Broader incubation temperature tolerances for microbial drinking water testing with enzyme substrate tests

Robert L. Matthews and Rosalind Tung

ABSTRACT

Microbiological testing is an integral part of measures to ensure safe drinking water. However, testing can be restricted in low-resource settings by the requirement for specialized laboratory facilities and testing procedures. Precisely controlled incubation temperature is one example. The effect of varied incubation temperatures on the performance of two enzyme substrate tests for the detection of *Escherichia coli* and total coliforms has been examined. The aim was to determine whether these tests would provide consistent and comparable enumeration over a broader temperature range than currently specified. Recovery of chlorine-injured and wild type *E. coli* was examined over a range of non-standard incubation temperatures in comparison to 37 °C ± 1. Colilert® and Aquatest, a new *E. coli*-specific detection medium, served as the two representative enzyme substrate media. Recovery of chlorine-injured *E. coli* in Colilert was not impaired within the range 33–39 °C; the equivalent range in Aquatest medium was 31–43 °C. Both these tests recovered *E. coli* without significant loss of performance over a wider range of temperatures than currently specified.

Key words | Aquatest, coliform, Colilert, drinking water, *Escherichia coli*, incubation temperature

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INTRODUCTION

Microbiological testing of drinking water is an increasingly important component of global efforts to reduce water-related disease (UNICEF & WHO 2011; Bain *et al*. 2012a). This is evident in the post-2015 targets for water, sanitation and hygiene, proposed recently by a UNICEF/WHO working group, which place greater emphasis on water quality, mentioning specifically the measurement of *Escherichia coli* in drinking water (JMP 2013). In many areas of developing countries, however, the ability to conduct microbial testing is constrained due to a shortage of laboratory facilities, trained personnel and essential infrastructure, such as electricity supply.

A recent review of currently available water quality tests found many to be of limited suitability for use in resource-poor settings (Bain *et al*. 2012b). A contributing factor is the difficulty of conducting tests in a manner that complies with regulations and standard operating procedures.

One standard that is difficult to adhere to in developing countries is tightly controlled incubation temperature. Tests for total coliforms and *E. coli* are generally incubated at 35 °C ± 0.5 in the USA and 37 °C ± 1 in the UK (APHA 2005; Standing Committee of Analysts 2009). The International Organization for Standardization encompasses both of these by specifying 36 °C ± 2 (ISO 2000, 2012). There are some differences when comparing *E. coli* incubation temperature standards used in other disciplines. For example, in the testing of dairy products using an enzyme substrate test (Petrifilm™), a temperature of 35 °C ± 1 is recommended (APHA 2004). A laboratory or testing facility in a developing country may not have an incubator capable of maintaining temperature within such tight tolerances.

In addition, the lack of a reliable electricity supply would limit the operation of an incubator in many low-resource settings. Access to electricity has been highlighted as a major constraint to the use of healthcare technology

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in developing countries (Malkin 2007; Howitt *et al*. 2012). International Energy Agency figures show that, as of 2009 approximately 1.3 billion people (20% of the global population) were without access to electricity, mostly in developing countries. Sub-Saharan Africa has the lowest electrification rate of only 30.5%, with 14.2% in rural areas (http://www.iea.org).

A review of the history of incubation temperature standards and tolerances demonstrates that those currently employed have been used since the development of water testing technology in the early to mid twentieth century. The American standard incubation temperature of 35 °C ± 0.5 first appears in 1955 in the 10th edition of the APHA's *Standard Methods for the Examination of Water, Sewage and Industrial Wastes* (later to become *Standard Methods for the Examination of Water and Wastewater*). It is applied in the context of testing for coliform bacteria using a multiple fermentation tube method followed by confirmation on solid media (APHA 1955). All subsequent editions of the manual apply the same temperature and tolerances even when new test methodologies are used, such as membrane filtration and, latterly, enzyme substrate methods (APHA 1965, 1971, 1976, 1985, 1992, 1995, 1998, 2005). Previous editions describe testing for the coliform group, or its predecessors *Bacillus coli* or the coli-aerogenes group, at 37 °C without temperature tolerances, the exception being the 9th edition which recommends 35–37 °C (APHA 1917, 1920, 1923, 1925, 1936, 1946).

