THE BACTERIOLOGICAL EXAMINATION OF MOLLUSCAN SHELLFISH

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(With 2 Figures in the Text)

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INTRODUCTION

There is no standard bacteriological test for shellfish in this country, and the various methods in use do not give readily comparable results. Further, few comparative studies have been made, and every test advocated hitherto has had one or more inherent errors; none has found general approval, and little, if any, progress resulted from a conference held at Middelburgh in 1932, attended by bacteriologists representing the governments of seven European countries, and having as its object the recommendation of a standard method of bacteriological examination of edible molluscs. The present paper is an attempt to meet this deficiency.

Oysters, and to a less extent mussels, are eaten raw, but other shellfish are generally 'cooked'. 'Cooking', in this respect, is not a precise term, and may mean boiling in water for a few minutes or

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merely heating in water, or steaming until the shells open. In the latter case the temperature of the stomach or rectal contents may scarcely be raised, and sewage bacteria, and any pathogens associated with them, remain unaffected. Shellfish that have been lightly steamed may be indistinguishable bacteriologically from raw shellfish. Thorough 'cooking' is not favoured by the trade as it frequently reduces palatability. Properly cooked shellfish cease to be a potentially dangerous food; consequently methods of bacteriological examination would be designed to reveal spoilage or unsatisfactory sterilization, as in other cooked foods.

This paper is concerned with the detection in shellfish of faecal pollution, whether primary, derived *in situ* from sewage, or secondary, from handling or other chance infection during conveyance to the consumer. A complete solution to the problem of pollution in certain large fisheries has been found in 'approved' purification installations. There is more than one type of installation for the purification of oysters and mussels, but only those constructed with the co-operation and guidance of the Ministry of Agriculture and Fisheries have been approved by the Ministry of Health under the Shellfish Regulations.

Purification at 'approved' installations depends on the natural functioning of the shellfish during a 48 hr. period in clean sea water, i.e. sea water which has stood overnight after addition of 3 parts per million of chlorine and has been dechlorinated with sodium thiosulphate before use. During this period their intestines are emptied. The shellfish are first spread two deep on wooden grids in a concrete tank and hosed to remove external dirt. After one night in sea water the shellfish are again hosed, and all faeces, etc., flushed from the tank. A second overnight period is followed by a final hosing, and the treatment concludes with an hour's shell-sterilizing bath in sea water containing 3 parts per million of chlorine.

The efficacy of this method of purification has been demonstrated by 30 years of satisfactory results. Hence shellfish thus purified may justifiably be assumed by health authorities to be clean when they leave the tanks, and there would be no necessity for bacteriological examination but for the possibility of secondary pollution, which makes it desirable to test purified as well as unpurified shellfish. Although all shellfish from approved purification installations are sent away in specially sealed receptacles, there is no check on their disposal once the seals have been removed; a dishonest fishmonger who dealt both in purified and unpurified shellfish could sell all as purified.

The object of this paper is to suggest a standard method for examination of shellfish. In order to make the requirements of such a test clear a complete review of the present position is given in § I.

I. THE REQUIREMENTS OF A STANDARD TEST FOR MOLLUSCAN SHELLFISH

A standard method for the bacteriological examination of molluscan shellfish should fulfil the following requirements:

(*a*) It must show the degree, expressed in numerical terms, of recent faecal pollution in samples of shellfish, whether tested individually or pooled together.

(*b*) It must be as accurate as possible without being unduly complicated or time-consuming.

(*c*) The result must be available overnight, and without need for confirmatory tests.

1. REVIEW OF EXISTING TESTS

Before the existing methods of bacteriological examination of shellfish are compared, it is necessary to consider the relative importance of the types of organisms found, the significance of which is a guide to the value of the various tests designed to indicate their presence.

Briefly, there are two general classes of micro-organisms in shellfish; water saprophytes and organisms associated with sewage pollution. The water saprophytes are probably of little importance, but amongst the organisms associated with sewage pollution are the pathogens. As in the bacteriological examination of water, they are all important, but because of the difficulties of determining their presence directly it is more convenient to test for indicators of sewage pollution. Coliform organisms are now generally regarded as the most suitable of such indicators in preference to faecal streptococci and organisms allied to *Clostridium welchii*.

In the examination of water supplies *Bacterium coli* type I (faecal *coli*) can be used to signify recent sewage pollution, and other members of the Intermediate-*aerogenes-cloacae* (I.A.C.) group (other coliforms) to indicate more remote pollution. Dodgson (1937) showed that multiplication of other coliforms may sometimes occur in purified mussels; presumably this may occur also in unpurified shellfish. Although the absence of all coliform organisms is to be desired in shellfish sold as food, members of the I.A.C. group are not reliable indices of sewage pollution in shellfish. Any test for revealing effectively the presence and extent of sewage pollution in shellfish must therefore distinguish and enumerate faecal *coli*.

Certain of the bacteriological tests formerly used and their defects were fully discussed by Dodgson in his *Report on Mussel Purification* (1928), and it is intended here to make only a brief survey of the

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methods hitherto used. They can be subdivided as follows:

A. Estimation of bacteria by observation of growth in tubes of liquid media:
  (1) No dilution, shellfish tested individually.
  (2) Serial dilution.
    (*a*) Shellfish pooled, bacteria recorded as present or absent.
    (*b*) Shellfish pooled, probability tables used.
    (*c*) Shellfish tested individually.

B. Counts of viable organisms in solid media.

A. *Estimation of bacteria by observation of tubes of liquid media*

(1) *No dilution, shellfish tested individually*

This method was first introduced by Klein (1916), but was later modified by Andrews, Hewlett & Eyre (1924) and again by Eyre (1924) and is now popularly known as the Fishmongers' Company's Test. In this method the assumption is made that if material from a shellfish inoculated into litmus, lactose, bile-salt, peptone water turns it red and full of gas in 24 hr. at 37°,* it can safely be concluded that the material contained *B. coli*. The number of shellfish examined by this method was originally six, but later this was increased to ten. A proportion of each shellfish (1/10 ml. for mussels and 1/5 ml. for oysters) is inoculated into a tube of MacConkey broth at 37°. After incubation the number of 'positive' tubes (i.e. showing acid and gas) is recorded and used to denote the percentage purity of the shellfish thus:

Result	Percentage clean	Action taken
All 10 shellfish devoid of *B. coli*	100	Allowed to be sold without further comment
1 of 10 shellfish containing *B. coli*	90	
2 of 10 shellfish containing *B. coli*	80	
3 of 10 shellfish containing *B. coli*	70	
4 of 10 shellfish containing *B. coli*	60	
5 of 10 shellfish containing *B. coli*	50	Held up pending a further analysis and, if necessary, a revision of the topographical examination of the layings
6 of 10 shellfish containing *B. coli*	40	
7 of 10 shellfish containing *B. coli*	30	Condemned forthwith
8 of 10 shellfish containing *B. coli*	20	
9 of 10 shellfish containing *B. coli*	10	
All of 10 shellfish containing *B. coli*	0	

This method called for further confirmatory tests of two or three shellfish for *Streptococcus faecalis* and 'Bacillus (enteritidis) sporogenes' (now known as *Clostridium welchii*). No standards were originally given for these organisms nor was any mention made as to how the results should be interpreted excepting that 'they added confirmation to the *B coli* test'. When the test was later modified (1924) the three 'microbes of indication'—*Bacillus coli*, streptococci and *B. enteritidis sporogenes*—were stated to have unequal value as indices of pollution. Streptococci were stated to die out very quickly and were thus of little value except as evidence of very recent pollution. Conversely the spores of such anaerobes as 'B. sporogenes' were said to persist indefinitely in soil and thus to be of little value except to denote remote pollution. It is strange after such a statement that this part of the test was continued, but it was retained on the grounds that the presence of all three organisms in a water or food is perhaps more convincing evidence than of one alone. The Fishmongers' Company's test is based on the assumption that if *B. coli* is present in 1/5 ml. of a shellfish there are at least 50–200 *B. coli* in the whole shellfish, but if the result is negative there are less than 50 such organisms per shellfish. It has been demonstrated by Dodgson (1928) that with replicate tests on some samples it is possible to obtain every verdict from 100 to 0 % clean. It is thus evident that with all but very high or very low concentrations of pollution the results from such a test are largely a matter of chance.

