The effect of sodium thiosulfate dechlorination on fecal indicator bacteria enumeration: laboratory and field data

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ABSTRACT

In microbiological water quality testing, sample dechlorination with sodium thiosulfate is recommended to ensure that results accurately reflect the water quality at sample collection. Nevertheless, monitoring institutions in low-resource settings do not always dechlorinate samples, and there is limited research describing how this practice impacts drinking water quality results. The effect of dechlorination on indicator bacteria counts was evaluated by spiking laboratory water with five *Escherichia coli* (*E. coli*) concentrations (10^4–10^8 CFU/100 mL), chlorinating at six doses (0–0.6 mg/L), holding samples with and without sodium thiosulfate for 5–7 hours, and enumerating *E. coli* by membrane filtration with m-lauryl sulfate media. Additionally, sub-Saharan African water suppliers enumerated thermotolerant coliform by membrane filtration in paired chlorinated water samples collected with and without sodium thiosulfate. Across all *E. coli* and chlorine doses in the laboratory, and all field tests, samples held without sodium thiosulfate had lower bacteria counts ($p < 0.001$). Additionally, chlorinated water supply samples held without sodium thiosulfate had an 87.5% false negative rate. Results indicate the importance of dechlorinating microbiological water quality samples, discarding data from chlorinated samples collected without dechlorination, and reinforcing dechlorination recommendations in resource-limited environments to improve water safety management.

Key words | dechlorination, drinking water, microbiological testing, sodium thiosulfate, standard methods, water quality monitoring

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INTRODUCTION

The provision of treated drinking water prevents disease and protects public health. To ensure water safety, agencies such as the World Health Organization (WHO) and United States Environmental Protection Agency (USEPA) recommend ongoing monitoring of physical, chemical, and microbiological contaminants in drinking water (USEPA 2009; WHO 2011). Microbiological water safety is widely assessed using bacterial indicator species, including *Escherichia coli* (*E. coli*) and fecal (thermotolerant) coliforms. WHO guidelines and USEPA regulations require drinking water to be free of detectable *E. coli* and/or fecal coliforms (USEPA 2009; WHO 2011), and indicator levels are often used to classify diarrheal disease risk (WHO 1997).

Most microbiological water quality test methods include the recommendation to collect chlorinated water samples in bottles containing sodium thiosulfate ($Na_2S_2O_3$), a dechlorinating agent (WHO/OECD 2003; CDC 2010; USEPA 2013; APHA/AWWA/WEF 2017). Dechlorination is recommended to ensure that microbiological test results reflect the actual water quality at the time of sample

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collection, by preventing ongoing disinfection during sample holding and analysis. Recommendations to dechlorinate water samples from treated water supplies with sodium thiosulfate have existed since at least 1939 (Ministry of Health 1939).

Research conducted in the 1950s shaped current recommendations to add sodium thiosulfate to all potentially chlorinated water samples and to complete analysis within 8 hours of collection (APHA/AWWA/WEF 2017). This research identified that sodium thiosulfate: (1) did not affect *E. coli* survival in unchlorinated samples stored up to 6 hours (The Public Health Laboratory Service Water Sub-committee 1953); (2) favored survival in unchlorinated samples stored for 18 hours (Noble & Gullans 1955); and (3) also effectively neutralized residual copper, another disinfectant originating from distribution pipes (Hoather 1957).

Due to the ubiquity of the sodium thiosulfate recommendation, few recent studies have investigated the effects of dechlorination. One study found that omitting sodium thiosulfate during sample collection resulted in false negative readings of legionella in hot water supplies held for 30 minutes before analysis (Wiedenmann *et al.* 2001). A second investigation of mycobacteria in chlorinated swimming pools identified varying effects (both positive and negative) of dechlorination on bacteria detection by different methods with holding time up to 2 weeks (Iivanainen *et al.* 1999). Lastly, a study on acidified sodium chlorite, a disinfectant used in poultry and meat processing, concluded that sodium thiosulfate had no deleterious effect on *E. coli* survival, and that disinfection continued in spiked water samples stored for 5 minutes without sodium thiosulfate (Kemp & Schneider 2000). These recent studies have not impacted the dechlorination recommendations.