The first regulatory manual for microbiological drinking water analysis in the UK is *The Bacteriological Examination of Water Supplies*, informally known as 'Report 71', first prepared in 1934 and revised in 1939. This gives an incubation temperature for coliform tests of 37 °C without a tolerance range (Ministry of Health 1934, 1939). Temperature tolerances appeared in the 1982 and 1994 revisions where 37 °C ± 0.5 was recommended for total coliform testing by multiple tube fermentation and by membrane filtration, the latter following 4 hours prior incubation at 30 °C ± 0.5 (DHSS 1982; Standing Committee of Analysts 1994). In the most recent revision, the temperature tolerance has been relaxed to 37 °C ± 1, and this also covers enzyme substrate techniques such as Colilert® (Standing Committee of Analysts 2009).

It is well established that the maximum growth rate for *E. coli* occurs at approximately 37–42 °C (Mhor & Krawiec 1980; Ingraham & Marr 1987). However, the influence of temperature on recovery of *E. coli* is less well documented. In this review, no studies could be identified which support the tolerances in any of the aforementioned standards. It is noted that these standards were established with reference to the coliform group using lactose fermentation tests (lauryl tryptose and brilliant green lactose bile broths in the USA, MacConkey broth in the UK), and it is not apparent that they have been adapted for *E. coli* (APHA 1955; DHSS 1982). There appears to be an absence of published literature examining the performance of specific tests at different controlled temperatures, and that the development of new methods has been performed to conform to pre-existing standards.

The current standards were originally established for methods involving gas or acid production from the fermentation of lactose. These processes require enzymes, such as lactose permease, that are not required in enzyme substrate media. The older standard methods also utilize inhibitory agents such as bile salts. Consequently, shifts in incubation temperature may have a different impact on the recovery of *E. coli* and coliforms with traditional tests compared to enzyme substrate methods.

If, for some tests, wider temperature tolerances were to be defined, incubators that are less accurate but cheaper than conventional laboratory models could be used. Such increased flexibility could enable more widespread and regular monitoring of microbial water quality, particularly in remote or impoverished settings (Bain *et al*. 2012b).

This study defines the incubation temperature range for Colilert and Aquatest media. Both tests employ enzyme substrate technology and so do not require specific temperatures to select for target organisms. Colilert was selected as a widely recognized and approved method. Aquatest medium is an *E. coli*-specific detection medium utilized within Aquatest, a device developed to enable reliable enumeration of *E. coli* in drinking and surface waters in low-resource settings. In parallel with the development of Aquatest medium was that of a latent heat storage incubator to maximize the applicability of Aquatest in low-resource settings (Aquatest 2012). Since incubation based on latent heat may not adhere to standard temperature tolerances, it

would be advantageous if Aquatest medium were suitable for use over a broad incubation temperature range.

MATERIALS AND METHODS

The incubation temperature ranges for Colilert and Aquatest medium were established by comparing the recovery of both chlorine-injured and wild type *E. coli*, when the tests were incubated at non-standard temperatures in comparison to a reference of 37 °C ± 1. Chlorine injury is used to approximate a water treatment process (USEPA 2010). It also presents a greater challenge to the test media, as recovery of chlorine-injured *E. coli* takes longer and is more sensitive to the test environment (McFeters *et al.* 1997). The effect of non-standard temperatures on total coliform recovery was also assessed for Colilert. Both media were used in combination with IDEXX Quanti-Trays™.