(2) *Serial dilution*

(*a*) *Shellfish pooled, bacteria recorded as present or absent.* Houston (1904), a pioneer investigator of shellfish pollution, introduced this method. He prepared decimal dilutions, made triple inoculations from each, and regarded organisms as present in any dilution only if two out of the three tubes were positive. Bacteria were recorded as present in 100, 10, 0·1, 0·01, 0·001 or 0·0001 ml. Here again the element of chance is considerable; whether bacteria are found to the extent of say 10 or 100 per ml. may depend on the result from one tube. Further, little attempt was made to prepare a homogeneous inoculum. Solely for comparative purposes in close relation to topography, Houston proposed tentative standards which concerned *B. coli* and *C. welchii*. An opinion has already been given about standards relating to the latter organism, and further reference to it is unnecessary.

For *B. coli* the standards were: 'stringent'—less than 100 *B. coli* per oyster, or less than 10 per ml.; 'lenient'—less than 1000 *B. coli* per oyster, or less

* Temperatures are in ° C. throughout.

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than 100 per ml. The condemnation of a batch of shellfish under either standard may depend on the result from one tube.

The American 'Score' method discussed by Perry (1928) is very similar in technique and method of assessing the result.

(*b*) *Shellfish pooled, probability tables used for recording results.* This method, although used in this country in some laboratories for the examination of shellfish, has not yet been described so far as the authors are aware. It is similar in principle to the method of examination of water recommended by the Ministry of Health (1934, 1939). The shellfish in a sample are pooled and inoculations generally of 1, 1/10 and 1/100 ml. are made into five tubes of MacConkey broth. These are incubated at 37° and the results interpreted from probability tables (McCrady, 1918). The new procedure proposed by the Standard Methods Committee for the examination of shellfish of the American Public Health Association (1942) is very similar, with the addition that all positive tubes are confirmed in accordance with the general principles laid down in the Association's *Standard Methods for the Examination of Water and Sewage* (1933, 1936). When applied to the examination of water this method is subject to a very large sampling error. It has been stated that this error may be + 260 % or − 70 % (see Halvorson & Zeigler, 1933; Swaroop, 1938; and Ministry of Health, 1939). With a pool of minced shellfish the error is likely to be still larger. Methods (*a*) and (*b*) are designed for use only with pooled shellfish. It would not be practical to attempt to modify them for testing individual shellfish, and for reasons given later the testing of pooled shellfish may fail to meet some requirements.

(*c*) *Shellfish tested individually.* The method proposed by Bigger (1934), in which individual shellfish are examined, is an advance on the two methods described above.

Bigger not only appreciated the significance of testing individual shellfish, but also sought to standardize the variable factor of shell water by discarding it and reconstituting the minced shellfish to 50 ml. with sterile water. From ten shellfish duplicate inoculations corresponding to 1/50, 1/250, and 1/1250 are made into tubes of lactose bile broth. The tubes are incubated at 37° and all positives confirmed. Although certain defects in the other tests described have largely been eliminated from this test, it still suffers from the defect of inaccuracy inseparable from the use of a liquid medium (see § II below, especially Tables 2–5). It is not considered that the method of reconstituting the shell water is sound, because no allowance is made for variation in the volume of body tissue in shellfish of different sizes. Furthermore, Bigger's method requires sixty tubes per sample.

B. *Counts of viable organisms in solid media*

References to the use of solid media for the examination of shellfish are few, apart from the work of Johnstone (1906, 1908, 1918, 1924) and the description by Dodgson (1928) of the technique of plating out portions of minced mussel juice in litmus, bile-salt, peptone agar (MacConkey agar). This method was used for many years at Conway in conjunction with confirmatory tests of red colonies in MacConkey broth. As explained by Dodgson, the occasional production of red colonies by non-coliform organisms made confirmation necessary. Regular confirmation being laborious, the method was simplified, and inoculations of 1·0 and 0·1 ml. were made into liquid medium in parallel with plate cultures. The results were interpreted in conjunction with the colony count on MacConkey agar. This modified test was intended for use with purified shellfish only, which seldom contained more than 5 coliforms per ml. and often none at all. It was adequate for its purpose, but is unsuitable as a general test for shellfish.

On the discovery of sporadic multiplication of coliforms in purified mussels (Dodgson, 1937), and realization that its occurrence was confined to the I.A.C. group, a test was clearly needed whereby the presence of numerous coliforms in purified shellfish could be distinguished from faecal contamination. Clegg & Sherwood (1939) showed that incubation in MacConkey broth at 44° was specific for faecal *coli*, and could be used for shellfish tests, but the adoption of this method did not meet the need for an accurate numerical test at 44° suitable for shellfish of all categories. The test used for water, with serial dilutions in replicate tubes incubated at 44° (Clegg, 1941), offered no solution because its inherent experimental error, in any case great, could hardly be diminished with samples less homogeneous than water. The inaccuracy of the test when applied to shellfish had already been demonstrated in replicate tests at 37°.

Incubation of MacConkey agar plate cultures immersed in a water-bath in watertight containers seemed a more promising method. It was tried, but although the water in which the containers were placed was accurately controlled to 44° ± 0·2°, the temperatures recorded inside them were not of the uniformity required for determination of faecal *coli*. This method also offered prospects of eliminating false positive results caused by glucose fermenting organisms (p. 512), most of which are inhibited by a high incubation temperature.

Wilson (1922) compared cultures in Esmarch or roll tubes with those made in plates with the same medium and incubated at 37°. His roll tubes gave slightly higher counts, and among other advantages which he described were rapidity and ease of counting. Similar comparisons by Thomas, Jones

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& Lloyd (1940) also indicated that roll tubes have many advantages. They found the main disadvantage of the roll tube to be the time taken to set up the tubes without an apparatus which would roll more than one tube at a time. One possible disadvantage of using 44° was realized by the present writers; while this temperature might be critical for production of gas by coliforms other than faecal *coli* in MacConkey broth, it might fail to prevent growth and colony formation of members of the I.A.C. group. Experiments were therefore devised to test the MacConkey roll-tube method at 44° both qualitatively and quantitatively. The results are given in § II, especially Table 6, which shows that the elimination of the I.A.C. group from the samples tested was practically complete.

2. Methods of preparing shellfish for bacteriological examination, with a discussion of difficulties

Current methods of preparing shellfish for examination differ considerably according to the steps taken to overcome certain frequent difficulties which arise from:

A. The possibility of external pollution and the presence of barnacles and other growths on the shell.

B. The necessity for complete liberation of bacteria from the gut and body tissues.

C. The need for a thorough mixing of bacteria and fluid so as to produce a homogeneous liquid inoculum.

D. The frequent partial or complete loss of shell water.

These difficulties are discussed separately below.

A. *Shell sterilization*

Sterilization of shells before bacteriological examination is usually unnecessary with unpurified shellfish; the concentration of pollution is generally far greater inside the shellfish than on the outside of the shells. The only exception is when pollution in transit from the beds is suspected.

Purified shellfish have their shells sterilized before leaving an approved purification plant; further treatment of the shells is unnecessary except in the case of mussels covered with barnacles, which should be scraped clean and scrubbed under a stream of sterile water. Barnacles differ from bivalves in their method of feeding, and cannot be relied upon to become cleansed along with the shellfish. During transport to market many barnacles on mussels become crushed, and their gut contents, possibly including sewage bacteria, may be washed by escaping shell water from one purified shellfish into another. There is little danger of pathogenic organisms being transmitted to the mussels by this means, for by virtue of their mode of feeding, barnacles are not able to take from the water such large quantities of pollution as bivalves.

