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Reduction of Infectious *Cryptosporidium* and Microbial Indicators in Wastewater Effluents by Disinfection with UV Irradiation or Chlorine

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Abstract

When properly treated, domestic wastewater should be considered a potential reliable water source in arid and semi-arid regions of the world for none-potable purposes. In Israel and other countries around the world, the main biological standards for water reuse are based on fecal coliform (FC) and turbidity. Furthermore, in secondary treatment, the Israeli standard for water reuse and for unrestricted irrigation comprises additional steps such as filtration and chlorination. The present study was conducted to compare the reduction efficiency of live *Cryptosporidium* oocysts in wastewater effluents by filtration and disinfection by either UV irradiation or chlorination. *Cryptosporidium* oocysts infectivity reduction was compared to those of the conventional microbial indicators (FC). The study was conducted in two full-scale wastewater treatment plants. The average concentration of FC and *Cryptosporidium* in secondary effluent was 2.8×10^5 cfu/100ml and 5.7 oocysts/10L, respectively. Infectious *Cryptosporidium* oocysts were detected in 2 out of 7 secondary effluent samples (28.5%). Infectious *Cryptosporidium* oocysts were not detectable in UV disinfected tertiary effluent. Conversely, 3 out of 7 (42.8%) tertiary effluent samples disinfected with chlorine were positive for infectious *Cryptosporidium* oocysts. The results of this study revealed that the application of a multi barrier treatment, including UV irradiation, for the reduction of *Cryptosporidium* oocysts and microbial indicators could improve tertiary effluent safety for unrestricted irrigation and other reuse purposes.

Keywords

Cryptosporidium, Infectivity, Wastewater Effluents, Disinfection, Indicators

1. Introduction

Wastewater treatment is essential for the production of an alternative water source, which can be utilized for restricted/unrestricted irrigation for crop production in arid and semi-arid regions. Domestic wastewater contains about 150 types of human pathogens comprising bacterial, viral and protozoal species [1]. Untreated or inadequately treated wastewater discharged into water sources may result in bacterial, viral and protozoal waterborne outbreaks [2] [3]. Wastewater treatment plants (WWTPs) are designed to reduce pollutants concentration to avoid direct discharge of wastewater into streams and oceans. However, conventional wastewater treatment is not entirely efficient to remove all pathogens (e.g. bacteria, viruses, and protozoa) [4] [5]. Faecal microorganisms, as well as pathogens, are released via WWTP effluents into rivers and assumed to be inactivated while being transported downstream [6] [7]. Additionally, adequate wastewater treatment can reduce pathogens levels at source and prevent public health risk when wastewater is discharged into rivers, oceans, and groundwater or directly used for irrigation. Production of high quality effluents relies mainly, on biological treatment followed by filtration and disinfection [8]. Though, biological and structural differences among pathogenic microorganisms may result in variable reduction efficiency of those microorganisms by wastewater treatment processes [9] [10] [11]. Even though, chlorine at low concentrations, is highly efficient in FC inactivation, it was found not efficient against *Cryptosporidium* oocysts at those values commonly used for water and wastewater effluents disinfection [12].

Cryptosporidium is a leading cause of waterborne outbreaks in developed countries [13]. For instance, the well-documented outbreak in Milwaukee, WI (1993) has been linked to an inadequate domestic water supply treatment [14]. The water treatment facility received water from Lake Michigan, which was contaminated with *Cryptosporidium* oocysts from heavy rain flooding, while oocysts were not efficiently reduced along the drinking water treatment process [14]. From 2004 to 2014, 239 cryptosporidiosis outbreaks had been reported worldwide [3]. Approximately 60.3% of those outbreaks were reported in developed countries such as Australia, Europe, and United States, besides many more cases that likely to have gone unreported or unnoticed [15].