Through the Monitoring for Safe Water (MfSW) program, the Aquaya Institute collaborated with 26 water utilities and public health agencies in six sub-Saharan African countries to evaluate microbiological water quality practices and build capacity for improved monitoring. All of these partners collected drinking water quality data, including indicator bacteria and free chlorine residual (FCR). A review of eleven water suppliers in July 2014 determined that five (45%) did not add sodium thiosulfate to dechlorinate water samples collected for microbiological analysis. This finding suggests that, in resource-limited contexts, sampling drinking water without dechlorinating may be common. However, there is a lack of research quantifying the extent to which this practice affects indicator bacteria counts (and therefore diarrheal disease risk levels) specific to drinking water quality monitoring.

The goal of this investigation was to evaluate the effects of dechlorination on the microbiological analysis of chlorinated drinking water supplies analyzed with current laboratory techniques. This was accomplished by measuring fecal indicator bacteria in both (1) *E coli*-spiked and chlorinated laboratory water samples and (2) water samples collected from chlorinated drinking water supplies across sub-Saharan Africa to determine if there was a detectable difference in indicator bacteria counts between samples held with and without sodium thiosulfate.

METHODS

Laboratory methods

Culture and water spiking

E. coli (ATCC® 25922) from pre-prepared, frozen stock was inoculated into Difco™ LB Broth (Difco Laboratories, Sparks, MD) and incubated overnight (18–24 hours) at 35°C to reach stationary growth phase, and then re-inoculated into growth media and incubated at 35°C to reach log-growth phase (2–3 hours). Cell concentrations in inoculated broth were estimated from OD-600 reading (GeneQuant 100 Spectrophotometer, GE Healthcare, Pittsburgh, PA) and a pre-developed conversion factor, and confirmed by plating serially diluted broth on Difco™ LB Agar, incubating at 35°C for 18–24 hours, counting colonies, and averaging plate counts within a countable range. Cells were washed from broth by centrifuging twice at 2,600 g at 4°C in an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) and re-suspending in sterile, 0.05 M phosphate-buffered saline solution by vortexing.

The *E. coli* suspension was added to 6 L of 0.001 M phosphate-buffered water (pH: 7.6; temperature: 20–24°C; total dissolved solids: 80–180 mg/L) to attain one of five target *E. coli* concentrations between 1×10^4 CFU/100 mL

and 1×10^8 CFU/100 mL. Spiked water was stirred with a magnetic stir bar at 300 RPM for a minimum of 30 minutes.

Chlorinating and sampling

E. coli-spiked water and unspiked buffered control water (600 mL samples) were chlorinated at each of five target FCR doses (0.0, 0.1, 0.2, 0.4, 0.6 mg/L) with diluted liquid sodium hypochlorite (Clorox® Bleach, The Clorox Company, Oakland, CA). Samples were stirred for 5 seconds with a plastic-coated magnetic stir bar at 300 RPM, and then samples were collected aseptically in sterile 120 mL glass bottles with and without sodium thiosulfate (0.1 mL of 3% solution (APHA/AWWA/WEF 2017)). Collected samples were immediately placed on ice in a cooler.

In all samples, FCR was measured in triplicate immediately after chlorine dosing, and again in all samples held for 4.0–5.5 hours with and without sodium thiosulfate. FCR was measured by the colorimetric method with DPD tablets and a Lamotte 1200 Colorimeter (Lamotte Company, Chestertown, Maryland), which has a 0.05 mg/L detection limit. Unchlorinated spiked water was assayed for *E. coli* within 1 hour of spiking to determine the starting *E. coli* concentration. All *E. coli*-spiked samples were assayed after holding 5–7 hours with and without sodium thiosulfate. Holding time was consistent between paired sets of samples in each experiment.

The entire protocol was repeated for a total of six spiked water tests: one at each of the five target *E. coli* concentrations, and a replicate experiment at the 1×10^5 CFU/100 mL *E. coli* concentration.

