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Comparison of two methods for assessing the bacteriological condition of the water supply in slaughter houses

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One hundred and five water samples of drinking water quality used in slaughter houses were examined according to The Netherlands' Standard N 3043 (1956) and by the new 'Differential Hydrobacteriogramme' (DHB) technique. The latter procedure allows for resuscitation of debilitated cells, uses one single selective enrichment medium and various isolation media and includes confirmation of isolates. Identification of isolates demonstrated that false positives as well as false negatives were frequently obtained by the N 3043 method. Rejection rate of samples by the N 3043 and DHB methods, respectively, was 63 vs. 72 for 81 samples taken from taps, and 4 vs. 10 for 24 samples drawn from wells and mains. Based on these results the DHB technique seems particularly suitable for the monitoring of re-used waters, because it provides more information and has a higher accuracy.

Key words: Water; Bacteriological examination; Differential hydrobacteriogramme; Slaughter houses

Introduction

Because of the increasing scarcity of drinking water, the rising costs connected with its use and the expenses involved in the disposal of waste water, research has been undertaken into the re-use of water in the food industry. This applies both to, for example, cooling water in the processing of foods and the water used more than once for the same purpose (Strauch, 1976; Mossel et al., 1978b; and Eichhoff, 1982). The possibility of contamination of such water demands a thorough inspection of its bacteriological condition after its primary use. The treatment to which water is sometimes exposed before re-use can result in sublethal lesions of bacteria surviving the treatment (Bonde, 1982; Mossel and Van Netten, 1984). These bacteria will again become fully active on, for example, meat and other products (Mossel and Van Netten, 1984). It is, therefore, necessary to include such debilitated bacteria, when assessing the bacteriological condition of recycled water (Allen et al., 1952, 1953). A new, simplified method was introduced for this purpose a few years ago, the so-called Differential Hydrobacteriogramme (DHB). It relies on (i) presence-absence (P-A) tests, subsequent to resuscitation, for Enterobacteriaceae, *Escherichia*

coli, Pseudomonas aeruginosa, Aeromondaceae, Lancefield D streptococci and (ii) identification of isolates (Mossel et al., 1977a).

In The Netherlands, monitoring of the bacteriological condition of drinking water until 1982 followed The Netherlands' Standard N 3043 (Nederlandse Norm, 1956). Examination according to this Standard includes (i) the determination of aerobic colony forming units at 20 °C and 37 °C; (ii) direct presence-absence tests in prescribed aliquots for specific bacteria i.e. *E. coli*, bacteria of the coli-aerogenes group and Lancefield group D streptococci.

The aim of this investigation was to compare the results of the DHB and the N 3043 techniques.

Materials and Methods

A total of 105 water samples were taken in a number of slaughter houses. They were drawn from: (i) in-flowing water: well water and water from the public drinking water mains; (ii) sampling points after treatment and buffering of water; (iii) various taps in the factory drinking water pipe system, such as continuously running sprinklers, hand showers, hand basin taps, organ rinsers, 'splitter' rinse water, etc.

The samples listed under (i) and (ii) were drawn under aseptic conditions as prescribed in The Netherlands' Standard N 3043. The samples listed under (iii) were taken during production in the following way. From the continuously open taps or sprinklers a sterile 500 ml bottle was filled with running water and immediately closed with a sterile screw cap. When sampling water from taps and sprinklers which

TABLE I

Mode of examination of drinking water according to The Netherlands' Standard N 3043

Bacterium type sought	Quantity of water examined	Medium used for enrichment	Growth temperature (°C)	Requirement
'Thermotolerant fermentative bacteria'; mainly *E. coli*	50 to 55 ml	buffered glucose peptone broth, modified after Eijkman	44	no gas formation nor bacterial growth
Mainly bacteria of the coli-aerogenes group	5 × 10 ml	peptone-bile-lactose-bromo-cresol purple broth according to MacConkey	37	in 1 × 10 ml no gas formation nor growth
Mainly 'faecal' streptococci, i.e. of the group D according to Lancefield (L.D.)	10 ml	sodium azide broth	37	no growth