Dodgson (1937) observed occasional multiplication of coliforms of the I.A.C. group, i.e. coliforms other than *Bact. coli* type I in barnacles and purified mussels. When this occurs in purified mussels, and bacteriological examination shows no *Bact. coli*, but a high count of 'other coliform' bacteria, the shellfish should not be regarded as showing evidence of heavy pollution. No multiplication of 'other coliforms' has yet been observed in over 400 samples of purified oysters examined at the Conway station. Examples of multiplication are likely to be very infrequent because oysters seldom carry many barnacles, and external growths on the shell are usually removed before marketing.

To summarize: except where pollution in transit is suspected, shell sterilization before examination is unnecessary as a routine measure, provided that adequate steps are taken to distinguish between faecal *coli* and coliforms of the I.A.C. group.

B. *Liberation of bacterial contents of gut and body tissues*

Three common methods of mincing the body of the shellfish to produce complete liberation of the bacteria are:

(*a*) Cutting up with scissors.

(*b*) Grinding with sand in a mortar.

(*c*) Cutting the rectum into small pieces, stroking the gills, and making a cruciform incision in the stomach.

Method (*a*) is applicable to all shellfish, both univalves and bivalves, but it is quite possible that a portion of the rectum may be left intact with the result that bacteria imprisoned in the faeces might not be released when shaken up with water.

Method (*b*) overcomes the difficulty in (*a*), is applicable to all shellfish, and gives complete release of all the bacteria from the gut. The examination of ten individual shellfish would, however, necessitate the use of ten separate pestles and mortars.

Method (*c*) is suitable only for bivalves, the rectum of which can be exposed in the half shell and cut up into small sections *in situ*, but it has been found to be more efficient than (*a*) for liberation of bacteria, and less cumbersome than (*b*).

Method (*c*) is therefore suggested for use with bivalves and method (*b*) for univalves.

C. *Mixing of body tissues with water*

Several methods are used, varying in the degree of effort made to attain a homogeneous suspension of the bacteria in the fluid, viz.:

(*a*) Pipetting the shell liquor and minced body tissue directly out of half shell.

(*b*) Stirring the shell liquor and body tissues in an enamelled cup or measuring cylinder.

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(*c*) Shaking the minced tissues and shell water in a tin with sterile granite chippings.

(*d*) Stirring or shaking the shellfish after grinding with sand in a mortar.

Methods (*a*) and (*b*) are considered to be inadequate. In methods (*c*) and (*d*) mixing is more thorough, but the operations of transferring to a sterile tin and shaking, or grinding in a mortar, are unnecessarily time-consuming and do not, in practice, give a more noticeably thorough mixing than the simple procedure described briefly below and in more detail in § III.

A stoppered 100 ml. cylinder containing a small amount of sterile water is used to measure the volume of the body tissue (by displacement), and further sterile water is added to bring the total fluid to twice the volume of the body tissue. Provided that the total volume of shellfish, plus fluid, is not more than three-quarters of the capacity of the cylinder, it has been found that a reasonably homogeneous mixing results from shaking with an up and down motion fifty times. This simple procedure is recommended for bivalves and method (*d*) for univalves.

D. *The problem of lost shell water*

In any sample of shellfish, whether taken directly from the beds or from the market, some individuals will be found to have lost some of their shell water. This loss is accentuated by some storage conditions and particularly by transport in airtight containers such as are of necessity frequently used. In samples taken for bacteriological examination the difficulty resulting from this partial or sometimes complete loss of shell water has long been recognized, and various suggestions have been made for overcoming or reducing irregularities in final results of examination due to this cause (Dodgson, 1928; Bigger, 1934). Rejection of shell water was among the recommendations proposed by Bigger which were adopted at the Middelburgh Conference.

This difficulty is particularly evident when individual shellfish are being examined. Two questions arise: is it necessary to make up the fluid in such individuals as have lost all or part of the shell water? If so, to what extent, e.g. should all the shellfish be made up to a standard volume before a portion of fluid is taken for inoculation?

The first question can be answered by the comparison of two shellfish of the same size and degree of pollution, but having different amounts of shell water. Suppose shellfish A and B to contain respectively 1 and 10 ml. of shell water. If 1 ml. of minced flesh and water from each were plated, the pollution in B would be considerably more dilute than that in A. Addition of sterile water is therefore essential, but the amount of such additions is not easily decided. Although the original shell water may have contained sewage or faecal organisms which would be absent from the substituted shell water, the number of such organisms is usually insignificant in comparison to the bacterial content of the body tissue, as shellfish do not defaecate when closed (Dodgson, 1928, p. 251). There is one important exception; shellfish taken from offshore beds not subject to pollution may be temporarily stored in polluted water at the port of landing before being sent to market. Dodgson (1928) has recorded the dipping of mussels in grossly polluted water in order to freshen them up. In such instances the shell water, which is merely a sample of the last water in which the shellfish opened, may be heavily polluted while the body tissue and gut is free from pollution. Here a separate examination of the shell water may give a clue to the source of the pollution.

In general, the organisms contained in the shell water, being greatly outnumbered by those in the gut, are unimportant, and the potential error attributable to rejection of shell water would be very small. The advantage of such rejection, which is recommended as a routine procedure, is that it provides the best means of eliminating variations in this small error, which inevitably result from loss of shell water in varying degree. When secondary pollution is suspected, the examination of pooled shell water is clearly advantageous. Whether it would be desirable always to pool the discarded shell water and to examine it separately is a matter on which opinions may differ, but the time involved would be insignificant, and a valuable check on unsuspected secondary pollution would result. It is therefore suggested that a routine examination of pooled shell water should form part of all tests of shellfish samples taken from the markets.

There remains the problem of how much sterile water to add to produce a sufficiently dilute fluid inoculum. It is clear that some notice must be taken of variations in the volume of body tissue among individual shellfish, and it would seem incorrect to make up the volume of a small and a large shellfish to the same amount, with a consequently greater dilution of the small shellfish. Comparison of the results from 1 ml. of minced flesh and fluid from small and large shellfish without addition would be equally erroneous, as would also be the dilution of a shellfish to a volume double that of its body tissue plus residual shell water, because loss of shell water varies in degree. Shell water varies in volume, from only a trace in univalves to between once and twice that of the body tissue in the oyster, mussel and cockle. The only technique which, though somewhat tedious, is apparently free from serious objections is the measurement of the volume of body tissue, and the addition of sterile water to produce a total volume proportional and simply related to that of the body tissue. The addition of a volume

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double that of the body tissue would thus serve the purpose.

The examples given in Table 1 illustrate the errors which would result by using various methods of restoring water to four marketable mussels of different size.

An examination of the first three methods, (*a*)–(*c*) in Table 1, reveals that in no case is the equivalent of body tissue in the 1 ml. of inoculum comparable in the four mussels. Method (*d*) of making the total volume up to three times the volume of the body tissue thus appears to be the best solution. This method involves dilution, but that in itself is not necessarily objectionable.

It could be argued that method (*d*) does not provide a conveniently rounded *volume* per shellfish on which to determine the number of bacteria. But there is a *standard* equivalent of body tissue contained in each standard amount (1 ml.) of inoculum, and from the recorded measurement of the body volume of the shellfish it is a simple matter to arrive at the total number of bacteria in the shellfish if desired. A direct relationship between size of shellfish and rate of filtering still awaits conclusive proof, but the work of Fox, Sverdrup & Cunningham (1937) strongly suggests this relationship. Thus mussel *Z* with a body tissue volume of 15 ml. should filter water at a greater rate than mussel *X* with a body tissue volume of 10 ml. If both these shellfish were in water of the same degree of pollution, *Z* would presumably contain a greater total number of organisms than *X*. Therefore, just as it would be erroneous to express the number of bacteria per ml. in both shellfish (without resorting to method (*d*) of dilution), so would it be misleading to record the number of bacteria per whole shellfish (using the method (*d*) of dilution, without stating the size of the shellfish. But to give two correlated figures such as 1000 *Bact. coli* per shellfish of 10 ml. body tissue is not only confusing but, as is shown below, unnecessary.