Bacteria enumeration

E. coli concentrations in water samples were enumerated using the membrane filtration method (APHA/AWWA/WEF 2017) with m-lauryl sulfate media (HiMedia Laboratories, Mumbai, India). This method was selected because it was identified as the most common microbiological test method among institutions in ten sub-Saharan African countries (Peletz *et al.* 2016). Samples were diluted appropriately with sterile buffered water, vacuum filtered aseptically through a 45-micron filter (Millipore, Billerica, MA), placed in a plastic petri dish with a media-soaked pad, and incubated for 6 hours at 25°C, followed by 14 hours at 35°C. Colonies were counted and concentrations calculated by averaging plate counts within a countable range (10–200 CFU/plate) after accounting for dilution factors. Ten percent of samples were duplicated, and a sterile water blank was run before each sample.

Field methods

Four African water suppliers from the MfSW program (a regional water supplier in Zambia, a town supplier in Kenya, and two water suppliers in Uganda) were selected to perform parallel testing of chlorinated drinking water. Suppliers were selected because they (1) delivered chlorinated water through a piped network, (2) demonstrated a strong microbiological water quality testing program including regular testing, yet (3) did not regularly use sodium thiosulfate in sampling, and (4) were willing to participate in the study. Two samples (one with sodium thiosulfate and one without sodium thiosulfate) were collected from sources including water treatment plants, storage tanks, and distribution network taps. Samples were placed on ice and assayed for thermotolerant coliforms within 8 hours using membrane filtration with m-lauryl sulfate media. Plates were incubated at 44°C for 18–24 hours and counted.

Data analysis

Data were entered into, and analyzed in, Microsoft Excel (Microsoft Corporation, Redmond, WA) and R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). Wilcoxon signed rank tests for paired samples were performed to determine if indicator bacteria concentrations differed between samples with and without sodium thiosulfate at 0.05 significance for: (1) chlorinated laboratory samples, (2) unchlorinated laboratory samples, and (3) field samples. A false negative rate for field-performed tests without sodium thiosulfate was also calculated.

RESULTS

Laboratory results

Laboratory results of FCR and *E. coli* concentration are tabulated by individual trial in Tables 1–6. Spiked water *E. coli*

Table 1 | Free chlorine residual (FCR) and *E. coli* counts in laboratory water samples (starting *E. coli* concentration 1.45×10^4 CFU/100 mL)

	FCR at collection (mg/L)		FCR after holding (mg/L)		*E. coli* after holding (CFU/100 mL)	
Target Cl dose	Unspiked	Spiked	Unspiked	Spiked	With $Na_2S_2O_3$	Without $Na_2S_2O_3$
0	0.01	0.02	0.01	0.00	2.00×10^3	2.20×10^3
0.1	0.06	0.09	0.01	0.01	48	0
0.2	0.21	0.19	0.11	0.07	0	0
0.4	0.41	0.39	0.26	0.23	0	0
0.6	0.59	0.53	0.51	0.39	0	0

Table 2 | Free chlorine residual (FCR) and *E. coli* counts in laboratory water samples (starting *E. coli* concentration 1.59×10^5 CFU/100 mL)

	FCR at collection (mg/L)		FCR after holding (mg/L)		*E. coli* after holding (CFU/100 mL)	
Target Cl dose	Unspiked	Spiked	Unspiked	Spiked	With $Na_2S_2O_3$	Without $Na_2S_2O_3$
0	0.00	0.00	0.01	0.00	8.26×10^4	1.15×10^5
0.1	0.07	0.07	0.04	0.02	190	0
0.2	0.18	0.17	0.12	0.09	8	0
0.4	0.41	0.37	0.35	0.24	5	0
0.6	0.58	0.61	0.59	0.45	6	0

Table 3 | Free chlorine residual (FCR) and *E. coli* counts in laboratory water samples (replicate test with starting *E. coli* concentration 6.98×10^4 CFU/100 mL)

	FCR at collection (mg/L)		FCR after holding (mg/L)		E. coli after holding (CFU/100 mL)	
Target Cl dose	Unspiked	Spiked	Unspiked	Spiked	With $Na_2S_2O_3$	Without $Na_2S_2O_3$
0	0.02	0.02	0.00	0.01	1.61×105	1.12×105
0.1	0.12	0.08	0.03	0.02	7,600	0
0.2	0.20	0.14	0.15	0.08	139	0
0.4	0.40	0.38	0.33	0.30	59	0
0.6	0.59	0.60	0.48	0.52	36	0