Microorganisms

For use in the chlorine-injury study, *E. coli* NCTC 9001 were obtained from the Health Protection Agency, Bristol, UK, and maintained on Microbank™ beads at −80 °C until required. For testing using wild type *E. coli* and total coliforms, a 500-ml sample of Bristol harbour water was collected in a sterile container from Prince Street Bridge, Bristol, and transported to the laboratory on ice. A fresh sample was obtained prior to each test. The initial concentration of *E. coli* and total coliforms in the sample was evaluated using Quanti-Tray and Colilert medium. Characteristics of the raw water were as follows: source temperature 9–15 °C; pH 7.89–8.36; total coliform concentration as most probable number (MPN) 10.76 MPN ml^{-1} to >200.5 MPN ml^{-1}; *E. coli* concentration 2–65.9 MPN ml^{-1}.

Growth media

The two growth media used in this study were Colilert and Aquatest growth medium. Colilert was obtained from IDEXX in individual test snap packs. Aquatest medium was obtained from the Aquatest project.

Chlorine injury

This chlorine-injury method was developed as a simpler alternative to the US Environmental Protection Agency (USEPA) chlorine-injury protocol (USEPA 2010), using a single strain of *E. coli* instead of wastewater samples. It is a less rigorous test than the USEPA method, but gives an indication of the ability of a test to recover injured organisms. The method of injury was similar to that applied by Walsh & Bissonnette (1989) and Bolster *et al.* (2005).

A 20-h culture of *E. coli* was grown in nutrient broth (Fluka No. 2) from a single Microbank bead. The resulting culture contained approximately 10^9 *E. coli* ml^{-1}; 1 ml of culture was added to 9 ml PBS and placed on ice. The *E. coli* were injured by exposure to chlorine, by adding 50 µl of a 10% dilution of technical grade sodium hypochlorite (Fisher) directly to the diluted culture to achieve a 2–4 log reduction in number of organisms; a level of injury recommended by USEPA (USEPA 2010). After 5 min exposure, the chlorine was quenched by the addition of 200 µl 20% sodium thiosulfate. Ten-fold dilutions were prepared in PBS to a concentration of 10^{-5}. To ascertain the level of injury, 100 µl of the 10^{-3}, 10^{-4} and 10^{-5} dilutions, and 100 µl of 10^{-7} unchlorinated culture, were added to 100 ml sterile deionized water containing Colilert medium and enumerated in Quanti-Trays following incubation at 37 °C ± 1 for 24 h. Injured organisms were refrigerated at 4 °C until use.

Test procedure

Tests using chlorine-injured *E. coli* were performed as follows: if a 2–4 log reduction in population had been achieved by the chlorination step, the tests were performed 24–30 h following injury, otherwise the test was aborted and the chlorination step repeated. Growth media (Colilert or Aquatest) were each suspended in 1.2 L sterile water at the concentration stated by the manufacturer. This was inoculated with injured *E. coli* to yield a population of approximately 10–30 *E. coli* per 100 ml, and dispensed in 100 ml volumes into 12 Quanti-Trays; a stack of six was placed in an incubator at 37 °C ± 1 and six were placed in an incubator at an alternative temperature: X °C ± 1. The test procedure was repeated for alternative temperatures in

the range 23–47 °C at 2 °C intervals. To facilitate accurate temperature measurement, an additional Quanti-Tray containing only sterile water and featuring a thermocouple (T-type) in one central well and one peripheral well was positioned in the centre of each stack. Temperature was recorded by means of a datalogger (Picotech TC-08, calibrated to ±0.5 °C), measurements were taken once per minute during the incubation period. The MPN *E. coli* per 100 ml was recorded at 24 h ± 0.5 h.

Tests using wild type *E. coli* were performed following enumeration of the sample and 24–30 h from sample collection. The procedure was similar to that for injured *E. coli*. The sample was diluted with sterile deionized water containing either Colilert or Aquatest medium, to give 1.2 L with an *E. coli* concentration of approximately 10–30 *E. coli* per 100 ml. The remainder of the test was performed as above. For raw water tests using Colilert, total coliform results were also recorded.

