Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(5): 1347 – 1357 (2024)
www.ejabf.journals.ekb.eg

Parasitological Assessment of Wastewater Reuse According to Egyptian Guidelines

Effat A. Morsy*, Ahmad Z. Al-Herrawy

Environmental Parasitology Laboratory, Water Pollution Research Department, National Research Centre, P.O. 12622, Dokki-Giza, Egypt

***Corresponding Author: effatali79@gmail.com**

ARTICLE INFO

Article History:
Received: Feb. 11, 2024
Accepted: Sept. 28, 2024
Online: Oct. 5, 2024

Keywords:
Nematode eggs,
Cryptosporidium,
Giardia,
Treated wastewater,
Guidelines,
Effluent

ABSTRACT

Twenty-four treated municipal wastewater samples were collected during one-year study period and examined for the presence of helminthes eggs, *Cryptosporidium* oocysts, and *Giardia* cysts to compare between their occurrences. Modified Bailenger method was used in this study to detect and enumerate nematode eggs in the treated wastewater samples, while polymerase chain reaction (PCR) technique was used to detect *Cryptosporidium* and *Giardia* in the same treated wastewater samples. Results revealed that *Ascaris* eggs were detected in only 8.33% out of 24 treated wastewater samples, while *Cryptosporidium* oocysts and *Giardia* cysts were detected in 20.83 and 12.5% out of the same 24 treated wastewater samples, respectively. The public's health is at risk since treated wastewater used for agriculture still contains *Giardia* cysts and *Cryptosporidium* oocysts, even if it satisfies the nematode criteria.

INTRODUCTION

Wastewater discharges are worldwide risk factors for the introduction of human pathogens into surface water used as drinking and recreational resources. Microbial pathogens, which can be potentially present in wastewater, are divided into three separate groups: viruses, bacteria, and parasites **(Dufour *et al.*, 2003)**. Pathogenic protozoa are more prevalent in wastewater than any other environmental source. These parasites are also characterized by their zoonotic transmission, low infective dose, and resistance in the environment. *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* are three of the major causes of parasitic induced diarrhea disease and the most common cause of infection worldwide **(Risebro *et al.*, 2007)**. Nematodes are common intestinal parasites that are usually transmitted by fecal route in humans **(Feenstra *et al.*, 2000)**.

Wastewater treatment is the most important way of ensuring that sewage is properly handled before discharge into the environment. The wastewater must be treated to remove pollutants such as organic matter and pathogens **(Robertson *et al.*, 2006)**. The

current World Health Organization and Egyptian guidelines for the microbiological quality of treated wastewater used for crop irrigation identify a standard of an arithmetic mean of ≤ 1 intestinal nematode egg per liter **(Blumenthal *et al*., 2000)**. However, **Grimason *et al*. (1996)** suggested that the removal of nematode eggs may not be a reliable indicator of the removal of *Giardia* cysts and *Cryptosporidium* oocysts in waste stabilization pond systems. Parasitic protozoans should be examined in treated wastewater intended to be used for agricultural purposes.

Therefore, the current study was carried out to investigate the presence of intestinal nematode eggs and protozoan parasites in treated wastewater as well as to suggest other protozoan parasitic stages to be used alongside nematode eggs in determining whether treated wastewater should be reused in agriculture.

MATERIALS AND METHODS

Treated wastewater samples were collected for one year from Zenin wastewater treatment plant, Giza, Egypt. Treated wastewater samples [8 liters volume each (4 liters for the detection of nematode eggs by using the modified Bailenger method, 2 liters for the detection of *Cryptosporidium* by using both Acid-fast trichrome (AFT) stain and PCR technique, and 2 liters for the detection of *Giardia* by using both Lugol's iodine stain and PCR technique)] were collected every two weeks in clean plastic containers and transported to the Parasitology Laboratory, Water Pollution Research Department, National Research Center, Giza, Egypt. The collected wastewater samples were subjected to parasitological analysis to concentrate and identify these parasites.

