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Impact of heat-treatment on wastewater analytical parameters

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ABSTRACT

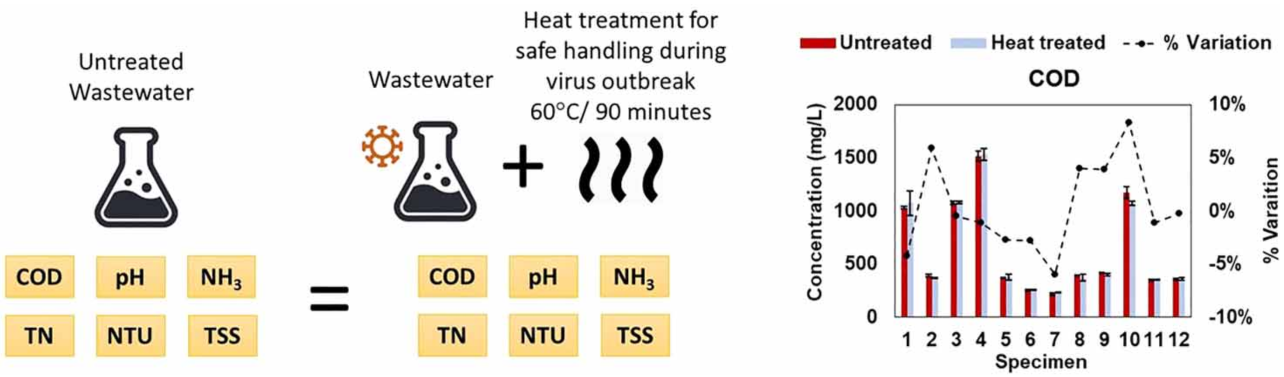
Raw wastewater analysis is an important step in treatment assessment; however, it is associated with risks of personnel exposure to pathogens. Such risks are enhanced during virus outbreaks, such as the COVID pandemic, and heat-treatment is a commonly used mitigation measure. We examined whether heat-treatment compromises wastewater analytical parameters results. We found that heat-treatment of blackwater at 60 °C for 90 min in capped containers yielded no statistically different values ($p > 0.05$) for pH, chemical oxygen demand (COD), ammonia (NH_3), total nitrogen (TN), total suspended solids (TSS), and turbidity for specimens from three different sources. This heat-treatment inactivated coliform bacteria (>4 $\log_{10}$ reduction) thus compromising the measurement of commonly used fecal contamination indicators. The observation of intact helminth eggs in heat-treated specimens suggests that the helminth egg enumeration assay is not compromised. These findings indicate that heat-treatment for the safe handling of wastewater, as may be needed in future virus outbreaks, does not affect the measurements of many common wastewater physico-chemical properties.

Key words: blackwater, COD, fecal coliform, heat inactivation, physico-chemical parameters, SARS-CoV-2

HIGHLIGHTS

- Heat-treatment of wastewater was used as a personnel safety measure during the COVID-19 pandemic.
- We examined whether heat-treatment compromises wastewater analytical parameters.
- Heat-treatment yielded no statistical difference in pH, organics, nitrogen, and solid content values.
- Heat-treatment significantly degraded the fecal coliform enumeration results, but helminth egg enumeration may be possible.

GRAPHICAL ABSTRACT



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INTRODUCTION

Human exposure to the virus through contaminated water and wastewater has long been a concern (Bertrand *et al.* 2012). Viruses reach wastewater through bodily fluid or washing, and whether enveloped or non-enveloped, viruses persist in water (Sobsey & Meschke 2003). Novel wastewater treatment technologies aiming to remove pathogens and pollution for safe onsite reuse, thus addressing the global sanitation crisis, are the objective of extensive research and development (Varigala *et al.* 2020; Welling *et al.* 2020; Castro *et al.* 2021; Cid *et al.* 2022). Many of these technologies have been proven effective at wastewater treatment and disinfection. However, conducting such evaluation on waste treatment requires handling by personnel of untreated waste for analytical purposes. This became a concern during the SARS-CoV-2 pandemic when it was discovered that the virus was present in wastewater (Crits-Christoph *et al.* 2021). While it was unclear whether the wastewater virus was a transmission source (Yeo *et al.* 2020), we adopted a heat-treatment protocol for raw wastewater for our operations to ensure personnel safety.