Statistical analysis

The Student *t*-test was used to assess difference in recovery between alternative temperatures and the control. A one tailed *t*-test was performed between the control Quanti-Trays incubated at 37 °C ± 1 and the alternative temperature Quanti-Trays. A one tailed *t*-test was selected as the objective was to determine whether the alternative temperature resulted in a lower recovery than the control, that is, H_0: alternative temperature recovery ≥ control recovery; H_1: alternative temperature recovery < control recovery. The null hypothesis was rejected for $p < 0.05$. Unless specifically mentioned, instances where the alternative temperature yielded higher recovery than the control are regarded as not significant and are marked $p > 0.5$.

Data of this nature can be assumed to be represented better by a lognormal distribution (Cochran 1950). The aforementioned analysis was also performed on log-transformed data, and in addition a non-parametric Mann–Whitney U test was conducted. In general, there was very good agreement between the three tests; whilst there were small differences in *p*-value, the nature of the results were consistent. For clarity, only the analysis of untransformed data is presented herein. Analysis was performed using SPSS Statistics version 19 and Microsoft Excel 2007.

RESULTS

The temperature ranges over which Colilert and Aquatest medium were used without loss of performance for the detection of wild type and chlorine-injured *E. coli* are presented in Table 1. These temperatures represent the maximum and minimum incubation temperatures that did not result in a significantly lower recovery of *E. coli* in comparison to a control incubation temperature of 37 °C ± 1 (Student *t*-test, $p < 0.05$). Percentage recovery at all non-standard incubation temperatures relative to 37 °C ± 1 is shown in Figure 1 for Colilert and Figure 2 for Aquatest medium. In general, all temperatures within the limits stated in Table 1 resulted in 80–120% *E. coli* recovery, none of which were significantly lower. All non-standard temperatures above and below the limits resulted in significantly lower recovery. Aquatest medium proved more resistant to non-standard incubation temperatures than Colilert for wild type *E. coli*, with a range of 18 °C in comparison to 12 °C for Colilert, and chlorine-injured *E. coli,* with a range of 12 °C compared to 6 °C for Colilert. This is particularly notable in the recovery of wild type *E. coli* at low temperatures, where Aquatest medium displayed good recovery even at 25 °C. Recovery values for all tests are presented in supplementary information (Tables S1 to S4, available online at http://www.iwaponline.com/wh/012/076.pdf).

Our results also suggest that temperatures exceeding the upper limit display a more rapid decline in recovery than temperatures below the lower limit. This general trend can be observed in the shape of the graphs in Figures 1 and 2. This is in accordance with analysis of temperature and growth rate by Ingraham & Marr (1987), which found a more rapid decline in growth rate at temperatures above 42 °C than at temperatures below 37 °C.

Table 1 | Maximum and minimum temperature for each test media and *E. coli* type not resulting in a significant difference in recovery ($p > 0.05$) in comparison to 37 °C ± 1

Test medium	Acceptable temperature range (°C)			
	Raw water *E. coli*		Chlorine-injured *E. coli*	
	Lower	Upper	Lower	Upper
Colilert	31	43	33	39
Aquatest	25	43	31	43

