1 mL of the 1 in 100 dilution to each of five tubes containing 10 mL of single strength MMGB. Each tube contains the equivalent of 0.01 g of tissue.
- If further dilutions have been prepared, inoculate 1 mL aliquots of each dilution to each of five tubes containing 10 mL of single strength MMGB.

1.5.2.2 Incubation

- Incubate the inoculated tubes at 37 ± 1°C for 24 ± 2 h.
- At the end of the incubation period, examine the tubes for the presence of acid, denoted by a yellow coloration of the medium. Note that the presence of any acid, regardless of quantity, is regarded as a positive result. Absence of acid denotes a negative result for *E. coli*.

1.5.3.1 Second stage tests

- At the time of detection, subculture all tubes showing the presence of acid to a section of BCIG agar and streak to separate colonies. Do not refrigerate tubes before subculture. Note that up to five sections per plate may be used.
- Incubate BCIG agar plates at 44 ± 1°C for 20-24 h.
- Examine the plates for the presence of blue-green colonies, which signify β-glucuronidase activity. Tubes of MMGB that grow blue-green colonies are deemed to contain *E. coli*. Absence

of blue-green colonies denotes a negative result for *E. coli*.

Control cultures

NCTC 9001 *Escherichia coli* (β -glucuronidase positive)

NCTC 9528 *Klebsiella aerogenes* (β -glucuronidase negative)

1.5.4 Calculation of *E. coli* count

- (j) After completing the second stage tests, determine the number of MMGB tubes/bottles positive for *E. coli*. Compute the most probable number (MPN) by reference to the MPN table for the appropriate dilution range. Report the results as the number of *E. coli* per 100 g shellfish.

If the positive tube combination obtained is not shown in the MPN tables, either report the test as void or repeat the test from the sample homogenate stored at 1-4°C.

2 Examination of shellfish for *Salmonella* spp.

Tests for salmonellas are required to be applied only to shellfish for immediate human consumption (final product) and not for the classification of harvesting areas. In certain circumstances it may be necessary to test for the presence of *S. typhi*. A general method for salmonella, which will recover a wide range of serotypes, is given below. This method is not suitable for the specific isolation of *S. typhi*, however, and an additional method for its isolation is also described.

2.1 General method for salmonella

2.1.1 Media

The use of commercially available dehydrated media is advised. Equivalent formulations from other suppliers may be used.

Buffered peptone water - e.g., Oxoid CM 509.

Rappaport Vassiliadis Soya (RVS) peptone broth - e.g., Oxoid CM 866.

XLD agar - e.g., Oxoid CM 469.

Brilliant green agar (BGA) - e.g., Oxoid CM 329 (modified).

2.1.2 Sample preparation

See Section 1.3.

2.1.3 Sample homogenisation and pre-enrichment

a) Stomacher used for *E. coli*

Remove 25 g of bulk homogenate prepared as in section 1.4.1 to a 250 mL or larger container. Add 225 mL of buffered peptone water (BPW). Mix well. Incubate at 37 \pm 1°C for 18-24 h.

b) Blender used for *E. coli*

Weigh out a separate 25 g amount into a sterile beaker. Add about 50 mL from a measured 225 mL volume of BPW and homogenise at high speed for a total of 60 s (see Section 1.4.2). Add remainder of BPW and mix well. Incubate at 37 \pm 1°C for 18-24 h.

2.1.4 Selective enrichment

Transfer 0.1 mL of incubated BPW to 10 mL sterile Rappaport Vassiliadis soya (RVS) peptone broth. Incubate at 41- 42°C for 48 \pm 2 h.

2.1.5 Subculture

Subculture the incubated RVS broth after 18 to 24 h and again after 48 h. Using a loop of 2-3 mm diameter, subculture to plates of XLD agar and brilliant green agar (BGA) and streak to obtain isolated colonies. Incubate the XLD and BGA plates at 37 \pm 1°C for 18-24 h. At the end of incubation examine the plates for suspect salmonella colonies. Investigate suspect colonies using standard biochemical and serological methods.

2.2 Method for *Salmonella typhi*

2.2.1 Media

Buffered peptone water - e.g., Oxoid CM 509

Selenite F broth - e.g., Oxoid CM 399 with sodium biselenite L121 to give 0.4% concentration.

Caution: sodium biselenite is hazardous.

Desoxycholate citrate agar (Hynes modification) - e.g., Oxoid CM227, Lab M LAB65

Bismuth sulphite agar - e.g., Lab M LAB13A and LAB13B.

2.2.2 Sample preparation

See Section 2.1.2.

2.2.3 Sample homogenisation and pre-enrichment

See Section 2.1.3.

2.2.4 Selective enrichment

Transfer 10 mL of incubated BPW to 100 mL of sterile selenite F broth. Incubate at 37 \pm 1°C for 24 \pm 2 h.

2.2.5 Subculture

At the end of the incubation period, subculture the selenite F broth to desoxycholate citrate agar (DCA) and bismuth sulphite agar. Use a loopful 2-3 mm in diameter and streak to obtain isolated colonies. Incubate the plates at 37°C for 18 to 24 h. Examine the plates for suspect salmonella colonies. Reincubate the plates for a further 24 h (48 \pm 2 h in total) and examine again at the end of incubation. Investigate suspect salmonella colonies using standard biochemical and serological methods.