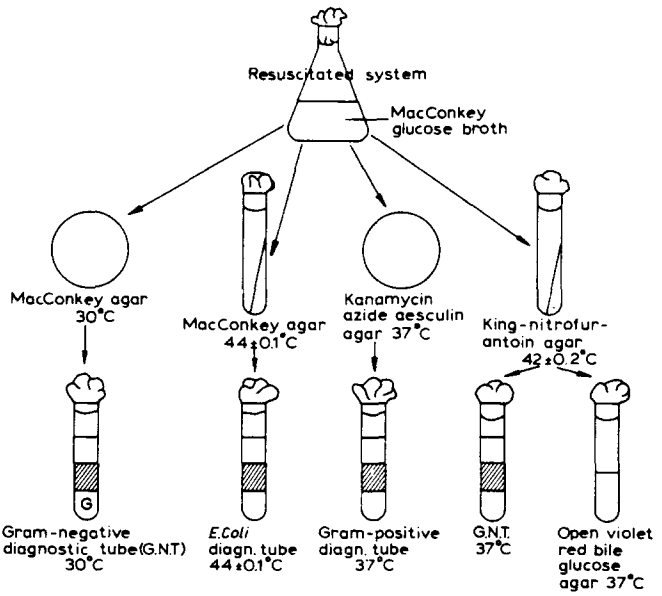
were not continuously running a sterile 500 ml bottle was filled with the first water flowing out when the tap was opened.

The samples were transported to the laboratory under refrigeration. They were examined within one hour according to the two procedures.

1. Examination according to The Netherlands' Standard N 3043, hereafter referred to as N 3043; cf. summary in Table I.

When positive results were obtained, the contents of the flasks or tubes concerned were examined for the pertinent organisms by the confirmation procedures, used in the DHB technique; vide infra (Mossel et al., 1977a).

2. Determination of the Differential Hydrobacteriogramme, hereafter referred to as DHB. This procedure is indicated in Fig. 1. It can be summarized as follows.



Target groups and criteria

Criterion	Entero-bacteriaceae	*Aeromonas*	*E. coli* at 44 °C	Lancefield D streptococci	*Ps. aeruginosa*
Mode of attack on					
Glucose	F(G)	F(G)	FG	F	Ox
Motility	V	+	+	..	+
H_2S	V	−	−	..	−
Indole	V	V	+	..	−
Oxidase	−	+	−	..	+
Catalase	+	+	+	−	+

F = fermentative attack
FG = Do with gas formation
Ox = Oxidase
V = variable
− = negative
+ = positive
.. = irrelevant

Fig. 1. The differential hydrobacteriogramme (DHB).

Aliquots of 100 resp. 10 ml are collected in sterile vessels of suitable size. An equal volume of tryptone soya broth is added and the mixture left at room temperature for 2 to 6 h, depending on the period of time that must be assumed to be required for complete recovery of the most severely stressed population present in the specimen (Mossel and Van Netten, 1984). Subsequently 20, resp. 2 ml of a concentrated bile salts bromocresol purple solution (Mossel et al., 1977a) is added and the system incubated overnight at 30 °C. Enrichment cultures that remain crystal clear are discarded and the result noted as negative. Flasks showing acidification or just growth are shaken and one loopful of their contents streaked onto the selective media shown in Fig. 1. Negative cultures are discarded, positive ones are further examined to assess the taxonomic position of the isolates by stabbing into the appropriate diagnostic single tubes as also indicated in Fig. 1 (Mossel et al., 1977b). This procedure therefore allows the assessment of the presence or absence of at least six taxa in the water sample under examination: (i) Enterobacteriaceae as a group, if required examined further for the presence of the coli-aerogenes group (Mossel et al., 1979); (ii) *Aeromonas* (Schubert, 1975; Mossel et al., 1979); (iii) *E. coli* (Mossel et al., 1980); (iv) Lancefield group D streptococci (Mossel et al., 1978a); (v) *Ps. aeruginosa* (Mossel et al., 1976); (vi) Gram-negative organisms not currently looked for but yet of occasional significance (Allen et al., 1983).