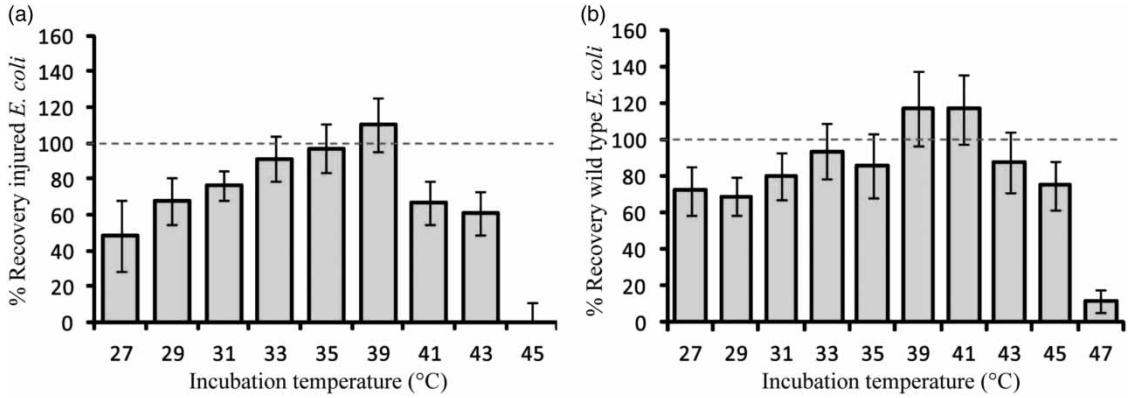

Figure 1 | Percentage recovery of *E. coli* using Colilert at non-standard incubation temperature relative to a control incubated at 37 °C ± 1. Error bars show combined percentage standard error. (a) Recovery of chlorine-injured *E. coli.* (b) Recovery of wild type *E. coli*.

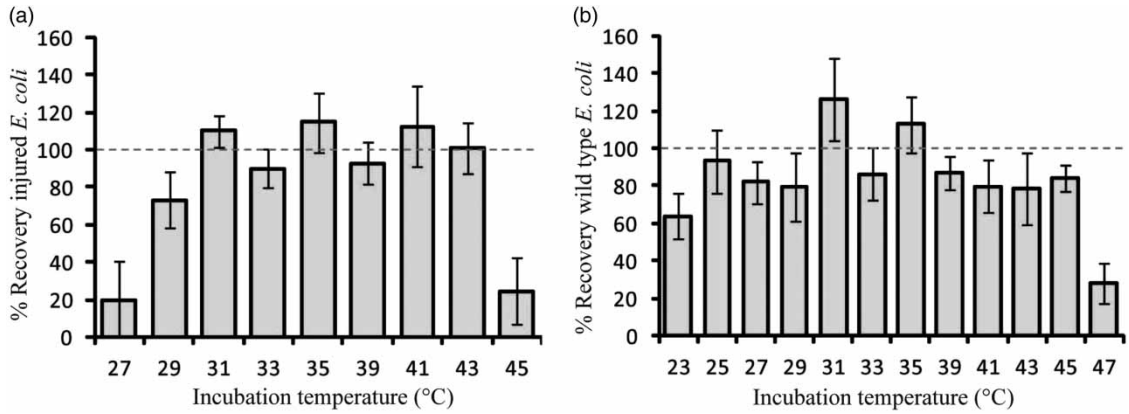

Figure 2 | Percentage recovery of *E. coli* using Aquatest medium at non-standard incubation temperature relative to a control incubated at 37 °C ± 1. Error bars show combined percentage standard error. (a) Recovery of chlorine-injured *E. coli.* (b) Recovery of wild type *E. coli*.

Data on total coliform recovery are only available for Colilert, as Aquatest medium does not contain a total coliform substrate. Percentage recovery of total coliforms using Colilert is presented in Figure 3. The minimum temperature at which Colilert could be used without producing a significantly lower recovery was 29 °C; however any temperature above the 37 °C control, i.e. 39 °C and above, gave a significantly lower recovery ($p < 0.05$). In addition 31 and 33 °C resulted in substantially higher recoveries than 37 °C, 143 and 188% respectively. To assess whether these were significantly higher, a one tailed *t*-test was performed with reversed hypotheses. Both of these were found to be significant, with *p*-values of 0.043 and 0.003, respectively.