Heat treatment, or thermal disinfection, is one of the simplest disinfection methods, especially in low-resource settings (Espinosa *et al.* 2020). The extent of pathogen reduction achieved in heat-treatment processes is influenced by the combination of time and temperature (Espinosa *et al.* 2020).

The heat-treatment condition was selected to ensure safety against SARS-CoV-2, and it is applicable to a variety of other viruses that may be present in wastewater. Our selection of 60 °C/90 min was based on the most conservative of the time/temperature combinations reported in the literature from researchers on wastewater SARS-CoV-2 surveillance in 2021 (Bivins *et al.* 2021; Crits-Christoph *et al.* 2021; Prado *et al.* 2021). This 60 °C/90 min treatment was used by some of this group of authors for the detection of SARS-CoV-2 in wastewater for epidemiological surveillance (Welling *et al.* 2022). A recent report of SARS-CoV-2 infectivity heat inactivation has confirmed the safety of the selected treatment conditions, finding that 60 °C/60 min treatment yields a 4.9 $\log_{10}$ reduction of virus titer, which is adequate for the diluted virus titer in raw wastewater (Burton *et al.* 2021).

As far as applicability to other viruses, temperatures at or below the 50–60 °C range have been reported to inactivate coronaviruses such as MERS (Leclercq *et al.* 2014), as well as polio (Ward *et al.* 1976), enteric viruses (Bertrand *et al.* 2012), and hemorrhagic fever viruses such as Ebola and Lassa (Hossain 2022).

While heat-treatment ensures personnel safety, it may also impact the analytical parameter of the wastewater that is sampled to assess a treatment process under test.

Literature studies on thermal disinfection of wastewater have focused on measurements of pathogens and have not reported on wastewater physico-chemical parameters of environmental interest. Separately, the role of temperature in organic and nutrient removal in wastewater biological treatment is well understood; however, there is a paucity of data on how heat treatment for virus inactivation impacts physico-chemical parameters.

This study examines the effect on analytical results from wastewater samples under heat-treatment conditions that can ensure the safety of handling for a variety of known and possibly future viral pathogens.

This manuscript reports an examination of heat-treated wastewater from different sources for common widely used physico-chemical parameters such as chemical oxygen demand (COD), pH, ammonia, total nitrogen (TN), and solid content. This study also reports the effect of heat-treatment on analytical microbiological analysis of wastewater for fecal coliform bacterial count and helminth eggs.

METHODS

Blackwater sources

Blackwater samples used for the study were collected from three different sources in the city of Coimbatore (India). Source 1 (S1) was a public toilet booth for male users connected to a 2,200-L four-chamber baffled anaerobic digester. Source 1 wastewater was collected from the first chamber of the digester. Source 2 (S2) was a 25,000-L, single-chamber septic tank collecting effluent from a women's bathroom facility with 10 toilet stalls in a private industrial site. Source 2 wastewater was the supernatant of the septic tank, collected with a pump into a 100-L container. Source 3 (S3) was the effluent of the single toilet in a shared bathroom facility on a college campus collected daily in a 200-L tank (Kachoria *et al.* 2023).

Samples from these sources were collected in an autoclaved 500-mL borosil bottle for physico-chemical and bacterial measurements and two 1,000-mL polypropylene bottles for helminth eggs analysis. The samples were then transported to the laboratory in a secondary container.

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Heat-treatment

The selection of 60 °C temperature and 90-min-long treatment was based on the most conservative of the time and temperature combinations reported in the literature from researchers on wastewater SARS-CoV-2 surveillance in 2021 (Bivins *et al.* 2021; Crits-Christoph *et al.* 2021; Prado *et al.* 2021). A serological water bath (Labtech, Coimbatore, TN) was used to carry out the heat-treatment process. The water bath temperature was stabilized, and then sample containers with closed lids and minimal headspace were immersed in water. After the heat-treatment, samples were removed from the water bath and allowed to reach room temperature, and then the analysis was performed.

All physico-chemical analysis was performed on heat-treated and matching untreated wastewater for comparison purposes. Bacterial and helminth egg concentration measurements were conducted on heat-treated specimens and followed by selected bacteria species confirmatory analysis on both heat-treated and matching untreated specimens.

Physico-chemical parameters

Ammonia (NH_3), TN, COD, total suspended solids (TSS), pH, and turbidity were analyzed at the same time for heat-treated and matching untreated samples.