Results

The data obtained in the comparative examination of various types of water sampled in slaughter houses according to DHB and N 3043 respectively are

TABLE II

Comparison of the results of the determination of the DHB and examination according to N 3043 of well and piped water tested before entering the industrial drinking water mains system. A total of 24 water samples was examined

Criterion	Number of positive results		
	N 3043	Both in N 3043 and in DHB	DHB
Thermotolerant fermentative bacteria	2 [a]	–	1 *E. coli*
Coli-aerogenes bacteria	1	1	7 [b] Enterobacteriaceae Aeromonadaceae
Faecal streptococci	–	–	3 L.D. streptococci
Other	–	–	no *Pseudomonas aeruginosa*

[a] 1 × *Serratia* and 1 × *Klebsiella* isolated from the thermotolerance test, instead of *E. coli*.
[b] 1 × *Xanthomonas* isolated.

TABLE III

Comparison of the results of the determination of the DHB and examination according to N 3043 of industrial water from taps in pork slaughter houses; 53 water samples

Criterion	Number of positive results		
	N 3043	Both in N 3043 and in DHB	DHB
Thermotolerant fermentative bacteria	9 [a]	6	19 *E. coli*
Coli-aerogenes bacteria	16	16	45 Enterobacteriaceae Aeromonadaceae
Faecal streptococci	4 [b]	2	19 L.D. streptococci
Other	–	–	no *Pseudomonas aeruginosa*

[a] 3 × N 3043 positive and DHB negative in the same sample; 5 × *E. coli* negative.
[b] 2 × N 3043 positive and DHB negative in the same sample.

summarized in the Tables II–V. The following gross results were noted as far as samples leading to positive results are concerned: 39 were positive according to both tests; 47 positive according to N 3043; 133 were positive in the DHB. In Tables VI and VII the results were summarized after elimination of the false positive results sometimes obtained in the N 3043 method.

The net results of the presence-absence tests for the pertinent bacterial types, as recorded in Table VIII, were particularly analysed for the number of times that a limit value was exceeded. It appeared that: (i) amongst the 24 water samples from

TABLE IV

Comparison of the results of the determination of the DHB and examination according to N 3043 of industrial water from taps in a beef slaughter house. A total of 22 water samples was examined

Criterion	Number of positive results		
	N 3043	Both in N 3043 and in DHB	DHB
Thermotolerant fermentative bacteria	4 [a]	3	6 *E. coli*
Coli-aerogenes bacteria	3	3	16 Enterobacteriaceae
Faecal streptococci	3	3	6 L.D. streptococci
Other	–	–	no *Pseudomonas aeruginosa*

[a] 1 × *Serratia* instead of *E. coli* isolated from the 44 °C enrichment test; DHB negative in this sample.

TABLE V

Comparison of the results of the determination of the DHB and the examination according to N 3043 of industrial water from taps in the remaining types of slaughter houses (calf, sheep, and chicken). A total of 6 water samples was examined

Criterion	Number of positive results		
	N 3043	Both in N 3043 and in DHB	DHB
Thermotolerant fermentative bacteria	3	3	4 *E. coli*
Coli-aerogenes bacteria	2	2	5 Enterobacteriaceae Aeromonadaceae
Faecal streptococci	–	–	2 L.D. streptococci
Other	–	–	no *Pseudomonas aeruginosa*

TABLE VI

Comparison of all gross results

Criterion	Number of positive results		
	N 3043 only	Both in N 3043 and in DHB	DHB only
Thermotolerant fermentative bacteria [a]	3	12	18 *E. coli*
Coli-aerogenes bacteria	0	22	55 Enterobacteriaceae Aeromonadaceae
Faecal streptococci	2	5	25 L.D. streptococci

[a] *E. coli* positive after confirmation.

TABLE VII

Analysis of the results obtained in testing for *E. coli* by the two methods

Type of water	Method	Result of the examination		
		Positive	Positive, confirmed as *E. coli*	Positive not confirmed as *E. coli*
Well and piped water (24)	N 3043	2	0	2
	DHB	1	1	0
Water from taps (81)	N 3043	16	8	8
	DHB	29	24	5

TABLE VIII

Net results of the N 3043 and DHB methods of bacteriological examination of water samples

	E. coli +ve		Coli-aer. +ve		Lancefield D streptoc. +ve	
	N 3043	DHB	N 3043	DHB	N 3043	DHB
Piped and well waters, 24 samples	0	1	1	7	0	3
Drawn from taps 81 samples	8	24	21	66	7	27
Total sub-samples	8	25	22	73	7	30