Detection of nematode eggs in treated wastewater samples

Nematode eggs analysis was performed using the modified Bailenger method applied to wastewater **(Ayres & Mara, 1996)**. Briefly, the collected treated wastewater samples (4 liters volume each (V)) were separately dispensed in glass containers and allowed to settle by gravity for one and a half hour. After sedimentation, ninety percent of the supernatant was removed. The recovered sediment was carefully transferred to tubes and centrifuged for 15min at 1000g. The deposited sediments were combined, transferred into one tube, and then centrifuged for another 15min at 1000g. After that, the obtained pellet was suspended in an equal volume of acetoacetic buffer (pH 4.5). Two volumes of ethyl acetate solution were added to the suspended pellet, and mixed in a vortex mixer before being centrifuged again at 1000g for 15min. After centrifugation, the contents of the tube were separated into three layers: the bottom layer contained all non-fatty heavier debris (including helminth eggs), the middle layer consisted of a clear buffer, and the top layer formed a thick dark plug of fatty and other materials in ethyl acetate. The volume of the pellet in the bottom layer, which may contain helminth eggs, was recorded, and the supernatant was poured off in one smooth motion. The determined pellet from each sample was then re-suspended in five volumes of zinc sulfate solution (ZnSO4, specific

gravity 1.3, density = 33%), and mixed thoroughly. Finally, the resulting solution (X mL) was transferred to a McMaster slide for further examination. The full McMaster slide was left to stand on a flat surface for 10min before the examination; this allows all eggs to float to the surface. The McMaster slide was microscopically examined using 10X magnification of the research microscope. All the nematode eggs seen within the grid in the slide were counted. The number of eggs per liter (N) was calculated using the following equation: $N = AX / PV$, where N = number of eggs per liter of the sample, A = number of eggs counted in the McMaster slide, X = volume of the final product (ml), P = volume of the McMaster slide (ml), and V = original sample volume (liters).

Detection of *Cryptosporidium* oocysts and *Giardia* cysts in treated wastewater samples

The collected treated wastewater samples (4 liters volume each) was vigorously shaked and divided into four equal parts (1- liter volume each) that were separately subjected to the following steps: membrane filtration [through a nitrocellulose membrane (1.2 µm pore size, 142 mm diameter, Millipore Crop., USA)], elution, and flotation [by a Percoll – sucrose gradient]. The final pellet of the 1st part was subjected to AFT stain to detect *Cryptosporidium* oocysts by the light microscope, while the 2nd part of the sample was subjected to Lugol's iodine stain to detect *Giardia* cysts. The final pellets of the 3rd and 4th parts were preserved at -20°C to be used later on for semi-nested PCR technique for both *Cryptosporidium* and *Giardia*.

Identification of *Cryptosporidium* and *Giardia* DNA by using PCR technique

For *Cryptosporidium*, the pellet of the 3rd part of each sample was subjected to DNA extraction according to the method of **Nichols *et al*. (2006)**. Briefly, 15 consecutive cycles of freezing in liquid nitrogen for 1min and thawing at 65°C for 1min were employed, with vortex for 30sec every 5 cycles. Samples were digested with proteinase K (200µg/ ml) at 55°C for 3hr, incubated at 90°C for 2min to inactivate proteinase K, and cooled on ice for 1min before being centrifuged at 14,000g for 5min. Supernatants were transferred to clean tubes and stored at -20°C until used in PCR reactions. For *Giardia*, the pellet of the 4th part was subjected to DNA extraction according to the method of **Nikaeen *et al*. (2003)**. This method involved six cycles of freezing and thawing, alternating between liquid nitrogen for 60 seconds and a 65°C water bath for 60 seconds.

Amplification of extracted DNA

Cryptosporidium DNA obtained from the collected treated wastewater samples was amplified by using PCR technique according to the method of **Hashimoto *et al*. (2006)**. This step was done to amplify a restricted fragment of DNA through specific primers and other additives that led to the continuity of the reaction. *Giardia* DNA obtained from treated wastewater samples was amplified by using PCR technique

according to the method of **Castro-Hermida *et al*. (2009)**. This step was done to amplify a restricted fragment of DNA through specific primers and other additives that led to the continuity of the reaction.

The primers used for PCR amplification of *Cryptosporidium* spp. and *Giardia* spp. DNA were first compared with published sequences on GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Nucleotide BLAST application. The results showed 100% homology with the sequences of the respective genera. The PCR assay primers and their target gene fragments used for detecting *Cryptosporidium* spp. and *Giardia* spp. are displayed in Tables (1, 2), respectively.

Table 1. Primer sets for detection of *Cryptosporidium* spp. **(Hashimoto *et al*., 2006)**

PCR	Primer	Sequence	Length (bp)
Initial	CPB-DiagR CPB-DIAGF1	TAAGGTGCTGAAGGAGTAAGG GCTCGTAGTTGGATTTCTGTTAA	435
Semi-nested	CPB-DIAGR1 CPB-DIAGF1	CCAATCTCTAGTTGGCATAG GCTCGTAGTTGGATTTCTGTTAA	400

Table 2. Primer sets for detection of *Giardia* spp. **(Castro-Hermida *et al*., 2009)**