If the arguments put forward above are correct, then if *X* contains 1000 *Bact. coli*, *Z* should contain 1500 *Bact. coli*. When these shellfish are diluted by method (*d*), 0·33 ml. equivalent of body tissue is contained in the 1 ml. of inoculum of reconstituted shellfish. Thus 1/30 of the equivalent of body tissue (and presumably the bacterial content) of *X* and 1/45 of *Z* would be contained in the 1 ml. inoculum. This would yield a result of 33 *Bact. coli* in each case per ml. of reconstituted shellfish.

Table 1. *Various methods of 'restoration' of shell water*

(Volumes are in ml. throughout.)

Method of dilution	Shell water	Volume of body tissue	Volume of shell water	Added sterile water	Total 'made up' volume	1 ml. of 'made up' volume equivalent to: Body tissue	1 ml. of 'made up' volume equivalent to: Water
(*a*) Making each fish up to a definite volume, i.e. 50 ml. (Bigger's method)	*W*	8	Discarded	42	50	0·16	0·84
	X	10	Discarded	40	50	0·20	0·80
	Y	6	Discarded	44	50	0·12	0·88
	Z	15	Discarded	35	50	0·30	0·70
(*b*) No addition of water except 10 ml. to those fish containing no shell water (Conway old method)	*W*	8	5	0	13	0·62	0·38
	X	10	10	0	20	0·50	0·50
	Y	6	10	0	16	0·37	0·63
	Z	15	0	10	25	0·60	0·40
(*c*) Doubling the volume of fish and shell water	*W*	8	5	13	26	0·31	0·69
	X	10	10	20	40	0·25	0·75
	Y	6	10	16	32	0·19	0·81
	Z	15	0	15	30	0·50	0·50
(*d*) Making the total fluid up to a volume twice that of body tissue	*W*	8	Discarded	16	24	0·33	0·67
	X	10	Discarded	20	30	0·33	0·67
	Y	6	Discarded	12	18	0·33	0·67
	Z	15	Discarded	30	45	0·33	0·67

Method (*d*) thus measures the degree of pollution regardless of whether it is contained in a large or small shellfish. It would be logical to refer to so many *Bact. coli* per ml. when method (*d*) of dilution is used, providing it is borne in mind that this refers to 1/15 of a 5 ml., 1/30 of a 10 ml., 1/45 of a 15 ml. and 1/60 of a 20 ml. shellfish.

Method (*b*) would give the same balancing effect (i.e. 1 ml. of 10 ml. fish equivalent to 1/10, or 1 ml. of a 15 ml. fish equivalent to 1/15) *if the shell water, which has to be replenished when lost, were constant and not variable in amount*, but without the correct replenishment of wholly or partly lost water, which is impracticable, this method would fail to give consistent results.

Method (*a*) of Bigger attempts to remedy this defect by discarding all shell water and making up the volume to 50 ml. by the addition of sterile water. The results would still be misleading. For example, mussel *X* with 10 ml. body tissue and 1000 *Bact. coli* would be made up to 50 ml.; 1 ml. of this would be

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equivalent to 0·20 ml. body tissue containing 20 *Bact. coli*. On the other hand, mussel *Z* with 15 ml. body tissue and 1500 *Bact. coli* would yield when diluted to 50 ml. 30 *Bact. coli* per ml.

Method (*c*) remains to be examined. This is inaccurate because shell water and body tissue amounts are regarded as a single factor, whereas they are two factors, which vary independently.

In the employment of method (*d*) in conjunction with adequate measures for releasing the gut content, and with thorough shaking, it is reasonable to assume for practical purposes that the bacterial content of each shellfish is distributed throughout the fluid. Thus 1 ml. of fluid represents the content of 0·5 ml. of body tissue.

3. A COMPARISON OF LIQUID AND SOLID MEDIA

One important advantage of a solid over a liquid medium is that an actual count is obtained with a solid medium, as against a 'most probable number', the error of which may be very large with a liquid medium unless limits of dilution considerably smaller than ten times are used. In favourable circumstances the coefficient of variation for the most probable number from liquid media with three dilutions each with five tubes is ±60%, while the corresponding figure for colony counts ranges from ±10 to ±20%. Some 50–100 fermentation tubes with each of three dilutions appear to be required to give an estimate equal in accuracy to a single satisfactory plate count (Prescott, Winslow & McCrady, 1946).

Advantages of a liquid over a solid medium are that (with minor exceptions) it is more specific for the organism tested, and can readily be incubated in a water-bath at an accurately controlled temperature. It can be accepted as reasonably certain that positive tubes of MacConkey broth have been fermented by organisms of the type sought for, i.e. coliforms. The same cannot be said for a solid medium, because organisms which ferment lactose without the production of gas may give colonies similar to those produced by organisms which ferment lactose with gas production. Issues such as this, however, cannot be decided entirely on theoretical grounds and it is necessary to consider test results. With regard to the accuracy of quantitative tests, Tables 2–5 give a few results showing differences between replicate counts from minced shellfish liquor or cultures of *Bact. coli* made in solid and liquid media. The numbers of colonies from solid media are counts of red colonies on single plates of MacConkey agar inoculated with 1 ml. of minced mussel 1 ml. of a culture of *Bact. coli*, and the numbers from liquid media represent the most probable number of coliform organisms per ml. determined from five replicate tubes each inoculated with 1/100, 1/1000 and 1/10,000 ml. of fluid. The incubation temperature was 37°.

Table 2. *Pool of ten mussels taken 5 hr. after beginning of purification (results of replicate tests)*

	In solid medium. Actual count of red colonies per ml.	In liquid medium. Most probable number of coliforms per ml.
	11	45
	19	25
	15	7
	22	2
	21	25
	14	13
	13	7
	11	13
	12	8
	15	13
Arithmetic mean	15·3	15·8
Standard deviation	4·0	14·6
Coefficient of variation	26·3	92·4

Table 3. *Pool of eleven purified mussels and one polluted mussel (results of replicate tests)*

	In solid medium. Actual count of red colonies per ml.	In liquid medium. Most probable number of coliforms per ml.
	266	250
	224	170
	224	170
	292	2
	246	5
	—	13
	—	2
	—	8
	—	8
	—	40
Arithmetic mean	250·4	66·8
Standard deviation	25·4	92·8
Coefficient of variation	10·2	138·9

It will be noted from Tables 2 to 5 that the coefficient of variation from results in liquid media is from four to ten times as great as in solid media. This is to be expected because with liquid media there are two inherent sources of error which cannot be avoided. First, the inoculum has to be diluted more than with solid media and, secondly, the results are not obtained directly but from probability tables. This comparison confirms the statement by Prescott *et al.* (1946) as to the superior accuracy of solid media.

Non-lactose-fermenting organisms may give colonies which appear similar to lactose-fermenting

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coliforms, with a resultant over-estimation of pollution, and Bigger (1934) reported unfavourably on the percentage of lactose-fermenting colonies on

Table 4. *Pool of lightly polluted mussels plus culture of* Bact. coli (*results of replicate tests*)

	In solid medium. Actual count of red colonies per ml.	In liquid medium. Most probable number of coliforms per ml.
	110	250
	112	80
	144	130
	119	35
	138	80
	108	—
	97	—
	130	—
	101	—
	109	—
Arithmetic mean	116·8	115·0
Standard deviation	15·4	82·6
Coefficient of variation	13·2	71·8

Table 5. *Water plus culture of* Bact. coli (*results of replicate tests*)

	In solid medium. Actual count of red colonies per ml.	In liquid medium. Most probable number of coliforms per ml.
	49	35
	44	70
	45	35
	45	45
	41	130
	47	—
	45	—
	38	—
	51	—
	53	—
Arithmetic mean	45·8	63·0
Standard deviation	4·5	40·0
Coefficient of variation	9·8	63·5

MacConkey agar plates, having found only seven lactose fermenters out of 145 isolated. This appears to be an unusual case, requiring further investigation, for data collected at this laboratory over a number of years show that out of 3711 colonies isolated from MacConkey plates 3170 or 85·4 % were lactose fermenters.