Reasons for rejection	N 3043	DHB
Piped and well waters	1	11
Taps	36	117
Total sub-samples	37	128

Percentages of rejected water samples	N 3043	DHB
Piped and well waters	8	37.5
Taps	79	89

well and piped water, 1 sub-sample exceeded the limits specified for the N 3043 examination, whereas based on the results of the DHB method, 10 sub-samples exceeded those limits; (ii) amongst the 81 water samples from taps, 36 sub-samples were exceeding the limit of N 3043, whereas based on the DHB, 117 sub-samples were above the standard. The low productivity of the N 3043 method in comparison with the new technique is striking.

Discussion and Conclusions

The samples of well and piped water taken before entering the industrial drinking water mains system exceeded the limit of N 3043 only in one case. The DHB applied to the same water samples gave results that exceeded the standard ten times. Clearly, therefore, the DHB test is much more sensitive than the N 3043 method, especially as far as the presence of Enterobacteriaceae, Aeromonadaceae and Lancefield D streptococci is concerned. The explanation for this must be sought in the fact that the DHB method (i) relies on a search for more types of Gram-negative bacteria than the N 3043 technique; (ii) allows for resuscitation and subsequent enrichment which gives sublethally damaged organisms a better chance of recovery as earlier observed by Bonde (1982). Our data substantiate those of Kampelmacher et al. (1976) who also found that the N 3043 technique was the least productive and those of Van den Broek et al. (1979) corroborating the validity of the DHB method.

As shown by the final data in Table VIII a total of 79% of the water samples from taps contained quantities of bacteria above the levels given in N 3043; 89% of the

water samples were above the level specified in the DHB assessment. The higher temperature of water in taps and the organic load of this type of water apparently exert a resuscitating effect on organisms carrying sublethal lesions (Mossel and Van Netten, 1984), so that the differences between the N 3043 and DHB results are smaller than those for the well and piped water samples.

The results in Table VII clearly indicate that the enrichment of *E. coli* as practised in the N 3043 method is both less productive and less selective than that used for the detection of *E. coli* in the DHB method. This, once more, is in agreement with the observations of Kampelmacher et al. (1976).

As shown in Table VI the method of detection of faecal streptococci in the N 3043 technique is also considerably less productive than in the DHB. Confirmation tests indicated that kanamycin aesculin azide agar (Mossel et al., 1978a) allows almost exclusively the growth of D streptococci. In addition, the method of resuscitation applied in the DHB is apparently much more effective than the immediate use of a selective culture-medium, as it is practised in N 3043.

Pseudomonas aeruginosa was never isolated by the DHB method. However, other Pseudomonadaceae were demonstrated in 22 of the 105 samples examined.

The following conclusions appear to be justified with respect to methodology: (i) the DHB method more often yields a positive confirmed result for the coli-aerogenes group and is hence more productive than the N 3043 technique; (ii) the DHB technique is more selective for *E. coli* and Lancefield D streptococci. These shortcomings may in part be rectified by the recent modifications of The Netherlands Standard method (Nederlandse Norm 1981/1982).

Irrespective of this the DHB method can be recommended for monitoring recycled water in the food industry. It recovers reliably all organisms that have been recommended in the past and more recently for including in markers which are normally used in monitoring water for safety (Habs and Langeloh, 1958; Kretzchmar, 1959; Lieb and Anschau, 1959; Bonde, 1966; Cabelli, 1977; Althaus et al., 1982; Müller and Mossel, 1982; Burke et al., 1983; Grabow et al., 1983). Furthermore, the DHB method avoids the pitfalls inherent in testing for the taxonomically ill-defined and ecologically unresolved group of 'coliform' (coli-aerogenes) bacteria (Henriksen, 1955; Seligmann and Reitler, 1965; Mara, 1973; Leclerc et al., 1977; Lupo et al., 1977; Austin et al., 1981; Müller and Mossel, 1982; Lechevallier et al., 1983; Leclerc et al., 1983; Burlingame et al., 1984). This markedly increases the precision of the results obtained.

