The effect of sodium azide concentration on the recovery of enterococci from water

C. R. Fricker and B. J. Eldred

ABSTRACT

The ability of Slanetz and Bartley medium to recover chlorine-stressed enterococci has been studied. Results showed that chlorine injury significantly affected the ability of Slanetz and Bartley medium to recover enterococci while lower concentrations of sodium azide in the same basal medium allowed their recovery. However, reducing the concentration of sodium azide considerably reduced the specificity making it unsuitable for use in the routine examination of water. A non-azide-containing medium, Enterolert®-DW appeared to be able to recover injured and non-injured enterococci with similar efficiency. The data presented here suggest that further work is required to improve the recovery of chlorine-injured enterococci by Slanetz and Bartley medium.

Key words | chlorine injury, drinking water, *Enterococcus* spp., selective medium

C. R. Fricker (corresponding author)
CRF Consulting Ltd,
Child's Acre, Church Lane,
Three Mile Cross, Reading,
RG7 1HD,
UK
and
School of Environmental Studies,
Queen's University,
Kingston, Ontario,
Canada
E-mail: *colinfricker@aol.com*

C. R. Fricker
B. J. Eldred
Analytical Services Inc.,
Allen Brook Lane,
Williston, Vermont,
USA

INTRODUCTION

The presence of streptococci in water has long been regarded as being indicative of contamination and, more recently, 'faecal streptococci' or members of the genus *Enterococcus* have been used as indicators of faecal contamination in drinking water. While often less numerous than *Escherichia coli* in fresh faecal material, the enterococci are thought to survive longer outside of the intestinal tract of animals and to exhibit greater resistance to chlorine (Miescier & Cabelli 1982; Narkis & Kott 1992; Tyrrell *et al.* 1995; Tree *et al.* 2003). This makes them good candidates for indicators of faecal contamination in conjunction with the more numerous *E. coli* and they have also been shown to be useful indicators of the likelihood of gastrointestinal disease associated with recreational water (Fleisher *et al.* 1993). Consequently, the EU Drinking Water Directive and the EU Bathing Water Directive both include numerical standards for enterococci. There is therefore a need for reliable and robust methods for the detection of these organisms in both matrices.

Early recognition of the value of enterococci as indicators of water quality led to efforts to develop suitable culture media for the detection. As efforts to develop methods proceeded, it was quickly recognised that the detection of the enterococci was often difficult because of the growth of other organisms, particularly Gram-negative bacilli. Some of the earliest attempts at inhibiting the growth of Gram-negative bacteria in culture media for the detection of Gram-positive cocci were described by Snyder & Lichstein (1940) who found that the presence of 0.01% (0.1 g/L) of sodium azide prevented the swarming of *Proteus* spp. and also inhibited the growth of *E. coli* and *Salmonella typhi*. Chapman (1944) developed a medium containing sodium azide at a concentration of 0.06 g/L for the detection of streptococci and noted that while not all coliform bacteria were completely inhibited, their growth was restricted and colonies were less numerous and smaller in size. Hajna & Perry (1943) developed a medium for *Streptococcus faecalis* in which they combined the addition of 0.5 g/L sodium azide to a nutrient basal medium with an incubation temperature of 44.5 °C. Based on their findings, this concentration of azide allowed the growth of *S. faecalis* while inhibiting that of Gram-negative organisms.

doi: 10.2166/wh.2013.422

Mallmann & Seligman (1950) reported that, in their hands, azide dextrose broth with a concentration of 0.2 g/L sodium azide was the most effective medium for enumerating streptococci in water and sewage. They concluded that the superiority of azide dextrose broth over the medium of Hajna and Perry was due in part to the fact that the latter detected only faecal enterococci and, more importantly, that it failed to recover all of the viable faecal streptococci. In 1955 Slanetz *et al.* (1955) proposed the use of a medium containing 0.4 g/L sodium azide in combination with membrane filtration for the enumeration of enterococci and, subsequently, Slanetz & Bartley (1957) reported a modification of the medium that they felt improved recovery of the target organisms. It is this medium, known either as Slanetz and Bartley medium or m-enterococcus agar, that is used widely for the detection of enterococci in water and forms the basis of the International Organization for Standardization reference procedure for these organisms (ISO 7899-2).