Table 4 | Free chlorine residual (FCR) and *E. coli* counts in laboratory water samples (starting *E. coli* concentration 1.45×10^6 CFU/100 mL)

	FCR at collection (mg/L)		FCR after holding (mg/L)		*E. coli* after holding (CFU/100 mL)	
Target Cl dose	Unspiked	Spiked	Unspiked	Spiked	With $Na_2S_2O_3$	Without $Na_2S_2O_3$
0	0.02	0.01	0.00	0.01	1.08×10^6	4.22×10^5
0.1	0.09	0.09	0.05	0.03	2,100	0
0.2	0.17	0.16	0.14	0.07	700	0
0.4	0.35	0.35	0.30	0.27	150	0
0.6	0.57	0.56	0.52	0.45	60	0

Table 5 | Free chlorine residual (FCR) and *E. coli* counts in laboratory water samples (starting *E. coli* concentration 1.44×10^7 CFU/100 mL)

	FCR at collection (mg/L)		FCR after holding (mg/L)		*E. coli* after holding (CFU/100 mL)	
Target Cl dose	Unspiked	Spiked	Unspiked	Spiked	With $Na_2S_2O_3$	Without $Na_2S_2O_3$
0	0.02	0.01	0.00	0.02	4.44×10^6	3.33×10^6
0.1	0.09	0.06	0.06	0.01	16,000	0
0.2	0.21	0.17	0.15	0.02	2,900	1
0.4	0.40	0.36	0.40	0.11	540	0
0.6	0.58	0.54	0.52	0.26	180	0

Table 6 | Free chlorine residual (FCR) and *E. coli* counts in laboratory water samples (starting *E. coli* concentration 1.53×10^8 CFU/100 mL)

	FCR at collection (mg/L)		FCR after holding (mg/L)		*E. coli* after holding (CFU/100 mL)	
Target Cl dose	Unspiked	Spiked	Unspiked	Spiked	With $Na_2S_2O_3$	Without $Na_2S_2O_3$
0	0.02	0.02	0.01	0.01	9.30×10^7	1.13×10^8
0.1	0.08	0.06	0.06	0.02	2,000	0
0.2	0.18	0.14	0.10	0.02	13,200	0
0.4	0.37	0.31	0.23	0.03	330	0
0.6	0.60	0.51	0.47	0.02	5,060	0

concentrations were: 1.45×10^4 CFU/100 mL, 1.59×10^5 CFU/100 mL, 6.98×10^4 CFU/100 mL (replicate), 1.56×10^6 CFU/100 mL, 1.44×10^7 CFU/100 mL, and 1.53×10^8 CFU/100 mL.

There were three general trends in the FCR readings (Tables 1–6): (1) spiked water FCR was less than or equal to unspiked water FCR both before and after holding, probably because *E. coli* exerted chlorine demand; (2) FCR readings in spiked samples after holding were similar with increasing *E. coli* concentration for samples below 10^7 CFU/100 mL (at which point values were lower likely due to higher *E. coli* chlorine demand); and (3) FCR declined over the holding time in all samples (comparing FCR at collection and FCR after holding). Additionally, FCR readings for samples held with sodium thiosulfate were $\leq$0.03 mg/L (below the detection limit), indicating that sufficient sodium thiosulfate was added to quench all chlorine (data not shown).

Across all trials, chlorinated samples collected without sodium thiosulfate had no detectable *E. coli* after the holding period, with the exception of one sample with 1 CFU/100 mL (Table 5).

At the lowest starting *E. coli* concentration (1.45×10^4 CFU/100 mL), *E. coli* was present in only one sample dosed with chlorine and collected with sodium thiosulfate (Table 1). In contrast, in all trials with *E. coli* concentrations $>1.45 \times 10^4$ CFU/100 mL, *E. coli* was detected in all samples dosed with chlorine and collected in bottles with sodium thiosulfate. Within each trial, *E. coli* concentrations in samples collected with sodium thiosulfate usually decreased with increasing chlorine dose. Across the trials, *E. coli* concentrations in samples collected with sodium thiosulfate generally increased with increasing starting concentration (Tables 1–6). For a starting concentration of $\sim 10^5$ CFU/100 mL, chlorinated samples had 5–190 CFU/100 mL in one trial (Table 2) and 36–7,600 in another (Table 3). For a starting concentration of $\sim 10^6$ CFU/100 mL, chlorinated samples had 60–2,100 CFU/100 mL (Table 4). For a starting concentrations of $\sim 10^7$ and $\sim 10^8$ CFU/100 mL, chlorinated samples had 180–16,000 CFU/100 mL and 330–13,200 CFU/100 mL, respectively (Tables 5 and 6).