Ammonia, TN, and COD were measured according to the HACH kit method (high range) with Multiparameter Portable Colorimeter (DR 900). Ammonia was measured with the Hach method 10031. TN was measured with the Hach method 10072 and COD with the Hach method 8000 (measuring range: 20–1,500 mg/L); both underwent digestion (Hach digester DRB 200) prior to colorimetric readout. Turbidity was measured in NTUs (Nephelometric Turbidity Units) with the Hach Turbidimeter part number: 2100 Q and TSSs were measured as per the TSS EPA method 160.2 by filtration with a filter having a pore size of 1.5 μm.

Microbial analysis

Most Probable Number (MPN) assay

Enumeration of viable microorganisms in wastewater was carried out using the Most Probable Number (MPN) method adapted from Blodgett (2010). Samples were serially diluted 10-fold (10^{-1} to 10^{-8}) with nutrient-rich medium in a 48-well culture plate and incubated at 37 °C for 24 h. Each dilution well was examined for turbidity, and positive well counts were scored against a statistical table value to obtain the MPN/mL values. The growth medium was lysogeny broth (10 g of Tryptone water, 10 g of NaCl, and 5 g of yeast extract in 1 L of deionized water), autoclaved at 121 °C for 15 min after preparation.

Gram staining

Gram staining was carried out according to standard methods (Rice *et al.* 2012; Tripathi & Sapra 2020). The assay includes four steps: (1) Staining, (2) Fixing the color with a mordant, (3) Decolorizing the cells with 95% ethanol, and (4) Applying a counterstain. The samples were blotted dry, and slides were examined under a 100× oil immersion objective microscope. Gram-positive bacteria retain crystal violet stains, thus appearing purple, whereas Gram-negative bacteria retain counterstains and appear pink.

Acid and gas production assay

The assay was conducted as described in Bartram & Ballance (1996). The medium was prepared by dissolving 80.02 g of MacConkey purple broth (Cat no: M796) in 1 L of distilled water. Single-strength medium (10 mL) was added to the test tubes with Durham's tube and autoclaved. The wastewater sample (10 mL) was added to the tubes and incubated at 37 °C for 24 h. Turbidity and gas production in Durham's tube indicate the coliform presence. Production of acid by the active microorganism lowers the media pH and results in a medium color change from purple to yellow. Positive control was *E. coli* culture (ATCC 25922) and negative control was distilled water.

Agar plating on a selective medium

Simultaneous detection of *Escherichia coli* and total coliforms was carried out on agar plates prepared with chromogenic media (Rice *et al.* 2012; Lange *et al.* 2013). HiCrome™ Chromogenic Coliform Agar (Cat no: M19911) was prepared with 30.9 g of CCA media in 1 L of distilled water and heated to boiling temperature and then poured into a Petri dish. A wastewater sample (0.1 mL) was spread on the surface of the medium using an L-rod and incubated at 37 °C for 24 h under aerobic conditions. *E. coli* forms dark blue to violet colonies and total coliforms produces pink colonies. The positive control was a plate spread with *E. coli* culture and the negative control plate was with distilled water.

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Helminth egg enumeration

Helminth egg enumeration was conducted according to a modified AmBic method by isolation and microscopic evaluation (Grego *et al.* 2018). Briefly, the method consists of three steps: (1) Filtration, using 100 and 20 μm sieve for separation of eggs from large and small particles, (2) Centrifugation, and (3) Flotation, using $ZnSO_4$ at a specific gravity of 1.3 for egg flotation.

Statistical analysis

Physico-chemical parameters data sets were tested for normal distribution using Shapiro–Wilk and Anderson–Darling tests. For normally distributed data sets, a two-tailed *t*-test was used, and for non-parametric data sets, the two-tailed Wilcoxon test was used to compare. Statistical analysis was performed with GraphPad Prism v 9.4.0. $p < 0.05$ was considered statistically different.

RESULTS

Physico-chemical parameters

Blackwater samples were collected between November 2020 and August 2022 from three separate sources on $n = 12$ separate days. No change in appearance was observed in any of the samples after heat-treatment as compared to the same untreated sample. Paired analytical measurements on the same sample before and after heat-treatment were carried out in triplicate for S1 samples and duplicates for samples from S2 and S3. The experimental results are reported in Figure 1. Percent variation, defined as (%) variation = (COD untreated – COD heat-treated)/(COD untreated), is also plotted relative to a secondary *y*-axis.

