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Discharges of Wastewater Treatment Plants Needed Further Monitoring to Minimize Potential Risk of *Entamoeba* and *Blastocystis* for Public Health

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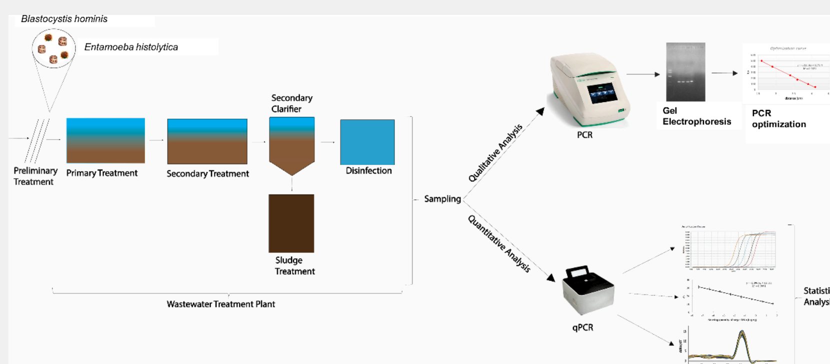

ABSTRACT: The protozoan parasites *Entamoeba histolytica* and *Blastocystis hominis* are responsible for causing human amebiasis and hominis infections, respectively. These infections are highly prevalent and are often linked to waterborne diseases. Due to the absence of regulations for monitoring these protozoa at the discharge points of wastewater treatment plants (WWTPs), the effluents reaching surface waters contribute to waterborne transmission. This underscores the significance of the removal capacities of WWTPs in reducing the spread of these infectious parasites. Therefore, this study examined five different types of WWTPs in Ankara, Turkey, over a year to assess their capacities to remove *E. histolytica* and *B. hominis*. The seasonal abundances of genes specific to these protozoa in both the influents and effluents of each WWTP were measured using a quantitative polymerase chain reaction. The reduction in the number of protozoan rDNA copies between the influent and effluent samples was evaluated as the removal capacity, expressed in $\log_{10}$ reduction (LRV) values. The results elucidated that the removal of *E. histolytica* and *B. hominis* was highly affected by the process used. Membrane bioreactor systems displayed the highest removal capacity with LRV > 3. Therefore, discharges of WWTPs with other processes could need further monitoring to minimize the potential risk for public health.

KEYWORDS: *Entamoeba histolytica, Blastocystis hominis, amebiasis, hominis, WWTP, protozoa removal*

■ INTRODUCTION

Entamoeba histolytica is believed to be pathogenic and causes amebiasis, also known as amoebic dysentery. It is often confused with its morphologically identical counterpart *Entamoeba dispar*.[1] However, based on genetic and biochemical analyses, it has been concluded that only nonpathogenic *E. histolytica* appears as a distinct species. Mostly tropical countries with poor sanitary conditions record *E. histolytica* and *E. dispar*. Each year, millions of amebiasis cases emerge and end up with thousands of deaths.[2] *Blastocystis* infections are also common among mammals,[3] and the prevalence is higher in developing countries.[4] Unlike *Entamoeba*, *Blastocystis* isolates display a great genetic distance. Among *Blastocystis* spp., only human isolates are designated as *Blastocystis hominis*. However, it has been recorded that there is no host specificity, since the species are transmitted zoonotically[5] and show a wide range of genetic diversity with varying virulence.[6]

Both *E. histolytica* and *B. hominis* are transmitted by fecal−oral spread, and infection occurs following ingestion of contaminated food or water.[7,8] Removal of these pathogenic protozoa from wastewater, therefore, is important from a public health viewpoint, especially in the locations where wastewater treatment plants (WWTPs) are vital for water safety sanitation and hygiene.[9] Wastewater treatment encompasses physical,