As well as being useful indicators of faecal contamination of water, the enterococci are also used as indicators in the food industry. Reinbold *et al.* (1953) described a medium containing sodium citrate (as a carbon and energy source) and 0.1 g/L sodium azide for the recovery of enterococci from raw milk. They noted that concentrations of sodium azide above 0.1 g/L inhibited the growth of some strains of enterococci resulting in smaller colonies and that at 0.3 g/L both the size and number of enterococcus colonies were reduced. Subsequently, Efthymiou & Joseph (1974) reported that several strains of *Enterococcus faecium* exhibited low azide tolerance and that 0.1 g/L appeared to be optimal for the recovery of *Enterococcus* spp. overall. The possibility that 0.4 g/L sodium azide could be toxic to 'resting' strains of enterococci was also raised by Litsky *et al.* (1953). The results of studies in our laboratory have also suggested that some strains of enterococci may be inhibited by 0.4 g/L sodium azide. We have noticed in our use of Slanetz and Bartley medium that it is very specific and hence we were concerned that the high specificity was leading to the inhibition of some strains of *Enterococcus* spp. This study was undertaken to determine the inhibitory effect of sodium azide on strains of enterococci and to explain the apparent lack of sensitivity of Slanetz and Bartley medium in recovering enterococci from some water samples.

MATERIALS AND METHODS

In order to determine the effect of sodium azide concentration on the recovery of enterococci from water, five media were compared namely, Slanetz and Bartley medium (Oxoid CM), Slanetz and Bartley medium made from individual components and with sodium azide concentrations of 0.1 and 0.2 g/L, Chromocult enterococcus agar (Merck) and Enterolert®-DW (Idexx Laboratories). The agar media were prepared, stored at 2–8 °C and used within 1 week of preparation. For the Slanetz and Bartley medium, filter-sterilised triphenyl tetrazolium chloride and sodium azide were added after the other ingredients had been dissolved by boiling and the medium cooled to approximately 55 °C . Enterolert®-DW was used in conjunction with QuantiTray™ according to the manufacturer's instructions.

Samples

Drinking water samples were collected from a variety of sites in the Vermont area as well as from other locations across the United States and preserved with sodium thiosulphate (sufficient to neutralize 20 mg/L free chlorine). Primary sewage effluents were collected from ten sites in the United States and shipped overnight to the laboratory on ice. Upon receipt, the sewage effluent samples were refrigerated and used within 48 h.

For production of spiked drinking water samples, the effluent samples were filtered through a cellulose coffee filter to remove large particulates and 1 L volumes were diluted 1:5 in deionised water. These samples were treated with a chlorine solution sufficient to give a theoretical initial chlorine concentration of 15 mg/L. Aliquots were then removed at 1 minute intervals, treated with sodium thiosulphate and the numbers of remaining enterococci were determined using Enterolert®-DW in conjunction with QuantiTray™ 2000. An initial sample of unchlorinated sewage was also examined to determine the initial concentration of enterococci in the samples. All chlorinated

samples were then held at 2–8 °C overnight until the results of the enterococcus estimations were available. The samples that gave a greater than 2 log reduction in enterococcus concentration were then used to spike drinking water samples to give an estimated enterococcus concentration of 5–15 cfu/100 mL. After spiking, the samples of drinking water were left for 4 h to equilibrate prior to examination by the five methods described above.

Additionally, a total of 126 samples of non-disinfected sewage were cultured using Enterolert®-DW and Slanetz and Bartley medium after dilution to achieve a target concentration of enterococci of 1–15 cfu/100 mL.

All Slanetz and Bartley media and Chromocult media were incubated at 37 °C for 48 h. The Chromocult plates were initially examined at 24 and 48 h but it quickly became apparent that 48 h of incubation was required for sufficient growth to be obtained. The Slanetz and Bartley plates that contained 0.4 g/L sodium azide were examined after 48 h incubation and then re-examined after 5 days incubation. Enterolert®-DW samples were examined after 24 h incubation and any degree of green coloration to the wells of the Quantitrays was deemed to be a positive result. For confirmation of the presence of enterococci, all membranes showing growth were transferred to bile aesulin azide agar and incubated for 4 h at 44 °C. The membranes from the Slanetz and Bartley medium were then replaced onto Slanetz and Bartley medium and reincubated for a further 3 days. Colonies demonstrating the ability to cleave aesculin were subcultured to tryptone soya broth containing 6.5% sodium chloride and incubated for 48 h at 44.5 °C. For the Enterolert®-DW, positive wells were subcultured onto bile aesculin azide agar and incubated at 44 °C for 24 h. Those cultures that cleaved aesculin were subcultured to tryptone soya broth containing 6.5% salt and incubated as described above. Organisms that cleaved aesculin and grew in 6.5% salt were considered to be members of the genus *Enterococcus*.

In order to determine the resistance of enterococci to sodium azide, 148 strains were selected at random from isolates obtained from Enterolert®-DW or Slanetz and Bartley containing 0.1 g/L sodium azide. These isolates were streaked to ensure purity, inoculated onto plates of Slanetz and Bartley medium containing 0.1 and 0.4% sodium azide and incubated at 37 °C for 48 h.