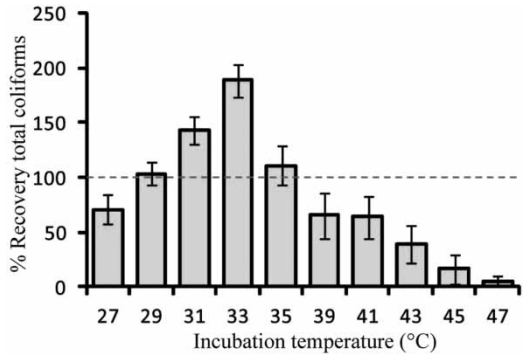

Figure 3 | Percentage recovery of total coliforms from a raw water sample using Colilert at non-standard incubation temperature relative to a control incubated at 37 °C ± 1 °C. Error bars show combined percentage standard error.

At these temperatures, recovery of *E. coli* was not significantly different, implying that the test was recovering more total coliforms that are not *E. coli*. Total coliform recovery values using Colilert are presented in supplementary information (Table S5, available online at http://www.iwaponline.com/wh/012/076.pdf).

DISCUSSION

E. coli test performance

This study demonstrates that two water quality tests can recover *E. coli* without loss of performance over a wider range of temperatures than current standards specify. This applies to both chlorine-injured *E. coli* and to wild type *E. coli* present in a raw water sample. Chlorine-injured organisms were more sensitive to non-standard incubation temperature than wild type. The raw water sample would have contained *E. coli* with an array of injury severity, whereas those in the injury study are all subject to severe stress and were used to represent a worst-case scenario for test performance.

Whether or not an organism is recovered within the test interval is dependent on both the growth rate and the lag phase duration of the organism. Over a temperature range of approximately 21–37 °C, the logarithm of the growth rate follows a linear relationship with the inverse of temperature (Ingraham & Marr 1987). In addition, organisms that have been subject to stress prior to inoculation can be expected to have longer lag phases and also more variability in lag phase duration (Stephens *et al*. 1997; Li *et al*. 2006). McFeters *et al*. (1997) observed that a substantial proportion of *E. coli* subject to chlorine injury were not recovered within a typical 24-h test interval using both enzyme substrate and conventional media at the standard incubation temperature. At low temperatures, the reduced growth rate may result in an increased number of cells with long lag phases not reaching a detectable concentration within the test interval.

Despite relying on the same enzymatic substrate for the detection of *E. coli*, 4-methylumbelliferyl β-D-glucuronide (MUG), the two media exhibited different acceptable incubation temperature ranges, which is presumably due to differences in media composition. This suggests that it would be possible to adjust the formulation of a test that utilizes β-D-glucuronidase to optimize robustness to incubation temperature. Previous work has shown that the rate of β-D-glucuronidase activity for *E. coli* is relatively stable over the range 25–45 °C, but potentially slightly faster at the higher temperature (Adams *et al*. 1990; Tryland & Fiksdal 1998).

This study did not include investigation of false-positive and false-negative rates; however, an independent evaluation (University of Surrey, UK) has found Aquatest medium to be at least as sensitive and specific as Colilert-18® in recovering *E. coli* from a range of water sources in South Africa (unpublished data). It follows that the composition of Aquatest medium enables better recovery of *E. coli* at extreme temperatures. It is possible that *E. coli* have a higher growth rate and/or shorter lag phase in Aquatest medium than in Colilert.

Total coliform test performance

One of the differences between the two media is the absence of a total coliform test in Aquatest medium. In Colilert this is provided by the galactosidase substrate ONPG, which releases the yellow-coloured nitrophenol dye when cleaved. The nitrophenol has a quenching effect on the blue fluorescence of the MUG and as a result *E. coli* counts can be more difficult to read. It is possible that some Quanti-Tray wells contained slow-growing *E. coli* that produced weak fluorescence, which was masked by the yellow colour when the results were read. This could mean that stressed bacteria are more detectable in the Aquatest medium, which is not subject to the quenching effect of ONPG. This offers one potential explanation as to why Colilert appears to be less robust to low temperatures where the *E. coli* can be expected to grow more slowly.