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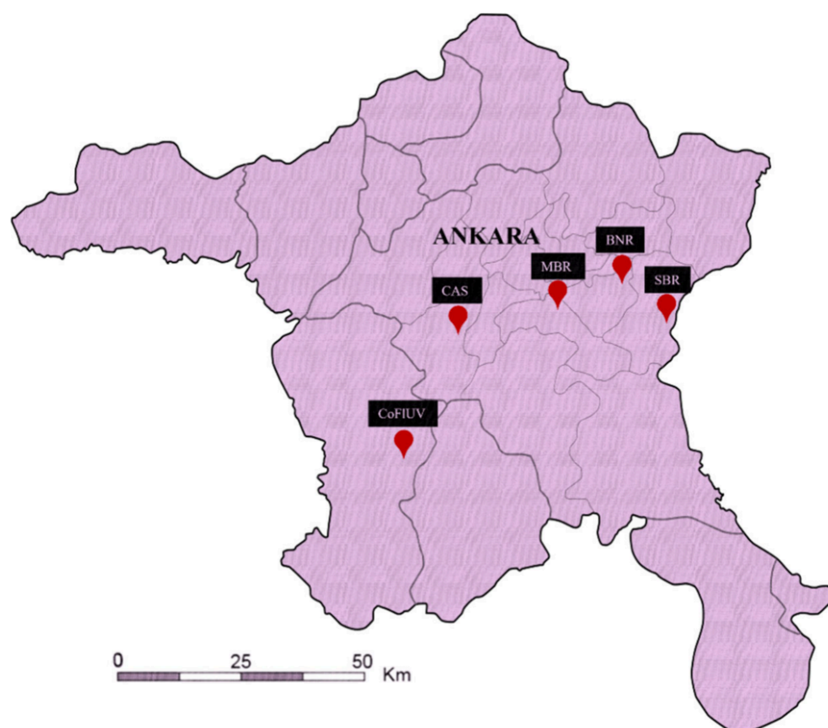

Figure 1. Locations of WWTPs sampled.

biological, and chemical processes for removing suspended solids, organic matter, and pathogenic microorganisms. Advanced treatment is particularly important in arid regions, where treated effluents are reused for irrigation and other nonpotable purposes. Efficient treatment is essential to preventing the spread of pathogenic protozoa through irrigated crops and water bodies that receive the treated effluents. With the conventional wastewater treatment processes and secondary sedimentation, only 0.3 $\log_{10}$ reduction (LRV) of *E. histolytica* is observed. While chemical disinfectants such as chlorine show 2 LRV reduction, UV disinfection displays more promising results.[1] *Blastocystis* is confirmed to be robust toward wastewater treatment techniques and should be included as a parameter when investigating parasites in wastewater.[10] However, there has been limited research or reviews on the protozoan quality of effluents and the impact of different types of WWTPs on the removal of protozoa such as *E. histolytica* and *B. hominis*.[11−13] For this reason, this study aimed to evaluate the removal capacities of different types of WWTPs for *E. histolytica* and *B. hominis*.

■ METHODOLOGY

WWTPs Sampled

Influent and effluent samples were collected from five WWTPs with different processes including conventional activated sludge (CAS), biological nutrient removal (BNR), sequencing batch reactor (SBR), membrane bioreactor (MBR), and a WWTP with coagulation−-flocculation and UV disinfection (CoFlUV) units located in Ankara, Turkey (Figure 1). Process details of the sampled WWTPs are as given in our previous study.[14] Average operational parameters and influent and effluent water qualities are presented in Tables S1 and S2, respectively.

Sampling and Pretreatment

The designs and sample collection points of the sampled WWTPs are represented in Figure 2. An amount of 1 L of water and sludge samples was taken from each WWTP in triplicate and placed in sterilized bottles as previously described.[14] DNA extractions were done within 24 h of sample collection, and the samples not used in the extraction were pretreated for storage. The pretreatment procedures to make the protozoa more concentrated were as follows: collected sludge and water samples were centrifuged at 16000*g* for 15 min and 1000*g* for 16 min, respectively, and the pellets were stored at −20 °C until DNA extraction.[15]