RESULTS

A comparison was made of five media using 122 samples of drinking water that had been spiked with low levels of chlorine-injured enterococci. The results shown in Table 1 demonstrate a clear trend in the behaviour of Slanetz and Bartley media with increasing concentrations of sodium azide. As azide concentration increases, specificity increases and sensitivity decreases. Enterolert®-DW, which contains no sodium azide, was the most specific of the media. While not all Enterolert®-DW or Chromocult isolates were 'confirmed', the confirmation rates were 95.0% and 87.1%, respectively. Only Slanetz and Bartley medium with 10 mg/L of sodium azide recovered more enterococci than Enterolert®-DW, but the specificity was too low for this medium to be used for the routine examination of water.

A total of 148 strains of enterococci were selected at random and tested for their ability to grow in the presence of 0.1% and 0.4% sodium azide. All but two strains grew equally well on the two media. Two strains failed to grow in the presence of 0.4% sodium azide.

Since the number of strains shown to be sensitive to 0.4% sodium azide was low and could not explain the difference in recovery efficiency between the different media, a comparison was made between Slanetz and Bartley medium and Enterolert®-DW with 126 non-chlorinated sewage samples that had been diluted to achieve a target concentration of 1–15 cfu/100 mL enterococci. The results are shown in Table 2. There was no significant difference in the recovery of organisms using the two methods when

Table 1 | Recovery of enterococci on different media from drinking water samples spiked with low numbers of enterococci from disinfected sewage

Method	Presumptive isolates	Confirmed isolates	Percentage confirmation rate
Enterolert®-DW	865	822	95.0
S&B 10 mg/L azide	2,337[a]	872	<37.3
S&B 20 mg/L azide	1,470	724	49.3
S&B 40 mg/L azide	516	482	93.4
S&B 40 mg/L azide 5 days	698	604	86.5
Chromocult™	659	574	87.1

[a]Excludes 21 samples where the number of presumptive isolates was recorded as 'too numerous to count'.

Table 2 | Recovery of enterococci from 126 drinking water samples spiked with low numbers of enterococci from non-disinfected sewage samples using Slanetz and Bartley medium and Enterolert®-DW

Method	Total presumptive isolates	Total confirmed isolates	Percentage confirmation rate
Enterolert®-DW	960	921	95.9
Slanetz and Bartley	954	910	95.4

non-chlorinated sewage was used to spike drinking water samples.

DISCUSSION

In previous work, we have demonstrated that the recovery of enterococci with Slanetz and Bartley medium and Enterolert®-DW were similar (Fricker & Fricker 1996). In recent years, a new product has become available, Enterolert®-DW, which was designed for the detection of enterococci specifically in drinking water. In our initial tests with this medium, it appeared that this medium was much more sensitive than the original Enterolert® medium. The choice of methods for the detection of microbes in any given sample type is usually made on a combination of sensitivity and specificity of the medium. There is often a compromise when choosing methodologies since methods that are very specific tend to be less sensitive. While a highly sensitive method is preferable, it is not a good choice if there is so much background growth that the target colonies are difficult to distinguish.

Experience gained in the routine use of Slanetz and Bartley medium has shown that the medium is very specific and that most organisms forming colonies on the medium are confirmed as enterococci. In this study, 93.4% of organisms growing on Slanetz and Bartley medium after 48 h growth from waters spiked with chlorine-treated sewage were confirmed as enterococci. The corresponding figure for non-disinfected samples was 95.4%.

There was a significant difference between the ability of Slanetz and Bartley medium and Enterolert®-DW to recover enterococci when chlorine-damaged organisms were used. Analysis of the data using the procedures described in ISO 17994 showed a significant difference between the two methods at the 10% level. While the difference between the two methods was reduced when the incubation period of the Slanetz and Bartley medium was extended to 5 days, it remained significant. However, reduction in the concentration of sodium azide in the medium did increase the recovery of enterococci as shown in Table 1. In fact, reduction of the azide concentration to 0.1% increased the sensitivity of the Slanetz and Bartley medium such that it was more sensitive than Enterolert®-DW. However, the specificity of the medium was significantly reduced with only 37.3% of the countable organisms growing on the plates after 48 h incubation confirmed as enterococci. In fact, the specificity was considerably lower than this since with many plates the number of colonies present on the medium was too numerous to count. Increasing the concentration of azide to 0.2% reduced the sensitivity of the medium but specificity improved to 49.3%. Packer (1943) reported that a concentration of 0.2% sodium azide was inhibitory to most Gram-negative organisms in a blood agar base but that the resistance of streptococci was reduced when a tryptose glucose agar was used. Furthermore, it was noted that there was a considerable difference in the inhibition of both streptococci and Gram-negative organisms depending on pH. Azide-containing media at a pH of 6.8 was much more selective than the same media at pH 7.4. Slanetz and Bartley medium should be at a pH of $7.2 + 0.2$.