References

Allen, L.A., S.M. Pasley and M.A.F. Pierce, 1952. Conditions affecting the growth of bacterium coli on bile salts media. Enumeration of this organism in polluted waters. J. Gen. Microbiol. 7, 257–267.

Allen, L.A., M.A.F. Pierce and H.M. Smith, 1953. Enumeration of *Streptococcus faecalis*, with particular reference to polluted waters. J. Hygiene 51, 458–467.

Allen, D.A., B. Austin and R.R. Colwell, 1983. Numerical taxonomy of bacterial isolates associated with a fresh water fishery. J. Gen. Microbiol. 129, 2043–2062.

Althaus, H., W. Dott, G. Havemeister, H.E. Müller and C. Sacré, 1982. Fäkal-Streptokokken als Indikatorkeime des Trinkwassers. Zbl. Bakt. Hyg. I, Orig., A, 252, 154–165.

Austin, B., D. Hussong, R.M. Weiner and R.R. Colwell, 1981. Numerical taxonomy analysis of bacteria isolated from the completed 'most probable numbers' test for coliform bacilli. J. Appl. Bacteriol. 51, 101–112.

Bonde, G.J., 1966. Bacteriological methods for estimation of water pollution. Health Lab. Sci. 3, 124–128.

Bonde, G.J., 1982. Resuscitation method for attenuated Enterobacteriaceae in drinking water. Antonie van Leeuwenhoek 48, 620–623.

Burke, V., M. Gracey, J. Robinson, D. Peck, J. Beaman and C. Bundell, 1983. The microbiology of childhood gastro-enteritis: *Aeromonas* species and other infective agents. J. Infect. Dis. 148, 68–74.

Burlingame, G.A., J. McElhaney, M. Bennett and W.O. Pipes, 1984. Bacterial interference with coliform colony sheen production on membrane filters. Appl. Environ. Microbiol. 47, 56–60.

Cabelli, V.J., 1977. Indicators of recreational water quality. In: Bacterial indicators/health hazards associated with water, edited by A.W. Hoadley and B.J. Dutka, Am. Soc. Testing Materials, Philadelphia, pp. 222–238.

Eichhoff, E., 1982. Die mikrobiologische Kontrolle von Wasser im Nahrungsmittelbereich. Lebensmitteltechnik 6, 291–292.

Grabow, W.O.K., V. Gans-Müller, O.W. Prozesky and F. Deinhardt, 1983. Inactivation of hepatitis A virus and indicator organisms in water by free chlorine residuals. Appl. Environm. Microbiol. 46, 619–624.

Habs, H. and H. Langeloh, 1958. Die Bedeutung der sogenannten atypischen Colibakterien für die Trinkwasserbeurteilung. Arch. Hygiene Bakteriol. 142, 401–414.

Hendriksen, S.D., 1955. A study of the causes of discordant results of the presumptive and completed coliform tests on Norwegian waters. Acta Pathol. Microbiol. Scand. 36, 87–95.

Kampelmacher, E.H., A.B. Leussink and L.M. Van Noorle-Janssen, 1976. Comparative studies of methods for the enumeration of coli aerogenes bacteria and *E. coli* in surface water. Water Res. 10, 285–288.

Kretzchmar, W., 1959. Die Bedeutung der Differenzierung von Coli und coliformen Keimen für die bakteriologische Wasseruntersuchung. Z. Ges. Hygiene Grenzgeb. 5, 73–91.

LeChevallier, M.W., S.C. Cameron and G.A. McFeters, 1983. Comparison of verification procedures for the membrane filter total coliform technique. Appl. Environ. Microbiol. 45, 1126–1128.

Leclerc, H., D.A.A. Mossel, P.A. Trinel and F. Gavini, 1977. Microbiological monitoring – a new test for fecal contamination. In: Bacterial indicators/health hazards associated with water, edited by A.W. Hoadley and B.J. Dutka, Am. Soc. Testing Materials, Philadelphia, pp. 23–36.

Leclerc, H., F. Gavini, D. Izard and P.A. Trinel, 1983. Les coliformes: mythe et réalité. In: Les bacilles à Gram négatif, d'intérêt médical et en santé publique: taxonomie – identification – applications. Colloques Inst. Nat. Santé Rech. Méd. 114, 597–617.