Worldwide water reuse guidelines relay on FC periodical monitoring as indicator of the microbial quality of treated effluents. Water scarcity in various arid regions dictates intensive application of treated effluents for indirect potable reuse and even for direct potable reuse. Therefore, stringent standards based on log reduction were issued based on a treatment train which complies with the "12-10-9" framework for viruses, *Cryptosporidium* oocysts and bacteria reduction, recommended by NWRI (2013) [16]. Although, wastewater treatment process, including filtration, generally removes oocysts at high efficiency, low levels of *Cryptosporidium* may be found in the final effluent. USEPA method 1623, used to monitor *Cryptosporidium* oocysts provides information on oo-

cysts occurrence in raw wastewater or treated wastewater but will not assess their infectivity [15]. To determine the real public health risk posed by waterborne *Cryptosporidium* oocysts, oocysts infectivity must be determined. The method based on cell culture that relies on microscopic detection provides fast results by enumeration of developmental stages of this human parasite [17]. Molecular-based infectivity detection methods Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR) to detect DNA from infected HCT-8 were developed with a reported sensitivity of less than five infectious oocysts [18]. The method was used to detect infectious *Cryptosporidium* oocysts from raw water and filter backwash samples [18]. Q-PCR detection of infection in HCT-8 cells was used to demonstrate that oocyst recovered from environmental water samples by immunomagnetic separation (IMS) and by USEPA Method 1622 retained their infectivity [19]. The same assay was also used to estimate UV inactivation efficiency [20]. Limited studies evaluated the reduction efficiency of infectious *Cryptosporidium* oocysts by advanced wastewater treatment processes (that consists chlorine and UV disinfection), that can provide accurate data on the efficiency of treatment process and health risks linked to treated effluents process. The present study was performed to determine the prevalence of infectious *Cryptosporidium* oocysts in wastewater effluents. Furthermore, the reduction efficiency of *Cryptosporidium* oocysts by wastewater treatment processes including inactivation by chlorine and/or UV irradiation was evaluated. In addition, sensitivity of detection of direct immune staining and microscopic enumeration of infected foci culture was compared with that of cell culture-PCR (CC-PCR) method. The suitability of fecal coliform as indicator of *Cryptosporidium* oocysts infectivity reduction by wastewater treatment processes was also evaluated.

2. Materials and Methods

2.1. Study Sites and Sample Collection

Samples of secondary (activated sludge) and tertiary (filtration and disinfection) treated wastewater samples were collected from two wastewater treatment plants in the central part of Israel. Plant A serves 240,000 inhabitants and Plant B serves 44,000 inhabitants. In both plants, the treatment process consists of primary settling, activated sludge, secondary settling, sand filtration and disinfection by either chlorine (Plant A) or UV irradiation (Plant B). In plant A, total suspended solids (TSS) were reduced from 344 mg/L to 4.5 mg/L and the biological oxygen demand (BOD_5) was reduced from 275 mg/L to 3.9 mg/L. In plant B, TSS was reduced from 430 mg/L to 4.4 mg/L and BOD_5 was reduced from 270 mg/L to 4.6 mg/L. The quality of the effluent produced must be within the Israel Standard for unrestricted irrigation [21] and most of the produced effluents are used for irrigation. The two volumes of collected wastewater samples to be analyzed were as follows: secondary treated effluent 10 L and tertiary treated effluents 50 L, depends on sample turbidity. Turbidity was measured with 2100p portable turbidity meter (Hatch, Loveland, CO, USA). Grab samples were collected once

a month and samples were transported to the laboratory within 2 hours and further analyzed for FC and for the detection of *Giardia* cysts and *Cryptosporidium* oocysts.

2.2. Fecal Coliform Enumeration

Fecal coliform (FC) were enumerated on highly selective medium mFC according to Standard Methods [22]. Blue colonies were enumerated and considered FC.