COD values covered a broad range of values from 200 to as high as 1,500 mg/L across all the specimens in the study and the % variation due to heat-treatment was within ±5% for all samples, as also plotted against a secondary *y*-axis (Figure 1(a)). pH values between 7 and 8.5 were measured and variation due to heat-treatment was within 5% (Figure 1(b)). Ammonia and nitrogen were higher than 100 mg/mL for specimens 1–5 and around 50 mg/L for the remainder of the specimens. A change in values of nearly 20% due to heat-treatment was observed in specimen 10, but all others were negligibly affected (Figure 1(c) and 1(d)). Turbidity exhibited high variation up to 40% due to heat-treatment while variation of TSS was modest, with the exception of specimen 9 (Figure 1(e) and 1(f)).

Statistical analysis was carried out on the physico-chemical results shown in Figure 1 to assess the significance of the measured differences due to heat-treatment.

Statistical tests for ammonia, TN, COD, and TSS result in *p*-values >0.05, indicating no statistically significant differences. The *t*-tests for pH and turbidity data also yielded $p > 0.05$, not statistically different (Table 1).

In summary, in 12 specimens from three different wastewater sources, no statistically significant difference due to heat-treatment in the condition of this study was observed.

Effect of heat-treatment on microbial growth

Blackwater samples from source 1 and source 2 were tested for fecal bacterial content with the MPN method after heat-treatment.

We reproducibly observed the MPN count as 10^2–10^3 MPN/mL in heat-treated samples across 10 specimens (each from duplicate wells) (Figure 2). These MPN values were well above the limit of detection of the assay, 3 MPN/mL, and several orders of magnitude lower than reference values (10^4–10^7 MPN/mL) previously recorded from untreated specimens of the same source.

A set of assays were conducted to identify the nature of the active bacteria present in heat-treated blackwater. Coliforms are gram-negative, rod-shaped, non-spore-forming and able to ferment lactose with the production of acid and gas when incubated under 35–37 °C. We thus conducted Gram staining, acid and gas production assay, and agar plating on a selective medium.

In the MPN assay, the presence of viable microorganisms is indicated by the turbidity of the MPN culture medium (Figure 3(a)). Gram staining assay was performed on samples from positive MPN wells and revealed consistently Gram-positive bacteria (long rods with and without spores) from heat-treated blackwater from both S1 and S2, whereas untreated samples exhibited a mixture of both Gram-positive and negative bacteria (rods, cocci).

Acid and gas production assays were carried out where the medium color change (purple to yellow) indicated acid production and visual observation of air bubbles indicated gas production (Figure 3(b)).