DNA Extraction

After sample collection, protozoan DNA extractions were done within 24 h following the methodology described in our previous study.[14] The quality and concentration of the isolated protozoan DNA was determined by a Colibri microvolume spectrophotometer (Titertek Berthold, Germany) and 1.5% agarose gel electrophoresis.[16] Before analyses, the spectrophotometer was blanked with the TE buffer that was also used to store the extracted DNA. In the spectrophotometric analyses, the purity of the extracted DNA was assessed using the 260/280 and 260/230 nm ratios. The ratio of absorbance at 260/280 nm should be around 1.8 for DNA to be accepted as pure. This ratio was around 1.8 for all of the samples measured for the extracted protozoan DNA. The 260/230 nm ratio should be in the range of 2.0−2.2, and for all of the samples measured this value was between 2.0 and 2.2. For the agarose gel electrophoresis analyses, a previously described method was used for the electrical conducting agent and to prepare the agarose gel.[14]

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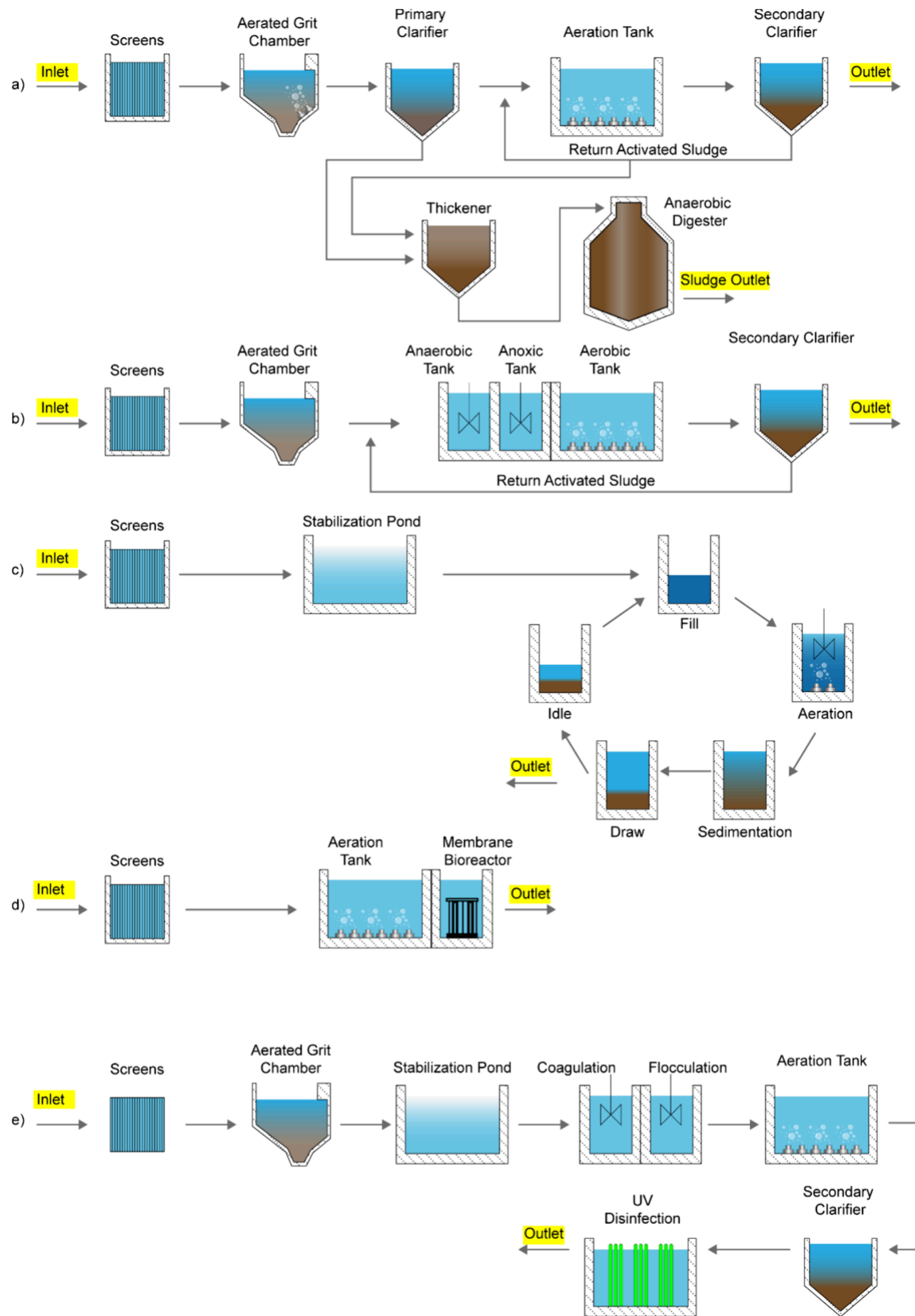