E. coli concentrations in chlorinated water samples collected without sodium thiosulfate were consistently lower

than concentrations in equivalent samples collected with sodium thiosulfate (Tables 1–6). Considering all paired samples, *E. coli* concentrations differed significantly between samples collected with and without sodium thiosulfate ($p < 0.001$). No significant difference was seen between unchlorinated samples collected with and without sodium thiosulfate ($p = 0.84$), indicating that sodium thiosulfate alone did not affect *E. coli* survival (Tables 1–6).

Field results

Seventy-nine paired water samples were collected from five water distribution systems managed by the four suppliers at: network taps ($n = 67$), network storage tanks ($n = 7$), and finished water from treatment plants ($n = 5$). The median FCR was 0.2 mg/L (range 0–0.8 mg/L, $n = 79$). Thermotolerant coliforms were detected in 24 samples (30%) collected with sodium thiosulfate, with median 2 CFU/100 mL (range 1–9 CFU/100 mL) (Table 7). Thermotolerant coliforms were detected in three samples (3.8%) collected without sodium thiosulfate, with median 2 CFU/100 mL (range 1–6 CFU/100 mL) (Table 7). Thermotolerant coliform counts in samples collected with sodium thiosulfate were statistically significantly higher than counts in samples collected without sodium thiosulfate ($p < 0.001$). Tests completed on samples without sodium thiosulfate had a false negative rate of 87.5% (21 of 24 samples had no detectable thermotolerant coliform, whereas paired samples with sodium thiosulfate had detectable thermotolerant coliform).

DISCUSSION

In both laboratory *E. coli*-spiked water and chlorinated drinking water supplies in sub-Saharan Africa, samples collected without sodium thiosulfate had statistically significantly lower indicator bacteria counts than paired samples dechlorinated with sodium thiosulfate. There was also no evidence that sodium thiosulfate alone affected *E. coli* survival in unchlorinated samples. It is important to note that the actual presence of false negative readings in drinking water samples depends on bacterial concentration, FCR levels, and sample holding time, but the false negative rate for tests performed on samples without sodium thiosulfate from five African chlorinated piped water distribution systems was 87.5%. These results demonstrate that unquenched chlorine can continue to disinfect during holding time and analysis, and highlight (1) the importance of dechlorinating all drinking water samples collected for microbiological analysis and (2) the difficulty in interpreting microbiological data collected without sample dechlorination. Our results are consistent with other published data (The Public Health Laboratory Service Water Sub-committee 1953; Wiedenmann *et al.* 2001) but are more comprehensive at quantifying the effect of sodium thiosulfate in terms of potential error magnitude and false negative rates in microbiological testing applied to treated drinking water quality monitoring.

While it is unlikely that contamination on the level of the laboratory-spiked water would be found in chlorinated drinking water supplies, observed differences up to 1.6×10^4 CFU/100 mL in laboratory samples illustrate the potential for large errors in microbiological test results if chlorinated water is collected without dechlorination. Additionally, field test results demonstrate that even small differences in indicator bacteria counts between samples collected with and without sodium thiosulfate can cause drinking water samples to be misclassified as complying with microbiological standards (non-detectable in 100 mL) when they do not. Because of these high potential errors, prior data collected without the use of sodium thiosulfate should not be used.