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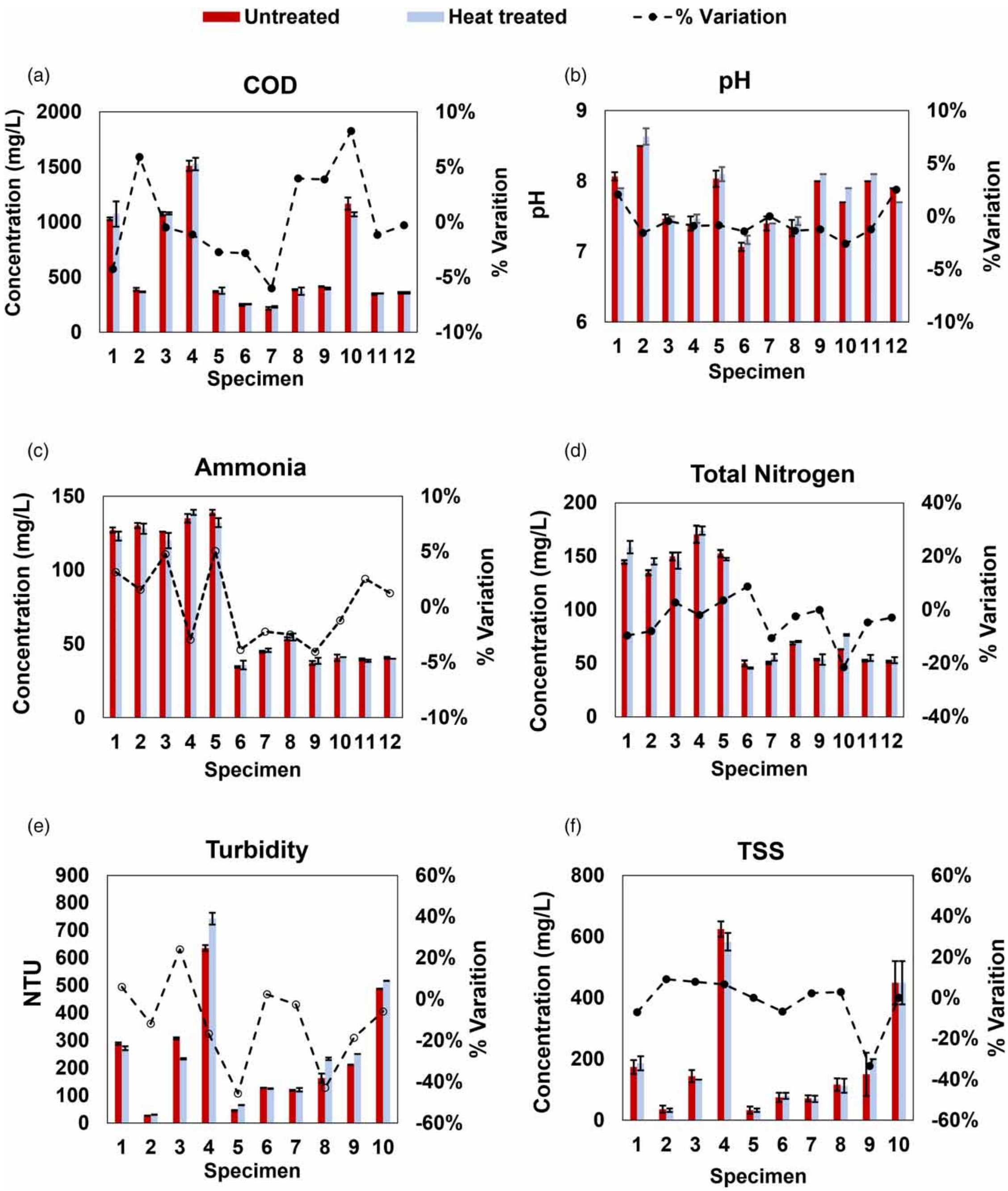

Figure 1 | Matched analytical results for six physico-chemical parameters on wastewater before and after heat-treatment. Specimens 1–5 are from blackwater source S1, 6–8 from source S2; 9–12 from source S3. Error bars are standard deviation from replicates.

The heat-treated wastewater samples were consistently negative for acid and gas production, while untreated samples were positive for both acid and gas production, as expected.

An agar plate culture on selective chromogenic media (Figure 3(c)), showed no isolated colonies of *E. coli* nor total coliforms in heat-treated samples while the untreated samples were positive for both.

Table 2 summarizes the results of these assays that are consistent with each other and lead to the conclusion that the heat-treatment inactivates coliforms bacteria but does not inactivate gram-positive spore-forming bacteria, and the latter is responsible for bacterial counts.

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Table 1 | Statistical comparison of untreated vs. heat-treated physico-chemical parameters

Parameter	Statistical test	*p*-value
Ammonia	Wilcoxon	0.6338
TN	Wilcoxon	0.2402
COD	Wilcoxon	0.8931
TSS	Wilcoxon	>0.9999
pH	*t*-test	0.2839
Turbidity	*t*-test	0.2845

Wilcoxon test: non-normally distributed parameters; *t*-test: normally distributed parameters.

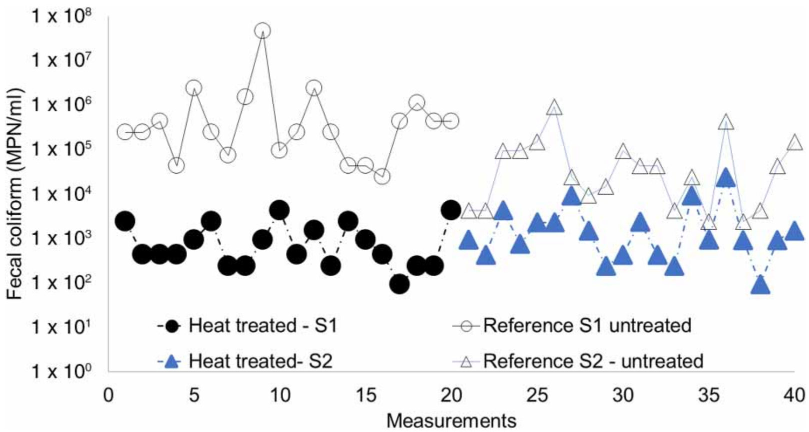

Figure 2 | Fecal coliform measurements (duplicates for each specimen) from heat-treated blackwater from source 1 and source 2 and reference values of untreated blackwater from the same sources.