There is another possibility of error which probably applies to both solid and liquid media similarly when used for the examination of shellfish, viz. the production of acid and gas by non-lactose fermenting organisms which presumably use glucose formed from glycogen in the shellfish tissue. This was first demonstrated by Dodgson (1928), and later confirmed by Webb (1928), who successfully demonstrated the production of glucose from the glycogen of shellfish. Dodgson showed that acid and gas were produced in a 10 ml. tube of carbohydrate-free medium by bacteria in polluted mussels by the introduction into the medium of an amount of minced mussel juice as small as 0·1 ml. Bigger (1934) showed that this drawback could be overcome in liquid media by increasing the volume of the medium, thus decreasing the concentration of the fermentable sugar to such an extent that any acid formed from the glucose was neutralized by the buffer in the medium. Bigger further showed that 0·075 % of glucose was the maximum that could be allowed in the litmus bile broth if acid reaction from the fermentation of this carbohydrate was to be avoided, and suggested the use of tubes containing 30 ml. of medium. The largest amount of minced shellfish juice inoculated into a tube by this method was 0·5 ml. of reconstituted mussel, representing 0·3 ml. mussel tissue; assuming a glucose content of 5 % for shellfish, this would give a concentration of 0·05 % in the medium. Bigger suggested that it would not be possible to increase the amount of solid medium used in this way. Incubation at 44°, however, appears to eliminate this difficulty altogether.

II. THE USE OF ROLL TUBES INCUBATED AT 44° FOR THE ENUMERATION OF FAECAL *COLI*

1. Preparation of the medium

The standard MacConkey agar is unsuitable for roll tubes incubated at 44° because during incubation it slips down the tube, and also condensation water collects at the bottom.

After trials of modified media containing various proportions of agar and gelatin, which were rolled in air, water, and on ice, a mixture of 5 % agar and 2 % gelatin was found to set when rolled under tap water at temperatures up to 25°. The medium was made up in 10 ml. quantities as used in the Petri-dish technique. By the use of 15 by 2·5 cm. tubes, rolling the medium until it had spread 10 cm. up the tube gave a surface of *c.* 70 sq.cm., i.e. slightly greater than that of a Petri dish of 9·0 cm. diameter.

The constituents of this special MacConkey agar are as follows:

Agar	50 g.	Sodium chloride	5 g.
Gelatin	20 g.	Sodium tauroglycocholate	5 g.
Peptone	10 g.	Distilled water	900 ml.

To prepare, dissolve the agar (washed shredded agar, soaked overnight is preferable) in 700 ml. of water by heating in an autoclave at 15 lb. pressure

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for 20 min. Dissolve the other ingredients (sheet gelatin is preferable) in 200 ml. of water by heating in a steamer for approximately 1 hr., and mix with the dissolved agar. Adjust the pH to 7·8 (keeping the 10 ml. test portion at boiling-point while adjusting). Steam for 1 hr. and filter hot through a thin layer of moistened absorbent cotton-wool. Replace any water lost during heating and filtration, and distribute in 9 ml. quantities in 15 by 2·5 cm. test-tubes. Sterilize by heating in a steamer for 30 min. Prepare a solution of 10 % lactose in 0·1 % neutral red, and sterilize in the same way. Add 1 ml. of this indicator-sugar solution to each tube at the time of inoculation. (Separate addition eliminates one avoidable heating of the sugar, and reveals any incompletely melted agar; the addition of the dye with the lactose is also a safeguard against the accidental omission of the lactose.)

To test whether the increased concentration of agar or the inclusion of gelatin would result in a lower count, replicate plate cultures were made from shellfish samples in standard MacConkey agar and in the special medium and arranged alternately in copper boxes in a 44° water-bath. In thirty-nine pairs of plates from five samples the total count on the MacConkey agar was 6172, while that on the special roll-tube medium was 5901 colonies, i.e. 4·5 % less. A statistical examination by the 't' test showed that this difference was not significant.

2. Description and use of mechanical roller

Although tubes can be rolled by hand the advantage of a mechanical roller (Fig. 1) are obvious. Gee (1932) devised a centrifugal machine for preparing tube cultures while at sea (the use of Petri dish cultures being inconvenient in a rolling ship). A power-driven experimental model to take five tubes (as shown in Fig. 2) was constructed at Conway and has given satisfactory service. Similar devices were used in the examination of milk by Mundinger & Wolckel (1934) and Prouty, Bendixen & Swensen (1944).

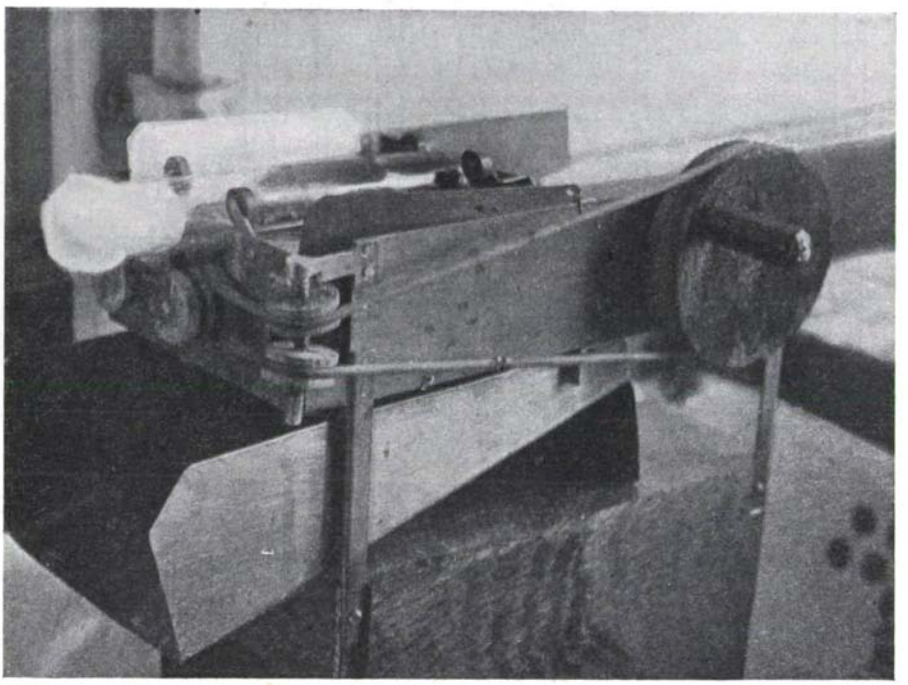

Fig. 1. Hand-driven rolling machine.

The machine now in use consists of six wooden rubber-covered rollers, 2·5 cm. diameter by 10 cm. in length (with central steel spindles 0·2 by 13 cm.) mounted side by side, 10 cm. apart, in a horizontal brass frame. The frame is tilted about 2½° to prevent flow of the medium to a greater distance than 10 cm. along the tube during rolling. The spindles are fitted with securing collars and cog wheels, which by means of similar intervening wheels, provide for rotation of the rollers in the same direction. The drive is from a 1/50 h.p. electric motor with a rubber belt to a pulley 7·5 cm. in diameter on one of the roller spindles.

Above the roller, supported by a stand, which also holds the frame, is a metal tank of 18 l. capacity fed from a tap. From the bottom of the tank five tubes of 5 mm. diameter, each with a tap controlled by

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a common handle, deliver jets of water over the middle of the culture tubes placed on the rollers, the jets being 3·0 cm. above the tubes. The rate of delivery of each jet is *c.* 600 ml./min. The rolling speed is adjusted by means of a resistance to 350–400 r.p.m.