Slanetz and Bartley medium is widely used for the examination of drinking water and, in fact, forms the basis of the International Organization for Standardization procedure (ISO 7899-2), which specifically states that the method is particularly suitable for drinking water and swimming pool water. The results presented here suggested that Slanetz and Bartley medium may not be well suited to the examination of water containing chlorine-damaged enterococci and further work is necessary to determine whether the damage caused to the enterococci using the chlorination procedure used here reflects the damage seen in drinking water samples. It is clear from the data produced using injured and non-injured organisms that there is a difference in the ability of enterococci to grow on Slanetz and Bartley medium depending on whether the cells have been exposed to chlorine. The mechanism of sodium azide inhibiting bacterial growth is presumed to involve inhibition of the enzyme catalase but the data presented here suggest that there may be an alternative mode of action since the

enterococci do not possess this enzyme. In any event, it appears that chlorine-injured enterococci may be susceptible to the level of sodium azide found in Slanetz and Bartley medium although uninjured cells are apparently unaffected. This may explain the results presented by several workers when using sodium azide as an inhibitor of Gram-negative flora. All other reports on the use of azide as an inhibitor have used non-injured enterococci and, consequently, the issue of increased sensitivity after chlorine injury has not been addressed. It is clear that the lower levels of sodium azide used during this study are not suitable for routine work as the confirmation rates were too low. However, modification of the Slanetz and Bartley medium to make it more suitable for the recovery of injured organisms would seem warranted.

REFERENCES

Chapman, G. H. 1944 The isolation of streptococci from mixed cultures. *J. Bacteriol.* **38**, 113–114.

Efthymiou, C. J. & Joseph, S. W. 1974 Development of a selective enterococcus medium based on manganese ion deficiency, sodium azide, and alkaline pH. *Appl. Environ. Microbiol.* **28**, 411–416.

Fleisher, J. M., Jones, F., Kay, D., Stanwell-Smith, R., Wyer, M. & Morano, R. 1993 Water and non-water risk factors for gastroenteritis among bathers exposed to sewage-contaminated marine waters. *Int. J. Epidemiol.* **22**, 698–708.

Fricker, E. J. & Fricker, C. R. 1996 Use of defined substrate technology and a novel procedure for estimating the numbers of enterococci in water. *J. Micro. Meth.* **27**, 207–210.

Hajna, A. A. & Perry, C. A. 1943 Comparative study of presumptive and confirmative media for bacteria of the coliform group and for fecal streptococci. *Am. J. Pub. Health* **33**, 550–556.

Litsky, W., Mallmann, W. L. & Fifield, C. W. 1953 A new medium for the detection of enterococci in water. *Am. J. Pub. Health* **43**, 873–979.

Mallmann, W. L. & Seligman, E. B. 1950 A comparative study of media for the detection of streptococci in water and sewage. *Am. J. Pub. Health* **40**, 286–289.

Miescier, J. J. & Cabelli, V. J. 1982 Enterococci and other microbial indicators in municipal wastewater effluents. *J. Wat. Poll. Cont. Fed.* **54**, 1599–1606.

Narkis, N. & Kott, Y. 1992 Comparison between chlorine dioxide and chlorine for use as a disinfectant of wastewater effluents. *Water Sci. Technol.* **26**, 1483–1492.

Packer, R. A. 1943 The use of sodium azide and crystal violet in a selective medium for Streptococci and Erysipelothrix rhusiopathiae. *J. Bacteriol.* **46**, 343–349.

Reinbold, G. W., Swern, M. & Hussong, R. V. 1953 A plating medium for the isolation and enumeration of enterococci. *J. Dairy Sci.* **36**, 1–6.

Slanetz, L. W. & Bartley, C. H. 1957 Numbers of enterococci in water, sewage and feces determined by the membrane filtration technique with an improved medium. *J. Bacteriol.* **74**, 591–595.

Slanetz, L. W., Dent, D. F. & Bartley, C. H. 1955 Use of the membrane filter technique to enumerate enterococci in water. *Pub. Health Rep.* **70**, 67–72.

Snyder, M. L. & Lichstein, H. C. 1940 Sodium azide as an inhibiting substance for Gram negative bacteria. *J. Infect. Dis.* **67**, 113–115.

Tree, J. A., Adams, M. R. & Lees, D. N. 2003 Chlorination of bacteria and viruses in primary sewage effluent. *Appl. Environ. Microbiol.* **69**, 2038–2043.

Tyrrell, S. A., Ripley, S. R. & Watkins, W. D. 1995 Inactivation of bacterial and viral indicators in secondary sewage effluents, using chlorine and ozone. *Water Res.* **29**, 2483–2490.

First received 14 July 2013; accepted in revised form 6 November 2013. Available online 5 December 2013