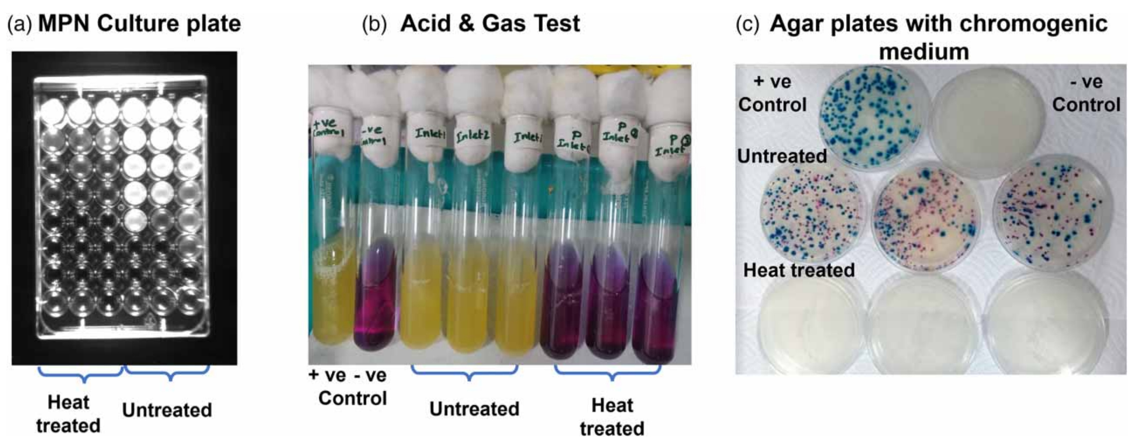

Figure 3 | (a) MPN test culture plate in a gel reader with triplicate wells for heat-treated and untreated specimen; (b) Photograph of acid and gas production assay; and (c) Agar plate with chromogenic medium for *E. coli* and total coliforms.

Effect of heat-treatment on helminth egg enumeration

Periodic helminth egg testing was conducted on wastewater from all sources of this study and the results were typically negative, i.e. absence of helminth eggs. In the period January–April 2022, a set of samples collected with weekly frequency exhibited a spike in helminth egg concentration. The presence of helminth in the specimens offered the opportunity to examine the effect of heat-treatment on helminth egg enumeration.

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Table 2 | Bacterial differentiation assays on blackwater from sources 1 and 2, each specimen in either duplicate or triplicate

Test	Heat-treated ($n = 4$ specimens)	Untreated ($n = 2$ specimens)
Acid and gas	Negative	Positive
Total coliforms (cfu/mL)	<10	$(2–15) \times 10^4$
E. coli (cfu/mL)	<10	$(0.5–1.2) \times 10^4$
Gram staining	Gram-positive rods	Both positive and negative rods and cocci

The vast majority of the eggs were identified as hookworm species, with a total count of 196 hookworm eggs, whereas a total of 11 additional eggs were identified as other species such as *Ascaris lumbricoides*, *Trichuris trichiura*, and *Hymenolepis nana*. The number of hookworms recorded over sampling dates is plotted in Figure 4(a), illustrating the peak concentration of 70 eggs in 2 L.

Only 13 of the 196 hookworm eggs appeared dead (e.g. shell was damaged), while the remaining 183 eggs were observed under optical microscope imaging to have intact shells and internal structures reflecting different stages of development. This suggests that heat-treatment does not cause damage to the morphology of common hookworm helminth egg species, and therefore, helminth egg enumeration is possible in heat-treatment samples. Additional studies are needed to assess the viability of the helminth eggs.

DISCUSSION

Heat-treatment of wastewater was conducted for the safety of the laboratory personnel while conducting field testing of wastewater technologies during the COVID-19 pandemic. This study examined how one common heat-treatment condition for virus inactivation compromises analytical wastewater measurements used in the assessment of wastewater treatment processes.