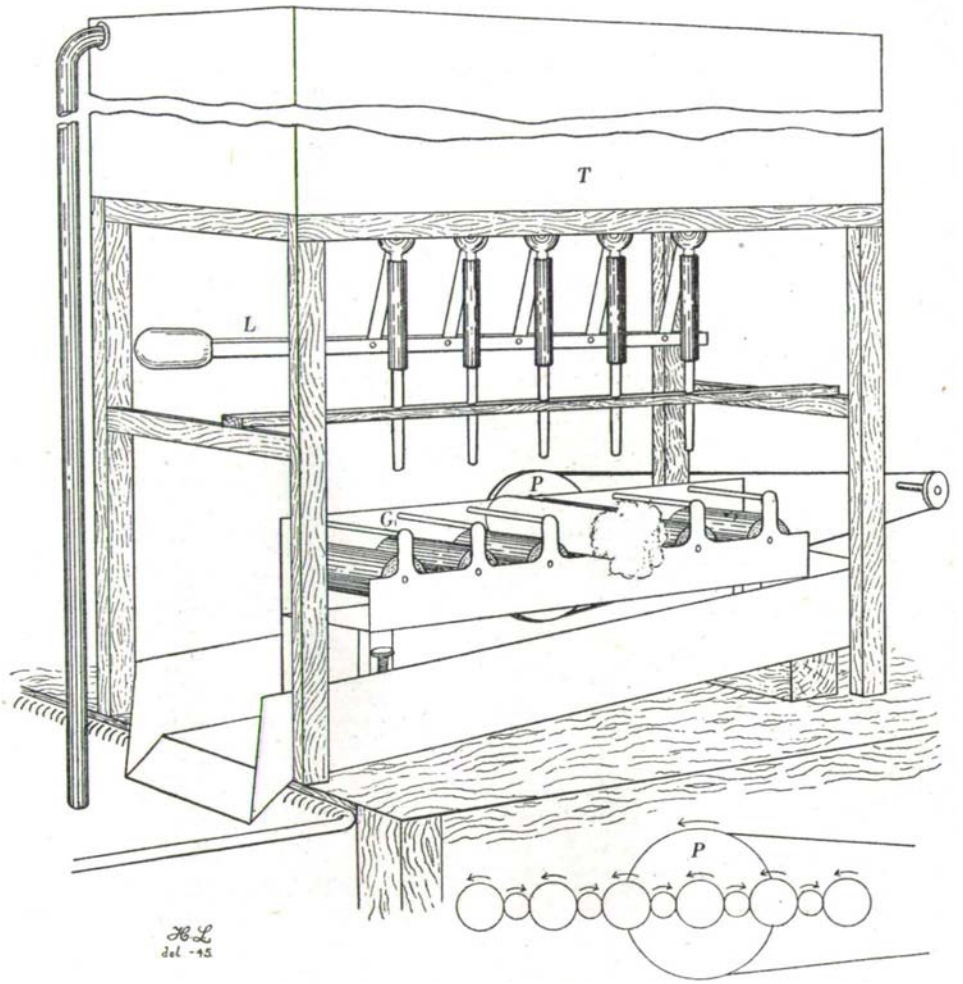

Fig. 2. Rolling machine for five tubes, showing one in position. Inset below: diagram of gears. *T*, water tank; *L*, lever controlling taps; *P*, driving pulley; *G*, guard rail to prevent displacement of tubes.

The inoculum is added to the tube containing the medium previously maintained at a temperature of 46° in a water-bath. After gentle shaking to mix the contents, the tube is placed on the rollers with the bottom against the frame carrying the driving gear, and left for at least 2 min. to ensure a complete 'set' before removal. If the speed of rolling falls much below 350–400 r.p.m. a short and thick coating is produced which makes counting difficult. Too high a speed causes the medium to spread too far with the resultant danger that it be above the level of the water in the bath during incubation. With the apparatus described there is hardly any reduction of speed when five tubes are rolled simultaneously instead of one. The tubes are placed in a partitioned wire basket for incubation.

3. Experimental results obtained with the special medium in roll tubes

A. *Types of organisms*

Roll-tube cultures of the modified MacConkey agar were made as previously described from a series of representative shellfish samples taken from widely separated beds and incubated in a water-bath at 44° ± 0·2°. From each sample 100 red colonies within a given area were chosen regardless of size, and tested for production of gas at 44° in MacConkey broth, and for production of indole at 37°. Cultures yielding positive results in both tests were recorded as *Bact. coli* type I. The remainder

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were tested further for methyl red, Voges-Proskauer and citrate reactions. It was thought unnecessary to test the complete 'IMViC' reactions of the lactose 44° positive, indole-positive cultures, because the results from a previous investigation of 1069 44° lactose positive failed to show any indole-positive samples would thus have exceeded the faecal *coli* content by 3·2 %. This figure is less than the experimental error of any plating or roll-tube method, though it may be an additional error. From this result it appears that use of the MacConkey agar roll tube would provide a specific test for faecal *coli*.

Table 6. *Sources of samples and types of organisms from* 1000 *red colonies isolated from MacConkey agar roll tubes at* 44°

	Reactions in MacConkey broth at 44°							
	Positive		Negative					
Source of sample	Irregular II	*Bact. coli* I	Irregular I	*Bact. coli* II	Intermediate I	*Bact. aerog.* I	Non-coliforms	Total
Conway Estuary (above bridge)	.	100	.	.	.	.	.	100
Conway Estuary (Junction sewer)	1	98	1	.	.	.	.	100
Conway Estuary (Morfa Channel)	3	93	3	.	1	.	.	100
Menai Straits (Bangor sewer)	2	95	2	.	.	.	1	100
Rhyl (foreshore near sewer)	2	97	.	.	.	.	1	100
Dee Estuary (Heswall)	5	92	.	3	.	.	.	100
Wash (Boston N. Lays)	.	100	.	.	.	.	.	100
Ribble Estuary (Lytham)	4	92	2	.	.	2		100
Medway oysters (Rainham)	.	97	1	.	1	.	1	100
Penclawdd cockles	3	95	1	.	.	.	1	100
	20	959	10	3	2	2	4	
	979		969		21			

Table 7. *Pool of six lightly polluted mussels (results of replicate tests)*

In solid medium		In liquid medium.
Plates at 37°.	Roll tubes at 44°.	Tubes at 44°.
Actual count of red colonies per ml.	Actual count of red colonies per ml.	Most probable number of faecal *coli* per 3 ml.
43	34	170
39	18	70
38	28	50
49	42	130
29	28	25
—	24	25
—	29	35
—	20	50
—	33	80
—	24	80
—	27	—
—	25	—
Arithmetic mean 39·6	27·7	71·5 (23·8 per ml.)
Standard deviation 7·1	6·5	47·0
Coefficient of variation 18·0	23·4	65·7

cultures other than *Bact. coli* type I (Sherwood & Clegg, 1942).

Table 6 gives the results of testing 1000 red colonies which grew at 44° from eight samples of mussels, one sample of oysters and one sample of cockles.

Of these cultures 96·9 % were of the 'IMViC' type + + − −, i.e. *Bact. coli* I and Irregular I, both of which are considered to be of intestinal origin. The number of red colonies at 44° from the ten

B. *Numbers of organisms*

To compare the error of the roll-tube method at 44° (with special MacConkey agar) with that of the colony count in MacConkey agar in Petri dishes, and also with the dilution method in liquid medium (MacConkey broth), experiments were made on pools of lightly polluted mussels and on water samples (Tables 7–10). In some of the tests in liquid medium the ten sets of dilution tubes have

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been read down as well as across, thus affording two sets of results.