The fact that the total coliform component of the Colilert test appears more sensitive to high temperatures is not surprising; the total coliform group consists of a number of different organisms, some of which are of environmental origin and will therefore be more acclimatized to lower temperatures than enteric bacteria (Leclerc *et al*. 2001). As a group of organisms, it is also possible that the optimum temperature range for total coliforms, being a composite of

several different temperature ranges, may be relatively narrow and it may also vary from source to source depending on the relative populations.

Implications

In demonstrating that the incubation range can be broadened for Colilert and Aquatest medium, the temperature requirements for other *E. coli* tests based on the β-glucuronidase enzyme could potentially be relaxed without compromising the sensitivity of tests. The fact that these two tests exhibited differing robustness to temperature implies that it would be difficult to establish universal expanded incubation temperature ranges for all such tests. However, even a conservative approach based on the data presented here would suggest 36 °C ± 3 could be adopted for β-glucuronidase *E. coli* tests, which is broader even than the range stated by the ISO. For combined *E. coli* and total coliform tests the picture is slightly less clear; however, our data tentatively indicate a temperature range of 35 °C ± 2 would enable Colilert to operate without loss of performance over the current protocol. Further testing using different water types and an increased number of samples would be required to verify this. Other water quality tests should be assessed individually to establish appropriate incubation temperature ranges where performance is not compromised.

The adoption of broader incubation temperature standards would benefit testing in low-resource settings, as it would enable the use of less accurate but more achievable incubation methods. For example, the use of an egg incubator, as suggested by Bluewater Biosciences in conjunction with their Coliplate™ and Watercheck™ tests. A similar incubator could also be used in conjunction with Aquatest or Colilert. Novel alternatives also include use of an incubator based on phase change material, such as the field incubator developed for use with the Aquatest device (Aquatest 2012). Another example is the D-lab Portatherm, which has been applied to tuberculosis testing using the QuantiFERON-TB Gold In-Tube assay (Dominguez *et al.* 2010), but can also be used for water quality testing. In being more robust to low temperatures and equivalent or better at high temperatures, it would appear that Aquatest medium is more suitable for use in conjunction with such incubators than Colilert.

Use of these tests at temperatures outside the limits suggested herein is likely to result in a reduction in sensitivity, indicated by the impaired recovery of injured organisms. This is in agreement with an analysis of ambient temperature incubation by Brown *et al.* (2011). This may still be preferable if the alternative is not testing, especially if untreated sources are being tested. In such cases the nature of the results for temperatures above or below the limits should be considered. Progressively lower temperatures appear to result in a gradual decline in the recovery, whereas increasing the temperature above the upper limit results in a more severe reduction, suggesting that erring on the low side is preferable. If testing for total coliforms is required then overheating is a more serious problem, as our results suggest that any increase above 37 °C ± 1 could have a detrimental effect on recovery.

CONCLUSIONS

Two enzyme substrate methods for the detection of *E. coli* in drinking water, Colilert and Aquatest medium, can be incubated over a broader range of temperatures than currently specified, without loss of performance in comparison to 37 °C ± 1. This is standard incubation temperature for *E. coli* test media, including enzyme substrate methods, as stated by the UK Environment Agency (Standing Committee of Analysts 2009). Aquatest medium is more robust to non-standard temperatures than Colilert, and for both media *E. coli* from a raw water sample were recovered over a wider temperature range than *E. coli* injured by exposure to chlorine. Relaxation of temperature tolerances would instil greater confidence in the use of alternative incubation methods; expanding water quality monitoring and reducing incidence of water-related disease in remote and impoverished areas.

CONFLICT OF INTEREST

Robert Matthews is a named inventor on an international patent application number PCT/GB2012/050452 entitled: Apparatus for testing the quality of a fluid sample.

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