Figure 2. Designs of WWTPs sampled: (a) CAS, (b) BNR, (c) SBR, (d) MBR, and (e) WWTP with CoFlUV.

QUALITATIVE AND QUANTITATIVE ANALYSES OF PROTOZOAN DNA

Qualitative Analyses

PCR reactions were performed in the T100 Thermal Cycler (Bio-Rad, USA) system in 25 μL reaction mixtures with a previously described PCR program.[14] The specific primers used in this study are given in Table S3. Negative controls were included in each PCR reaction. Reproducibility of the reactions was verified by performing duplicate PCR reactions. The amplicons were analyzed through 1.5% agarose gel electrophoresis.[17] The copy numbers of protozoan DNA per μL were calculated according to eq 1 as previously described.[14,18]

$$\frac{\text{Copy number of DNA}}{\mu\text{L}} = \frac{b \times c}{L \times a \times 10^{12}} \quad (1)$$

In eq 1, variables a, b, c, and L represent the weight of kb DNA/pmol (with 1 kb DNA equating to 0.66 μg/pmol), Avogadro's number (6.022×10^{23} mol^{-1}), the concentration of the template in μg/μL, and the length of the template that includes the target gene, respectively.

Quantitative Analyses

Quantitative PCR (qPCR) was used for quantitative analyses of the two protozoan parasites. The qPCR reactions were performed with a Coyote Mini8 real-time PCR (Coyote Bio, Columbia) in a 20 μL reaction mixture as described in our

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previous study.[14] Negative controls were also used in each qPCR assay to determine nonspecific amplifications. The standard curves for the qPCR were constructed by using Lambda DNA (New England Biolabs, USA).[19] Data were analyzed with the Mini8 Plus qPCR software (version 2.0.13; Coyote Bio, Columbia). The amounts of target DNA in the samples were calculated based on the standard curves that are constructed with Lambda DNA. The qPCR program included the steps previously described in our study.[14] The DNA samples were analyzed in triplicate. R^2 values, qPCR efficiencies, and melting curves were used to check the specificity of the products. R^2 values for all of the standard curves were higher than 0.99.

Data Analyses

Removal efficiencies of parasitic protozoa for each WWTP were measured with LRV.[20] LRVs were calculated by taking the logarithm of the ratio between protozoan DNA concentrations in the influents and effluents of the wastewater treatment plants, as illustrated in eq 2.

$$\mathrm{LRV} = \mathrm{Log}_{10}(C_{\mathrm{influent}}/C_{\mathrm{effluent}}) \tag{2}$$