In Table 11 are reproduced the arithmetic means and coefficients of variation for each sample by both plate and roll-tube methods.

one of the samples the average is well below 30, hitherto regarded as the lowest value to give consistently reproducible results (Wilson, 1935, p. 123). If allowance be made for the roll-tube counts falling within this unfavourable range, they suggest an

Table 8. *Pool of six lightly polluted mussels (results of replicate tests)*

	In solid medium		In liquid medium. Tubes at 44°.	
	Plates at 37°.	Roll tubes at 44°.		
	Actual count of red colonies per ml.	Actual count of red colonies per ml.	Most probable number of faecal *coli* per 3 ml.	
	16	14	25	35
	12	13	80	35
	15	14	25	35
	20	12	25	25
	14	19	35	35
	—	16	35	35
	—	9	13	80
	—	12	45	30
	—	7	52	25
	—	9	17	11
Arithmetic mean	15·4	12·5	35·2 (11·7 per ml.)	34·6 (11·5 per ml.)
Standard deviation	3·0	3·6	19·7	17·7
Coefficient of variation	19·3	28·7	56·0	51·2

Table 9. *Sample of water from heavily polluted stream (results of replicate tests)*

	In solid medium		In liquid medium. Tubes at 44°.	
	Plates at 37°.	Roll tubes at 44°.		
	Actual count of red colonies per ml.	Actual count of red colonies per ml.	Most probable number of faecal *coli* per 10 ml.	
	57	15	600	600
	52	22	600	600
	63	18	350	350
	71	26	170	900
	59	15	900	350
	56	25	350	350
	62	18	250	600
	67	15	250	250
	44	23	350	900
	56	12	350	350
Arithmetic mean	58·7	18·9	417 (41·7 per ml.)	525 (52·5 per ml.)
Standard deviation	7·7	4·8	220·1	234·8
Coefficient of variation	13·1	25·5	52·8	44·7

These figures suggest, as would be expected, that the higher the arithmetic mean (subject to the recognized limit) the lower is the coefficient of variation.

The errors of the three methods are reasonably consistent. The roll-tube counts, through elimination of the I.A.C. group at the higher temperature, are much lower than those on the Petri dishes; in all but accuracy of the same order as the Petri-dish counts, both series showing greater consistency than the most probable number from the liquid medium.

It is accordingly believed that the roll-tube method may be an advance on other methods for the numerical examination for faecal *coli*, applicable not only to shellfish, but to other material containing sufficient faecal *coli* to be present in 1 ml. amounts.

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Table 10. *Sample of water from heavily polluted stream (results of replicate tests)*

	In solid medium		In liquid medium. Tubes at 44°.	
	Plates at 37°. Actual count of red colonies per 2 ml.	Roll tubes at 44°. Actual count of red colonies per ml.	Most probable number of faecal *coli* per 5 ml.	
	132	9	80	600
	121	16	130	130
	105	7	80	80
	84	14	170	170
	119	19	80	250
	134	15	110	350
	126	7	80	250
	109	10	50	350
	115	21	80	80
	142	—	250	110
Arithmetic mean	118·7 (59·3 per ml.)	13·1	111 (22·2 per ml.)	237 (47·4 per ml.)
Standard deviation	16·7	5·1	39·3	322·8
Coefficient of variation	14·1	39·2	35·4	136·3

Table 11. *Comparison of arithmetic means and coefficients of variation from Tables 7 to 10*

	Colony count in Petri dish at 37°		Colony count in roll tube at 44°	
	Arithmetic mean	Coefficient of variation	Arithmetic mean	Coefficient of variation
From Table 7	15·4	19·3	12·5	28·7
From Table 8	39·6	18·0	27·7	23·4
From Table 9	58·7	13·1	18·9	25·5
From Table 10	118·7	14·1	13·1	39·2

III. PROPOSED BACTERIOLOGICAL TEST FOR SHELLFISH

1. SAMPLING AND CHOICE OF METHOD: GENERAL CONSIDERATIONS

A. *Bivalves*

For research or advisory purposes bivalve shellfish should be examined individually, but for routine checking of purity this method may not be sufficiently convenient or speedy and the examination of batches or pools may be substituted.

Mussels from a given bed usually become polluted uniformly and are therefore good indicators of pollution. In most instances therefore the examination of pools is likely to yield all the information required. There is one notable exception; mussels, initially clean, which have become polluted in transit by standing the bags in places which have been made insanitary by human or animal faeces may be very unevenly polluted. Where such pollution in transit is suspected, individual examination is recommended.

Oysters from the same bed have repeatedly been observed to be unevenly polluted, and for this reason individual examination is necessary, the most polluted oyster being taken to indicate the conditions to which they have been exposed. Uneven pollution due to exposure to insanitary conditions during transit is rare because oysters are usually protected to a large extent by the wooden containers in which they are marketed.

The control of shellfish by market tests is not in general recommended; the best indication whether shellfish should be accepted at the markets is to be derived from comprehensive surveys of shellfish beds. Evidence of pollution on the beds will vary considerably according to the state of the tide and the proximity of sewer outfalls. A pool of five or ten shellfish from one part of a bed would yield less information than one made up of individuals taken from different parts of the bed. More reliance can be placed on results obtained from the examination of individual shellfish than from a pool, while still more precise information can be obtained if sampling is repeated under different tidal conditions.

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B. *Univalves*

These shellfish do not feed by filtration but by 'grazing', and therefore the examination of individuals may give no clue to the general pollution of others from the same source, their intestinal contents being indicative only of the bacterial flora of the small area of the beds grazed during the few hours before the collection of the sample. Thus it is recommended that a number of individuals should be examined together. Little work has been done however, and special methods of sampling and examination may be required.

2. Preparation for examination

The shellfish is held in a double layer of sterile grease-proof paper *c.* 18 by 13 cm., which has an 8 cm. turn-up along one of the narrower sides, within which the shellfish is placed so that fluid draining from it, and the flesh when released from the shell, passes down the gutter thus formed into the appropriate receptacle. The shell water is either poured away or, if required for separate examination, collected from each shellfish in turn in the same sterile cup, which is then covered with a sterile saucer until the contents are required. Small enamel-ware cups and saucers are recommended for this purpose.

A. *Mussels*

Mussels are opened by inserting and slightly twisting a sterile oyster knife or scalpel at the point whence the byssus threads emerge; this permits escape of the shell water. The adductor mussel is severed with a sterile scalpel, and the flesh detached from the upper half shell by running the scalpel round the margin. The half shell is then levered up with the scalpel. Next, the body is scraped and chopped with the scalpel to expose the contents of the alimentary canal, particular attention being given to the rectum. The gills are stroked several times with the back of the scalpel to free any masses containing bacteria which may be adhering to them. Finally the mussel is cut free from the lower shell and tipped into a sterile cup and covered.

B. *Oysters*

Oysters are opened by inserting the point of a sterile oyster knife or scalpel on one side and severing the adductor muscle. If this presents unusual difficulty, a notch is cut in the shell with sterile bone-forceps. The subsequent procedure is similar to that used for mussels, except that the upper shell is already free as soon as the adductor has been cut. A deep incision is necessary to release the contents of the stomach.

In purified shellfish the rectum should be empty, but with unpurified shellfish the rectum can usually be seen to contain faeces.

C. *Cockles*

Cockles are opened similarly, except that there are two adductor muscles to be severed.

D. *Univalves*

Few univalves have been examined and the technique has not been perfected, but the following method is suggested: the shellfish are held in the same way as mussels; the point of a sterile blood needle, dissecting needle or pointed forceps is forced behind the horny disk covering the entrance to the shell and the flesh is gently withdrawn. If difficulty is experienced, and this frequently occurs, it is necessary to crush the shell with sterile pliers and extract the body with forceps.

3. Methods of examination

A. *Bivalves—long method (individual examination)*

From 5 to 10 ml. of sterile water (in proportion to the size of the shellfish to be examined) is placed in a sterile 100 ml. measuring cylinder to provide for measurement of the shellfish body tissue. The fluid which exudes after laceration should be added to the sterile water in the cylinder and the total volume measured. The flesh is then added and its volume measured by displacement. After measurement of the body tissue, sterile water is added to make the total volume equal to three times that of the body tissue. A sterile rubber bung is then placed in the mouth of the cylinder and the contents are shaken fifty times with an up-and-down motion. The shellfish 'liquor' is then ready for inoculation. This procedure is repeated with ten shellfish, a separate cylinder being used for each.