This study measured the effect of heat-treatment on physico-chemical wastewater parameters, namely, COD, pH, ammonia, TN, TSS, and turbidity. Our study of specimens from three different sources found no significant difference in these parameters associated with heat-treatment. These results were surprising because an effect was expected given the dependence of pH on temperature and the potential volatilization of organics with increased temperature.

We note that the heat treatment was conducted with a closed lid and the sample was allowed to return to room temperature prior to measurements. This implementation of heat-treatment appears to preserve the physico-chemical properties of the blackwater specimens. Results were consistent across specimens from three different sources that covered a range of values for organic, nitrogen, and solid content. The fact that heat-treatment does not compromise these environmental parameters is significant because it indicates that monitoring of wastewater treatment processes and technologies can be carried out under the circumstances of a virus outbreak that warrants heat treatment before laboratory analysis for personnel safety.

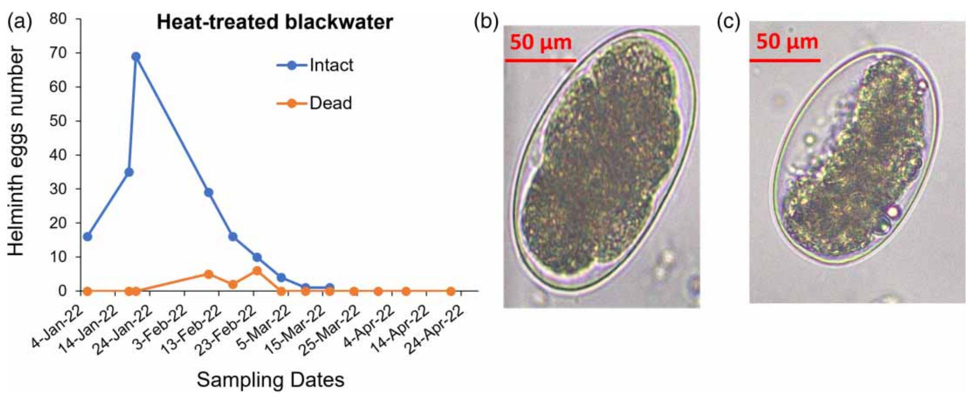

Figure 4 | (a) Number of helminth eggs (hookworm species) in heat-treated sample in blackwater source 2 in the period January–April 2022, (b) Optical microscope image of intact hookworm species egg, and (c) Dead (globular) hookworm species egg.

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Our study found that the heat treatment introduces a significant bias in coliform and *E. coli* enumeration and it reduces the bacterial count by at least 4 $\log_{10}$ cfu/mL to below the detection limit of the assay.

Fecal coliforms contained in blackwater are Gram-negative bacteria able to withstand temperatures up to 45 °C (Bartram & Ballance 1996) and heat-treatment at 60 °C was expected to inactivate fecal coliforms. Thus, heat treatment in this study compromises the ability to measure established fecal contamination indicators of wastewater.

As a separate observation, we found that heat-treated wastewater contained Gram-positive bacteria, so heat-treated samples, while not containing coliforms, were not disinfected.

Enumeration of helminth eggs on heat-treated wastewater appears to be possible. Helminth eggs are known to be resilient and able to withstand harsh environmental conditions, and heat-treatment for their inactivation has been investigated. Temperatures above 55 °C have been reported to be sufficient to inactivate helminth eggs in minutes; however, morphological damage was not necessarily observed with heat-treatment (Naidoo *et al.* 2020). That literature report is consistent with our findings of mostly intact helminth eggs in heat-treated specimens. Many blackwater treatment technologies remove helminth eggs by physical filtration method; thus, enumeration is the critical assay for determining the performance of a process, and we found that enumeration of intact eggs could be carried out.

CONCLUSION

Heat-treatment of blackwater for virus inactivation was conducted for personnel safety while field testing of treatment technologies during the COVID pandemic. This study found that heat-treatment at 60 °C for 90 min in a capped container in a water bath yielded no significant difference in six physico-chemical parameters. We also found that heat treatment compromises coliform/*E. coli* bacteria enumeration (>4 $\log_{10}$ reduction); thus, this assay is not meaningful on heat-treated specimens. Observed enumeration of helminth eggs appears to be possible after heat-treatment.

In conclusion, we report that it is possible to accurately measure a number of wastewater analytical parameters on specimens that are heat-treated for the safety of handling. Future studies are needed to assess heat-treatment impact on additional wastewater parameters such as phosphorus and total solids that were not included in this work.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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