In laboratory testing, samples with positive FCR readings had no detectable *E. coli*, as would be expected. However, among historical data collected by water suppliers participating in the MfSW program, there were samples that exhibited *both* positive FCR readings *and* positive thermotolerant coliform counts (data not shown). There are several explanations for why this could occur in the field: (1)

Table 7 | Thermotolerant coliform results for paired samples collected with and without sodium thiosulfate from chlorinated water supplies in sub-Saharan Africa

	With $Na_2S_2O_3$ ($n = 79$)	Without $Na_2S_2O_3$ ($n = 79$)
Samples with detectable thermotolerant coliform, N (%)	24 (30%)	3 (4%)
Thermotolerant coliform in positive samples, median (range)	2 (1–9)	2 (1–6)

visual colorimetric FCR tests could have been misread by users (Murray & Lantagne 2015); (2) interference from other components, such as bromine, could have inflated FCR readings; (3) microbiological contamination during sampling or analysis could have inflated coliform counts; or (4) biofilms in distribution systems could allow bacteria to resist disinfection (LeChevallier 1990; Percival & Walker 1999; USEPA 2002). These conditions, however, are not easily replicable in the laboratory setting.

Additionally, laboratory-prepared water had little chlorine demand, which is not realistic of natural water conditions. *E. coli*-spiked laboratory tests were also performed with water containing various amounts of chlorine demand in the form of bacteria growth media (data not shown). Similar trends in paired *E. coli* samples were observed in those tests; samples collected with sodium thiosulfate dechlorination had consistently higher *E. coli* concentrations than those collected without sodium thiosulfate. This was true even when all free chlorine was consumed and *E. coli* remained in all tested samples. These results suggest that trends exhibited here would also apply across other, more realistic, water conditions.

This work has some limitations. First, rapid chlorine disinfection rates required spiking with high *E. coli* concentrations, sampling precisely, and diluting highly-concentrated samples, which can result in measurement uncertainties. Disinfection rates up to 4.5-log *E. coli* removal in 5 seconds in this study were consistent with those previously reported (Rice *et al.* 1999; Zhao *et al.* 2001), and presented a challenge for developing laboratory methods. Additionally, potential aggregation of *E. coli* cells could have affected disinfection rates or detection accuracy. Nevertheless, consistent trends between trials and the application of quality control measures contribute to the internal validity of these laboratory test results. Second, inherent differences between prepared laboratory water and natural waters prohibit direct data extrapolation. As such, we do not estimate a magnitude of difference in laboratory *E. coli* counts taken from samples collected with and without sodium thiosulfate that is directly applicable to natural waters or treated drinking water supplies. Future research is recommended to confirm these results with *E. coli*-spiked natural waters, which may have slower disinfection kinetics and other test interferences; however, those results would be unlikely to change this study's conclusions. Future research is also recommended to investigate dechlorination effects with varying sample holding time and sodium thiosulfate dose, to better understand potential implications of these factors.

Existing international water quality monitoring and drinking water sampling recommendations recommend dechlorination of samples before microbiological analysis. National policies often mandate routine drinking water quality testing (Steynberg 2002; Rahman *et al.* 2011), but the prevalence of dechlorination recommendations in national policies is unknown. The MfSW program has identified several monitoring and surveillance institutions in sub-Saharan Africa that do not dechlorinate samples from chlorinated supplies collected for microbiological analysis. These institutions may not have testing protocols that specify dechlorination, or they may lack sufficient quality control procedures to ensure that protocols are followed. Our results indicate that dechlorination recommendations should be reinforced to improve water safety management in all settings providing chlorinated supplies, as artificially low or false negative microbiological test results may cause incorrect classification of disease risk from drinking water, provide a false sense of security, and prevent institutions from taking necessary actions to remedy potentially unsafe water supplies.

CONCLUSIONS

In microbiological water quality testing, it has long been recommended to dechlorinate samples with sodium thiosulfate upon collection. Despite this, research shows that many drinking water quality monitoring institutions in sub-Saharan Africa do not dechlorinate samples, and the impact of this practice on drinking water safety is unclear. We tested fecal indicator bacteria in paired samples collected with and without dechlorination in two settings: *E. coli*-spiked, chlorinated, laboratory water; and drinking water supplies in sub-Saharan Africa. In all cases, bacteria counts were lower in samples that were not dechlorinated with sodium thiosulfate before processing. These results highlight the importance of adhering to sample dechlorination guidelines due to the risk of artificially low or false

negative bacteria counts. There may also be a need to reinforce microbiological test procedures among resource-limited monitoring institutions to improve water safety management.

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