Lieb, F. and H. Anschau, 1959. Ueber einige erfahrungen mit den IMVC-Reaktionen. Arch. Hygiene Bakteriol. 143, 429–441.

Lupo, L., E. Strickland, A. Dufour and V. Cabelli, 1977. The effect of oxidase positive bacteria on total coliform density estimates. Health Lab. Sci. 14, 117–121.

Mara, D.D., 1973. Coliform counts of polluted waters: a comparison of media and methods. Water Res. 7, 1899–1903.

Mossel, D.A.A., H. de Vor and I. Eelderink, 1976. A further simplified procedure for the detection of *Pseudomonas aeruginosa* in contaminated aqueous substrata. J. Appl. Bacteriol. 41, 307–309.

Mossel, D.A.A., A.J.W.M. van Ekeren and I. Eelderink, 1977a. A simplified procedure for the examination of drinking water for bacteria of Public Health significance: the differential hydrobacteriogramme. Zentralbl. Bakteriol. Parasitenkd., Abt. I, Orig., B, 165, 498–516.

Mossel, D.A.A., I. Eelderink and J.P. Sutherland, 1977b. Development and use of single, 'polytropic' diagnostic tubes for the approximate taxonomic grouping of bacteria isolated from foods, water and medicinal preparating. Zentralbl. Bakteriol. Parasitenkd., Abt. I, Orig., A, 238, 66–79.

Mossel, D.A.A., P.G.H. Bijker and I. Eelderink, 1978a. Streptococci of Lancefield groups A, B and D and those of buccal origin in foods: their Public Health significance, monitoring and control. In: Streptococci, edited by F.A. Skinner and L.B. Quesnel, Academic Press, London, pp. 315–334.

Mossel, D.A.A., E.J.C. Paardekooper, M.J.C. Schoenmakers and G. Tuynenburg Muys, 1978b. Principles governing the ecological-microbiological assessment of water used in the food industry. Lab. Pract. 27, 13–14.

Mossel, D.A.A., I. Eelderink, M. Koopmans and F. van Rossem, 1979. Influence of carbon source, bile salts and incubation temperature on recovery of Enterobacteriaceae from foods using MacConkey-type agars. J. Food Protect. 42, 470–475.

Mossel, D.A.A., B. Wijers and S. Bouwer-Hertzberger, 1980. The enumeration and identification of stressed *E. coli* in foods using solid medium repair and Eijkman's thermotrophic criteria. J. Appl. Bacteriol. 49, No. 3, xvii–xviii.

Mossel, D.A.A. and P. van Netten, 1984. Harmful effects of selective media on stressed micro-organisms – nature and remedies. In: The revival of injured micro-organisms, edited by A.D. Russell and M.H.E. Andrew, Academic Press, London, pp. 329–369.

Müller, H.E. and D.A.A. Mossel, 1982. Observations on the occurrence of *Salmonella* in 20000 samples of drinking water and a note on the choice of marker organisms for monitoring water supplies. Antonie van Leeuwenhoek 48, 641–644.

Nederlandse Norm, 1981, 1982. Bacteriologisch onderzoek van drinkwater, NEN 6557 and NEN 6565.

Schubert, R.H.W., 1975. Die Relation von aerogenen zu anaerogenen Aeromonaden der 'Hydrophila-Punctata-Gruppe' in Fliessgewässern in Abhängigkeit von der Abfallstoffbelastung. Zentralbl. Bakteriol. Parasitenkd., Abt. I, Orig., B, 160, 237–245.

Seligmann, R. and R. Reitler, 1965. Enteropathogens in water with low *Escherichia coli* titer. J. Am. Water Works Assoc. 57, 1572–1574.

Strauch, D., 1976. Trinkwasser und Brauchwasser für Lebensmittelbetriebe. Neue Rechtsverordnung in der Bundesrepublik Deutschland. Fleischwirtschaft 56, 666–668.

Van den Broek, M.J.M., D.A.A. Mossel, A.E. Eggenkamp, I. Eelderink and M.E. Verouden, 1979. The microbial association of bivalve shellfish intended for consumption in the raw state, with particular reference to safety. Arch. Lebensm. Hyg. 30, 98–103.