An LRV of 1 indicates a 90% removal efficiency of the target protozoa, an LRV of 2 indicates a 99% removal efficiency, and an LRV of 3 indicates a 99.9% removal efficiency. This pattern continues as the LRVs increase. In their paper, Teel and co-workers state that the Nevada Administrative Code allows a treatment process to be credited with an LRV of 1−6.[21] The World Health Organization recommends a 4-log reduction for protozoa for potential agricultural reuse.[22] Due to the lack of specific regulations for protozoan removal, an LRV of 3 was chosen as the baseline for effective removal in this study. Wastewater treatment plants (WWTPs) with LRVs below 3 were considered ineffective and likely to release protozoan parasites into discharge points. Seasonal variations in the removal of protozoan DNA were evaluated using One-Way Analysis of Variance (ANOVA) followed by Tukey's posthoc tests (SPSS Statics for Windows ver. 28.0; IBM Corp., Armonk, NY). The limit of detection for the experiments was determined to be 9.87 log DNA copies/μL. To calculate the maximum LRVs, samples below this detection limit were set to 0, as they could range between 0 and the detection limit. In another analysis, samples below the detection limit were assigned a value of 9.87 log DNA copies/μL to compute the minimum LRVs. The limit of quantification was found to be 11.30 log DNA copies/μL, and values below this limit but above the detection limit were set to the average of these limits, 10.58 log DNA copies/μL.[12]

RESULTS AND DISCUSSION

Removal of Both Protozoa in CAS

With the CAS process, LRV > 3 was achievable for *B. hominis* in spring and summer, while the highest LRV of 2.93 was only obtained in summer for *E. histolytica* (Figure 3). *E. histolytica* removal in CAS, in particular, was shown to be relatively poor (LRV often <2). LRV 2−3, on the other hand, was readily achievable for *B. hominis* in CAS. However, seasonal changes for the removal of both parasites in CAS were significant ($p < 0.05$). The LRVs observed with the CAS process in this study were in agreement with those observed in previous studies for *E. histolytica*.[12,23] The main mechanism for removing protozoan cysts in CAS is high retention time.[24] Temperature, solar radiation, and high pH in sludge are also involved in the removal

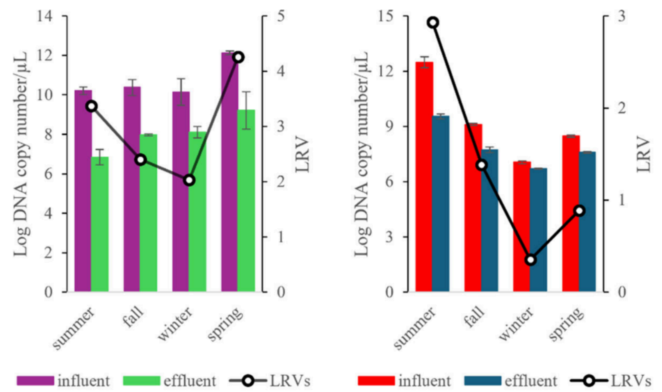

Figure 3. LRVs for *B. hominis* (left) and *E. histolytica* (right) in CAS. Error bars represent standard deviations.

of protozoan cysts.[25] Annual average removal rates of TSS, COD, BOD, TN, and PO_4−P of the sampled CAS process are given in Table S1. However, no correlation was found with the characteristics of the treatment process and the low LRV for *E. histolytica*. The removal could be impacted by the small size of *E. histolytica* cysts (12−15 μm) or by the nature of the treatment process.

According to total suspended solids (TSS), sludge treatment enriched the amount of *E.histolytica* in winter/spring and *B. hominis* in winter (Figure 4). For sludge adsorption, the attachment of cysts to the sludge is mainly affected by surface characteristics, hydrophobicity, pH, and ionic strength.[26] The adsorption of pathogens onto settleable solids is not always conducive to pathogen reduction.[27] In sludge treatment, protozoa are thought to be enriched due to adsorption.[28] Therefore, further treatment of sludge for both protozoa should be considered before sludge disposal.

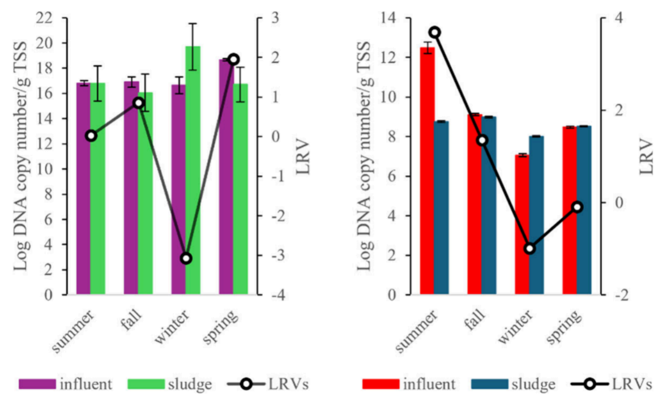

Figure 4. LRVs for *B. hominis* (left) and *E. histolytica* (right) in CAS sludge. Error bars represent standard deviations.