2.3. Sample Processing for *Giardia* Cysts and *Cryptosporidium* Oocysts Detection

Secondary and tertiary effluent samples were filtered through an Envirochek HV (Pall Laboratories, Washington, USA) filter followed by procedures described in Method 1623.1 of the U.S. Environmental Protection Agency (EPA) [15]. Trapped material on filter was eluted with 250 mL eluate solution (10 ml Laureth −12, 10 ml Tris 1M, pH = 7.4, 2 ml EDTA 1M and 0.15 ml Anti-foam in one liter of distilled water) and then the eluate was centrifuged for 15 min at 1100 × g to concentrate cysts and oocysts. Concentrated parasites were then affinity purified and re-concentrated by immuno-magnetic separation using magnetic beads coated with anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies according to manufacturer-recommended procedure. At this stage, 50% of the purified sample was examined by immuno-fluorescent staining and microscopic enumeration with an epi-fluorescent microscope (Zeiss, AxioCam MRc). In parallel, the other 50% concentrated sample was used to determine *Cryptosporidium oocysts* infectivity in cell culture.

2.4. Detection of Infectious *Cryptosporidium* Oocysts in Cell Culture

Infectious *Cryptosporidium* was cultivated in human intestinal adenocarcinoma cell-lines (HCT-8) (ATCC CCL 244). HCT-8 cells were cultured and maintained in 25 cm^2 tissue culture flasks at a constant CO_2 atmosphere (5%) at 37˚C. Cells and HCT-8 cells grown in RPMI medium supplemented with calf serum (5%) were passaged once a week. HCT-8 cells were grown on sterile glass coverslips for *Cryptosporidium* oocysts infectivity determination. Cells were grown up to 70% to 95% confluence before infection. Experimentally separated oocysts, already attached to magnetic beads were incubated in fresh HBSS (pH = 2) 198 µl, containing 2 µl of trypsin (10%), 9 µl HCl (1N) while a positive control (approx.10^5 oocysts/ml) was incubated in 20 µl (HBSS, pH = 2) containing 2 µl trypsin (10%), 9 µl HCl (1N) for 1 hour at 37˚C with thorough vortex every 15 minutes. Warm RPMI medium (300 µl) was added carefully to oocysts suspension and sporozoites were concentrated by centrifugation for 4 minutes at 2080 × g. The supernatant was aspirated and the pellet containing sporozoites was suspended in 600 µl RPMI medium for further wash. Centrifugation step was repeated for 4 minutes at 2080 × g and finally washed sporozoites were suspended in 600 µl RPMI medium.

Cryptosporidium infection of HCT-8 cell cultures was accomplished as follows: 1) aspiration of maintenance medium from the cell monolayers; 2) experimental sporozoites samples were added to cells and incubated for 1 hour under CO_2 (5%) atmosphere at 37˚C; 3) then warm growth medium (1.5 ml) was added to infected cell layer and incubated further for additional 72 hours.

To detect *Cryptosporidium* infected HCT-8 cells, monolayers were dried at room temperature (24˚C ± 2˚C) and fixed with absolute methanol. Then, dried cells were incubated with anti-sporozoite antibodies (Waterborne, Baton Rouge, Lo) for 1 hr at room temperature. The infected cultures were then washed with sterile PBS (0.01 M) and finally air-dried and a 10 µl aliquot of DABCO (2%) was added to each sample and a cover slip was mounted. For the enumeration of infected cells (foci), epi-fluorescent microscope was applied.

2.5. DNA Extraction from Cell Culture Infected with *Cryptosporidium* Sporozoites

Infected cells were washed with warm PBS (10 mM) and warm EDTA-trypsin solution (2 ml), then washed cells were incubated in EDTA-trypsin (2 ml) for 5 minutes in CO_2 atmosphere (5%) at 37˚C. After incubation, cells were transferred to an Eppendorf tube (1.5 ml) and centrifuged for 15 minutes at 6000 × g, the supernatant was discarded and the pellet (concentrated cells) were resuspended in PBS buffer (200 µl). DNA was extracted from those infected cells using QIAamp DNA mini kit (QIAamp DNA blood mini kit, Qiagen GmbH, Germany) according to manufacturer's instructions with minor modifications. Purified DNA samples were stored at −80˚C for future analysis.