B. *Bivalves—short method (shellfish pooled)*

Ten shellfish are opened as described, and put into a sterile container after draining off the shell water. The volume of the flesh from all the ten shellfish is measured by displacement in a sterile cylinder of suitable capacity, and sterile water is added to make the total volume equal to three times that of the body tissue. A sterile rubber bung is inserted in the mouth of the cylinder and the contents shaken fifty times with an up-and-down motion. The shellfish 'liquor' is then ready for inoculation.

C. *Univalves*

Ten shellfish are opened as described, and placed in a sterile cylinder containing a measured amount of sterile water sufficient to cover them. The volume of the body tissue of the ten shellfish is measured by displacement, and additional sterile water added to produce a total volume three times that of the body tissue. The contents of the cylinder are then poured into a sterile mortar, and the liquid decanted back into the cylinder to facilitate grinding with a sterile

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pestle the flesh left in the mortar. After the flesh has been ground for 3 min. the water from the cylinder is added, a few ml. at a time, stirring with the pestle being continued until all the water has been added and the sides of the mortar are free from adhering tissue. The flesh/water suspension is then tipped back into the cylinder, the rubber bung is fitted in the mouth and the cylinder shaken with an up-and-down motion fifty times.

4. Inoculation of roll tubes

With bivalves (mussels, oysters, etc.) it is usually sufficient to use 1 ml. of inoculum of the fluid from each shellfish, although 1 ml. of 1 : 10 dilution can also be inoculated if heavy pollution is suspected.

With uncooked whelks and periwinkles it is preferable to inoculate duplicate tubes with 1 and 0·1 ml. quantities. It may be necessary to dilute inocula up to 1/100 of the original as this type of shellfish may sometimes be excessively polluted; they have been observed to ingest crude faeces.

When melted, the special MacConkey agar (9 ml./15 cm. by 2·5 cm. tube) should be placed in a water-bath at 46° to prevent solidification. Immediately before inoculation of each tube 1 ml. of lactose solution is added, followed by the inoculum. The tube is then placed on the rolling machine and left for 2 min. until the medium has set. It will be found that the first tube has set by the time the fifth tube has been inoculated. When pools of shellfish are being examined by the short method and it is necessary to make only one or two roll-tube cultures, these can be prepared by hand rolling under a tap, or on the small hand-operated machine (Fig. 1). Condensation water in the upper portion of the tube can be removed by warming over a Bunsen flame.

With the short method, duplicate tubes should be inoculated each with 1 ml. of shellfish fluid from the pool, and the combined counts taken as the content per ml. of body tissue.

Tubes are incubated at 44° in a thermostatically controlled water-bath with a variation of not more than ± 0·2° (see Clegg & Sherwood, 1939). The water in the bath must completely cover the agar roll in the tube. Tubes inoculated the previous evening are ready the next morning for a preliminary examination of the red colonies if an early result is desired, but to ensure standardization all tubes should receive a full 24 hr. incubation.

The counting of colonies is not different but can be facilitated by use of a slot to fit 15 by 2·5 cm. tubes, cut in the face of a counting chamber. With plates incubated at 37°, often containing colonies of a size range from 2 or 3 mm. across down to those barely visible to the naked eye, doubt arises as to the dividing line between colonies of probable lactose fermenters with gas, and of other organisms, but with roll tubes of special MacConkey medium at 44° all red colonies may be included confidently in the count. This alone is a considerable advantage.

5. Determination of results

A. *Bivalves—long method*

As the volume of each 'reconstituted' shellfish (R.S.) is known, the results can be expressed either as so many faecal *coli* per shellfish or per ml. of body tissue. It is suggested (p. 510) that it would be more rational to use the second alternative, viz. to express the result as a *degree* of pollution regardless of the size of shellfish. To arrive at this figure, the number of red colonies from 1 ml. of R.S. is multiplied by the total volume of liquid and divided by the volume of body tissue. For example, the calculation for a shellfish with 8 ml. of body tissue made up to a volume of 24 ml. with sterile water, which gave a count of 100 red colonies per ml. of R.S. would be $(100 \times 16)/8 = 200$ *Bact. coli* per ml. of body tissue. More simply the figure is obtained by multiplying the count per ml. of R.S. by two.

Such a figure records the degree to which shellfish of any size have been polluted, and will give more consistent results than have hitherto been possible. It should have considerable value in the survey of shellfish beds in supplementing topographical information.

B. *Bivalves—short method*

The number of red colonies per ml. of the pool is multiplied by two to give the number of faecal *Bact. coli* per ml. of body tissue, or if two tubes have been prepared the sum of red colonies on both tubes is taken as equivalent to 1 ml. of body tissue.

C. *Univalves*

If 1 ml. of the R.S. has been inoculated, the method is the same as for bivalves examined by the short method, otherwise the number of red colonies per ml. of the pool must be multiplied by twice the diluting factor.

6. Interpretation of results

The interpretation of results is a matter for the authority concerned, all the circumstances having been considered. A few observations may, however, be helpful as a working basis.

It should be borne in mind in assessing danger to public health that during the last 30 years there has been no confirmed evidence of conveyance of disease-producing organisms by shellfish from approved purification installations. Such installations can no longer be regarded as on trial, to be closed temporarily as insanitary areas for an uncertain period, pending the operation of some

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unknown beneficial factor, should there be an unfavourable bacteriological result. There is scope for scrutiny of management, but not for bacteriological control of this sort, which would involve suspension of operations.

A standard of purity originally reached by purified shellfish was that of not more than five coliforms per ml. of minced flesh and shell water, cultures being incubated at 37°. It is now suggested that with the proposed test, the desideratum for all shellfish should be absence of faecal *coli* from 1 ml. quantities of body tissue. This should not be an inflexible standard, and any slight divergence from it should not result in drastic action. It is suggested that from any one source four out of five samples should reach this standard, and that the presence of more than two or three faecal *coli* per ml. of body tissue calls for investigation.

To those who on reading this paper may consider the proposed test to be too elaborate, it can be said in conclusion that the less homogeneous is the material to be examined the greater are the precautions needed in testing.

SUMMARY

Section I

A standard bacteriological test for molluscan shellfish should fulfil certain requirements stated: briefly, the test should show the degree of pollution, be accurate and rapid, and self-sufficient, not requiring subsequent confirmation. Review of the subject leads to recommendations, first, to overcome the technical difficulties of preparing samples for testing; secondly, on the nature of the test. In preparation: external shell sterilization can usually be omitted, shell water should be discarded and replaced by sterile water to make a total volume three times that of the body tissues. Pooling of individual shellfish into one sample is acceptable in routine examinations. In the test: a solid medium is preferable to a liquid medium, giving more accurate results, and review of existing tests leads to the conclusion that the use of roll tubes of MacConkey agar incubated at 44° should meet the requirements of a standard test.

Section II

A modification was found necessary in the MacConkey agar: a mixture of 2% gelatin and 5% agar is used instead of the normal 2% agar. Mechanical rolling devices for tubes are described and figured.

Among other critical experiments, 1000 roll-tube colonies grown at 44° from shellfish included 969 with + + − − 'IMViC' reactions and 979 acid and gas producers at 44°. The coefficient of variation among replicate tests of samples of shellfish and water in roll tubes was not seriously greater than that for colony counts in Petri dishes at 37° with ordinary MacConkey agar.

Colonies in roll tubes incubated at 44° can be counted as conveniently and accurately as those on Petri dishes, and, in general it is concluded that the new method is more satisfactory for estimation of faecal *coli* than other methods at present in use.

Section III

Directions are given for the preparation of shellfish and inoculation into roll cultures, both for individual and for pooled examination, and the method of determining results is described.

The interpretation of results is discussed, and it is suggested that shellfish which in four out of five samples from the same source are free from faecal *coli* in 1 ml. quantities of body tissue should be regarded as satisfactory for food. The presence of more than two or three faecal *coli* per ml. of body tissue in any one sample calls for appropriate action according to the number present.

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