Removal of Both Protozoa in BNR

Wide-ranging LRVs up to 4 were observed for the removal of *B. hominis* in the BNR process. The highest LRV of 3−4 was recorded in spring/winter, and the seasonal change was also found to be significant ($p < 0.05$). Like in the CAS process, the BNR process also displayed poor removal efficiency for *E. histolytica* (LRV often <1) (Figure 5). Nutrient removal systems appeared to have the potential for reducing or completely removing *B. hominis* due to the various physical, chemical, and biological processes that they encompass. The BNR process is influenced by various factors such as temperature, mixed liquor

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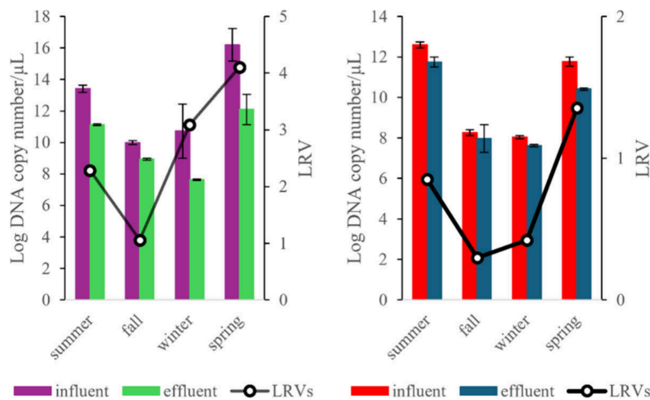

Figure 5. LRVs for *B. hominis* (left) and *E. histolytica* (right) in BNR. Error bars represent standard deviations.

suspended solids, influent BOD, and volatile fatty acids.[29] Annual average removal rates of TSS, COD, BOD, TN, and PO_4−P of the sampled BNR process are given in Table S1. However, the characteristics of the treatment process were not attributed to the removal of the protozoan parasite, in particular, *E. histolytica*. The relatively low specific gravity and smaller size of *E. histolytica* cysts might lead to a lower settling velocity and less removal.[30] Wang and his co-workers[31] also recorded low LRVs with the BNR process. Protozoa vary in size, which affects their tendency to attach to solid particles.[32] This could explain the different LRVs of both protozoa by the BNR process sampled in the current study.

Removal of Both Protozoa in SBR

LRV > 3 was only achievable for *B. hominis* in the summer in the SBR process. In the rest of the seasons for *B. hominis* and all seasons for *E. histolytica*, the SBR process displayed a very inadequate removal efficiency, with LRVs often <1−2 (Figure 6). However, seasonal variations in SBR were applicable for both protozoan parasites ($p < 0.05$). The SBR process consists of cyclic operations, including fill, react, settle, decant, and idle phases, which provide alternating anaerobic, anoxic, and aerobic conditions in defined sequences and durations.[33] Table S1 presents the annual average removal rates of TSS, COD, and BOD for the SBR process used in this study. Supha and co-workers[34] carried out a study on the long-term exposure of protozoan communities to TiO_2 in SBR. The research revealed that even addition of TiO_2 exhibited poor removal efficiencies (LRV 1) for protozoan communities. Similar efficiencies in SBR was also found by Chaudhary and co-workers.[35] The performance of SBR depends on several parameters like wastewater characteristics, cycle time, aeration rate, contact time, temperature, hydraulic retention time (HRT), and the retention time of solids.[36] The shorter HRT (8 h) and absence of the secondary clarifier in the SBR process used in the current study might explain the lower removal efficiencies for both protozoa.