Cell Culture PCR (CC-PCR)

PCR primers specific to *C. parvum* hsp70 gene were used, which resulted in a 300-bp product. The primer sequences were as follows: forward primer 5'AGTGACAAGAATAACAATACAGG3' and reverse primer 5'CCTGCTTTAAGC ACTCTAATTT3' [23]. PCR was performed with a SimpliAmp model thermo-cycler. The 20 µl PCR mixtures contained 10 µl PCR Mixture, 2 µl reverse primer, 2 µl forward primer, 4 µl Nuclease-Free Water and 2 µl DNA sample. *C. parvum* template DNA was used as PCR positive control, and molecular grade water was used as negative control. The amplification conditions were as follows: initial denaturation at 96˚C for 5 min; 35 cycles of denaturation at 95˚C for 30 s and annealing at 60˚C for 30 s; extension for 30 s at 72˚C and then a final extension at 72˚C for 7 min. Amplification products were separated by horizontal gel electrophoresis in agarose gel (1%) (CSL-Cleaver Scientific) and visualized under UV light. Gel images were captured using a gel documentation system.

2.6. PCR for the Detection of *C. parvum* in Medium of Infected Cultures

Cell culture growth medium was transferred to Eppendorf tube (1.5 ml) and centrifuged for 15 minutes at 6000 × g. Concentrated sporozoites from medium

of infected cultures were suspended in 200 µl buffer (PBS). DNA was extracted and then subjected to PCR as previously described for infected cell culture.

3. Results and Discussion

3.1. Prevalence of *Cryptosporidium* Oocysts, *Giardia* Cysts and Fecal Coliform in Secondary and Tertiary Effluents Disinfected with either Chlorine or UV

Giardia cysts were present in all secondary effluent samples at an average concentration of 213.8 ± 44.0 cysts/10L, while average concentration of *Cryptosporidium* oocysts was lower compared to *Giardia* cysts at an average concentration of 6.9 ± 4.0 oocysts/10L. Gravitational filtration was found to be more efficient compared to rapid sand filtration in the removal of *Giardia* cysts: 1.2 ± 0.7 cyst/10L and 45.7 ± 29.3 cysts/10L, respectively. Since *Cryptosporidium* oocyst levels in secondary effluent were very low, the observed difference observed between their removal efficiency by gravitational filtration and rapid sand filtration are negligible. Fecal coliform detected levels in effluents treated by rapid sand filtration and UV disinfection were significantly higher compared to those subjected to gravitational filtration and chlorine (Table 1). These results indicate that the majority of analyzed samples, treated by means of filtration and chlorine disinfection were within the limit of guidelines of effluents used for unrestricted irrigation. On the other hand, the levels of FC in effluents treated by filtration and UV were higher and exceeded the standards for unrestricted irrigation. It is worth noting that the turbidity levels of effluents treated by gravitational filtration or high rate filtration were comparable and averaged 1.7 ± 0.3 NTU, indicating that turbidity removal may be correlated with *Cryptosporidium* oocysts removal but not to *Giardia* cysts and fecal coliform. Previous studies had shown that wastewater secondary treatment based on activated sludge, did not reduce *Cryptosporidium* oocysts levels nor its infectivity; hence this study relied on

Table 1. Prevalence of *Cryptosporidium* oocysts, *Giardia* cysts and fecal coliform in secondary and tertiary effluents disinfected with chlorine or UV irradiation.

Sampling site	Microorganism concentration	Maximum	Minimum	Avg ± SD
Secondary effluent	*Giardia*/10L	299	180.5	213.8 ± 44.0
	Cryptosporidium/10L	13	2	6.9 ± 4.0
	Fecal coliform (cfu/100mL)	5.8×10^5	4.7×10^4	$1.8 \times 10^5 \pm 5.4 \times 10^4$
Tertiary effluent (UV)	*Giardia*/10L	85.8	7.2	45.7 ± 29.3
	Cryptosporidium/10L	5.6	0.2	1.2 ± 0.2
	Fecal coliform (cfu/100mL)	1.8×10^4	40	$4 \times 10^3 \pm 6.5 \times 10^3$
Tertiary effluent (Chlorine)	*Giardia*/10L	2.4	0.2	1.2 ± 0.7
	Cryptosporidium/10L	9.2	0.0	2.5 ± 0.4
	Fecal coliform (cfu/100mL)	5.7×10^2	0.0	52 ± 133

established data it was mainly designed to determine *Cryptosporidium* infectivity reduction by advanced treatment processes of wastewater effluents [12] [24].

3.2. Factors Affecting Detection Effectiveness of Infectious *Cryptosporidium* from Wastewater Effluents

The effect of secondary effluent on the detection of infectious *Cryptosporidium* in tissue culture was compared with their detection in deionized water. *Cryptosporidium* oocysts were seeded simultaneously in secondary effluent and in deionized water. Following excystation enhancement process, oocysts were inoculated onto HCT-8 cells. Twenty-five infectious zones were detected from seeded deionized water samples, while only 10 infectious zones were detected in seeded secondary effluent. These results indicate that secondary effluent constituents reduce infection efficiency of *Cryptosporidium* in HCT-8 cell culture. The effect of organic matter concentration coupled with oocysts centrifugation process on infection efficiency was compared to *Cryptosporidium* oocysts concentrated and purified by IMS method. Oocysts purified by IMS method revealed 20 infectious zones while after simple pelleting (containing higher organic matter) only one infectious zone was detected. Finally, in order to determine the highest efficient solution that can successfully release attached oocysts from the immune magnetic beads, Hank's Balanced Salt Solution Buffer (pH = 2) (HBSS) was compared to HCl (0.1N solution). Application of HBSS resulted in 15 infectious zones, while using HCl (0.1N) 55 infectious zones were detected.

3.3. Prevalence and Infectivity of *Cryptosporidium* Oocysts in Secondary Effluent Measured by IF Staining or CC-PCR

Cryptosporidium oocysts were detected in 7 out of 7 (100%) secondary effluent samples by IFA at an average concentration of 6.8 ± 4.4 oocysts/10L. Furthermore, infectious oocysts were detected by cell culture in 7 out of 7 (100%) secondary effluent samples at an average concentration of 1.7 ± 0.7 foci/10L. However, with cell culture PCR method (CC-PCR), only 2 out of 7 (28.6%) samples were found positive indicating that infection detection by cell culture and IFA is far more sensitive compared to CC-PCR (**Table 2**). PCR method was applied to detect *Cryptosporidium* in infected HCT-8 cells [18] [25]. Although these methods are highly specific, sensitive and able to screen a large number of samples, PCR based detection had been reported to yield false positive results from mock infections [26]. These authors have also demonstrated IFA superiority over PCR in detecting *Cryptosporidium* infectivity based on qualitative and quantitative measures [26]. These observations are compatible with results obtained in same study concerning comparison between IFA and PCR for the detection of infectious *Cryptosporidium* originating from secondary effluent (**Table 2**).

3.4. Prevalence of Infectious *Cryptosporidium* in Tertiary Effluent Disinfected with Either Chlorine or UV Irradiation

Even though *Cryptosporidium* levels in secondary and tertiary effluent were

Table 2. Prevalence of *Cryptosporidium* in secondary effluent as measured by IF and infectivity determined by IF staining or CC-PCR.

Date	Sampling site	*Cryptosporidium* concentration IF oocysts/10L	*Cryptosporidium* infectious foci IF/10L	*Cryptosporidium* positive by CC-PCR
13.02.17	SE	1.5	2	0
06.03.17	SE	4.5	1	0
03.04.17	SE	4.5	2	0
29.05.17	SE	8.5	2	0
28.06.17	SE	13.0	2	+
24.07.17	SE	3.5	2.5	+
07.08.17	SE	12.0	0.5	0
Prevalence %		100	100	28.6
Avg		6.8	1.7	
STD		4.4	0.7	

relatively low, infectious *Cryptosporidium* foci were detected in 5 out of 7 (71.4%) samples of tertiary effluent samples disinfected by chlorine while no infectious *Cryptosporidium* foci was detected in UV disinfected effluent samples (Table 3).