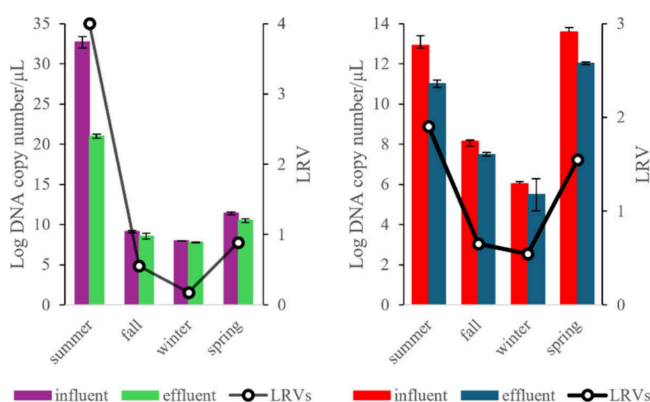

Figure 6. LRVs for *B. hominis* (left) and *E. histolytica* (right) in SBR. Error bars represent standard deviations.

Removal of Both Protozoa in CoFlUV

The removal of *E. histolytica* with LRV > 3 was only obtained mainly in the summertime in the CoFlUV process. In the rest of the seasons for *E. histolytica* and all seasons for *B. hominis*, the LRV was less than 3, although seasonal variations were significant for both protozoa ($p < 0.05$). Except for autumn, LRV > 2 was also achievable for *E. histolytica* (Figure 7). The annual average TSS, COD, and BOD removal of the CoFlUV system sampled in this study is illustrated in Table S1. For the coagulation−flocculation process, a ferric coagulant ($FeCl_3$) was used in the current study. Similarly, 1−2 LRV for protozoan removal was observed in coagulation-flocculation systems with ferric coagulants by Fewtrell and Bartram.[37] According to a previous study,[38] coagulation−flocculation along with secondary sedimentation also leads to LRVs of 2−3 for *E. histolytica*. For *E. histolytica*, LRV > 2 has also been reported.[39,40] This removal is explained by the precipitation of electronegative protozoan cysts with metal hydroxides during coagulation, which relies on accurate dosing and mixing. Types of microorganisms, disinfecting agents, and water composition also play roles in disinfection. UV lights with doses of around 30 mWs/cm^2 are usually suggested for pathogen inactivation; however, the UV dosage of the sampled system was 15 mWs/cm^2. The low LRVs for *B. hominis* observed in this study may be due to the coagulant used and the UV doses applied.

Removal of Both Protozoa in MBR

LRV > 3 was achievable for *B. hominis* for all seasons in the MBR process, and seasonal changes were found to be significant ($p < 0.05$) (Figure 8). However, this efficiency was obtained only in the summertime for *E. histolytica*. The rest of the seasons, the LRV for *E. histolytica* was often less than 2. Table S1 represents the annual average COD and BOD removal of the MBR process used in this study. For TSS, TN, and PO_4−P removal, the data were not available. Membrane filtration, as a wastewater treatment approach, is usually found to be reliable and has potential for pathogen removal. Since protozoan cysts are substantially larger than the pores in membrane filters, efficient removal is expected.[41] With membrane technologies, 3−4 LRV was also recorded by Ayed and Sabbahi for removing *E. histolytica*.[1] Although membranes with 0.1 μm pore size remove many pathogens effectively under test conditions, due to membrane integrity failures and membrane fouling problems under operational conditions, this removal efficiency can dramatically deteriorate.[42] This may account for the low LRVs found for *E. histolytica* in MBR systems in the current study.