Along the train of multi-barrier wastewater treatment, disinfection is considered an important barrier for the reduction of pathogens levels in order to avoid their environmental transmission through wastewater effluents reuse or discharge to receiving water bodies. For decades, chlorination was applied in water and wastewater disinfection, as the final step in multi-barrier train of water treatment plants. The presented results, clearly demonstrate that FC, the conventional microbial water quality indicator is sensitive to chlorine applied for effluent disinfection. FC concentrations were below the standard for unrestricted irrigation (<10 cfu/100ml) when chlorine had been applied. Nevertheless, infectious *Cryptosporidium* foci were detected in 5 out of 7 effluent samples disinfected by chlorine (Table 3). The present results are in agreement with previously reported data concerning the resistance of *Cryptosporidium* oocysts to chlorine disinfection. Hirata *et al.* 2001 reported that CT (C = the concentration of chlorine X multiplied by T = exposure time) in the range of 800 to 900 mg min/L is needed to yield one log reduction in *Cryptosporidium* infectivity; and further estimation that a CT of 2700 mg·min/L will be needed for a 3 log reduction of *Cryptosporidium* oocysts infectivity in animals [27]. High resistance of *Cryptosporidium* to free chlorine and monochloramine was already demonstrated by Rennecker *et al.* (2000), who reported a CT of approximately 2000 mg min/L and 12,000 mg min/L will be required to reach an inactivation of 99.9% of *Cryptosporidium parvum* by chlorine and monochloramine, respectively [28].

Results of the present study demonstrated that UV irradiation is highly

Table 3. Prevalence of infective *Cryptosporidium* in tertiary effluent disinfected with either chlorine or by UV irradiation (measured by IFA and infectivity-determined by foci or CC-PCR).

Treatment process	IF *Cryptosporidium* concentration oocysts/10L (average)	Foci *Cryptosporidium* concentration oocysts/10L (average)	*Cryptosporidium* Positive by CC-PCR (%)
Secondary effluent	6.9 (100%)	1.6 (100%)	28.6
Tertiary effluent (UV)	1.2(100%)	0.0	0.0
Tertiary effluent (chlorine)	2.5 (45.5%)	1.4 (70%)	28.6

efficient in the reduction of infectious *Cryptosporidium* oocysts present in filtered effluents. These results are compatible with previously reported data [29] [30] [31]. Different studies demonstrated the effectiveness of low UV irradiation doses to inactivate *Cryptosporidium* oocysts in various water environments. For example, Morita *et al.* (2002) showed that a UV dose of 1.0 mWs/cm^2 at 20˚C was needed to yield a 2-log10 reduction in oocysts infectivity (99% inactivation). Following fluorescent-light irradiation treatment, no infectivity was observed after exposure to or storage in darkness, indicating that the effect of UV irradiation on *Cryptosporidium* oocysts (determined by animal infectivity) can be conclusively considered irreversible [30].

4. Conclusions

1) *Cryptosporidium* and *Giardia* parasites were constantly present in secondary effluent indicating that limited reduction of these parasites is accomplished by activated sludge treatment.

2) Regular chlorine levels applied to effluents were sufficient to produce adequate quality effluents for unrestricted irrigation according to the guidelines of water reuse based on FC indicator levels; however, infectious *Cryptosporidium* was still present in the majority of effluent samples.

3) Full-scale UV irradiation step was found to be highly efficient in reduction of infectious *Cryptosporidium* after effluents filtration, resulting in no detectable infectious *Cryptosporidium*.

4) It may be suggested that advanced effluent treatment train may entail beside chlorine also UV irradiation as a residual disinfectant to preserve microbial quality in unrestricted irrigation effluents.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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