CONCLUSIONS

WWTPs utilize long-standing removal processes with various configuration of the treatment units. These various processes were especially effective in reducing pathogens typically high in particles and were rarely sufficient to meet the high pathogen removal standards (LRV ≥ 3) required for effective health protection. Optimized systems might achieve LRVs of 1−2; however, since maintaining optimized conditions was difficult,

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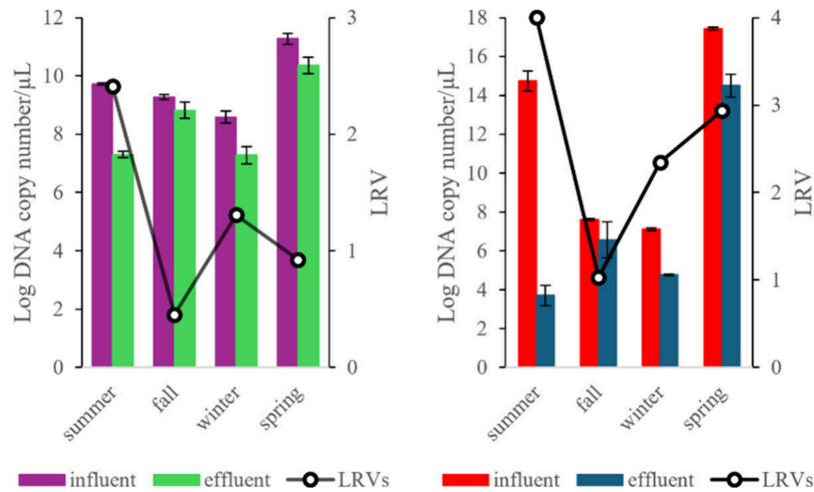

Figure 7. LRVs for *B. hominis* (left) and *E. histolytica* (right) in CoFlUV. Error bars represent standard deviations.

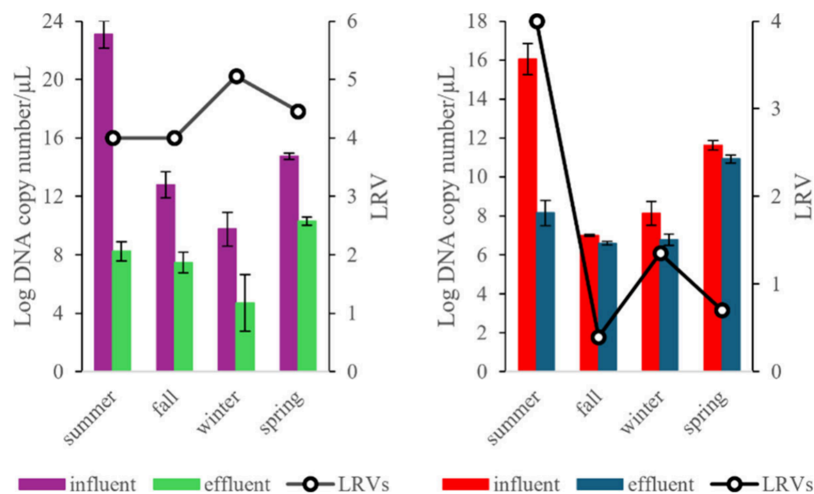

Figure 8. LRVs for *B. hominis* (left) and *E. histolytica* (right) in MBR. Error bars represent standard deviations.

this led to highly variable pathogen removal efficiencies. The current study revealed that the wastewater treatment processes studied reduced both protozoan parasites in the effluent with seasonal changes but also concentrated them in the sludge. Some of these processes like MBR displayed promising results with LRV > 3 for both parasites. However, regardless of the treatment processes employed, the removal efficiencies for *E. histolytica* were often below an LRV of 3. In most seasons, the removal efficiencies for this protozoan were typically around LRVs of 1−2. Therefore, discharge points of WWTPs need to be monitored and installed with robust membrane material systems to effectively remove *E. histolytica* and *B. hominis*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00113.

Characteristics and average operational parameters of WWTPs sampled and list of primers used in the study (PDF)

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Author Contributions

A.O.: Investigation, writing−original draft, methodology, validation, writing−review and editing, visualization, data curation, conceptualization. B.I.: Conceptualization, writing−original draft, methodology, validation, visualization, writing−review and editing, project administration, data curation, supervision.

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Notes

The authors declare no competing financial interest.

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