PCR	Primer	Sequence	Length (bp)
Initial	G7 G759	AAGCCCGACGACCTCACCCGCAGTGC GAGGCCGCCCTGGATCTTCGAGACGAC	753
Semi-nested	G376 G759	CATAACGACGC CATCGCGGCTCTCAGGAA GAGGCCGCCCTGGATCTTCGAGACGAC	384

PCR mixture consisted of 2.5µl Taq buffer, 1µl of 200µM deoxynucleoside triphosphate (dNTPs), 1.25U Taq polymerase, 1µl of 200nM (each) primers, and 5µl of extracted DNA, as a template for the first step, and 2.5µl of primary PCR product for the second step. The final volume of the PCR reaction was adjusted to 25µl by the addition of DEPC- treated water. In all cases, for each reaction, a negative and a positive control were added. Temperature conditions for *Cryptosporidium* spp. and *Giardia* spp. DNA amplification are shown in Tables (3, 4), respectively. In both amplifications, samples were incubated in a PCR thermal cycler apparatus.

Table 3. Temperature conditions for *Cryptosporidium* spp. DNA amplification.

Pre-denaturation	Cycles	Final extension	References
94 °C for 5 min	40 cycles: 94 °C for 60 sec. 54 °C for 30 sec. 72 °C for 30 sec.	72°C for 7 min	Hashimoto *et al*., 2006

Table 4. Temperature conditions for *Giardia* spp. DNA amplification

Pre-denaturation	Cycles	Final extension	References
94 °C for 5 min	40 cycles: 94 °C for 30 sec. 55°C for 30 sec. 72 °C for 60 sec.	72°C for 7 min	Castro-Hermida *et al*., 2009

Agarose gel electrophoresis

For both *Cryptosporidium* and *Giardia* DNA, electrophoresis on agarose gel was performed to separate DNA fragments, as described by **Helling *et al*. (1974)**. Eight micro-liters of PCR product were mixed with 2µl of loading dye and analyzed by electrophoresis using 2% (w/v) agarose gel containing 0.5µg/ µl ethidium bromide. The PCR product was compared with a 100 bp DNA ladder and run at 100 volts for 30 minutes, then visualized under ultraviolet (UV) light. The gel was subsequently photographed under UV transillumination.

RESULTS AND DISCUSSION

Examination of 24 collected treated wastewater samples revealed that *Ascaris* eggs were detected in only 2 samples, while *Cryptosporidium* oocysts and *Giardia* cysts were detected in 5 and 3 samples, respectively. The five treated wastewater samples were microscopically positive for *Cryptosporidium* oocysts and also yielded positive results in the initial PCR (Fig. 1). When the initial PCR products from these positive samples were used as templates for the semi-nested PCR technique, the same positive results were obtained (Fig. 2).

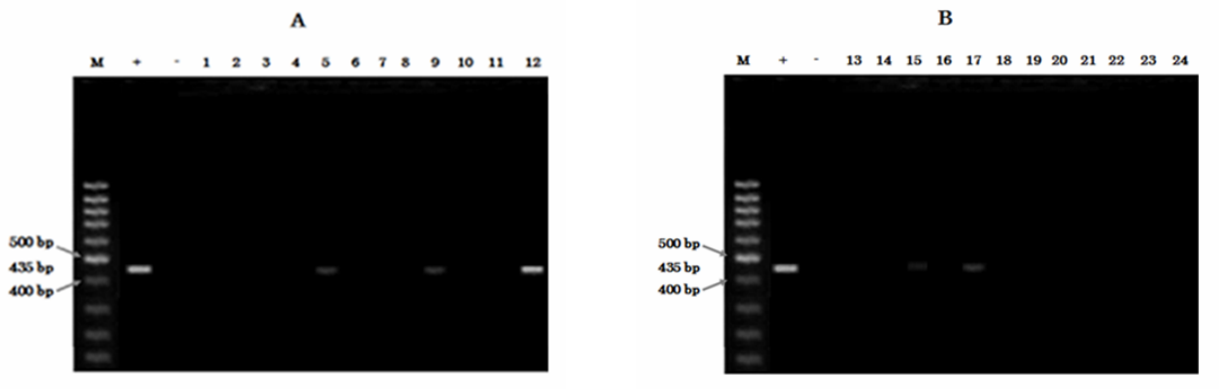

Fig. 1. Ethidium bromide stained 2% agarose showing initial *Cryptosporidium* PCR amplified product of DNA extracted from the collected treated wastewater samples, the specific band length is 435bp. **(A):** Treated wastewater samples from 1 to 12, **(B):** Treated wastewater samples from 13 to 24, M: 100bp DNA ladder, +: positive control, and –: negative control

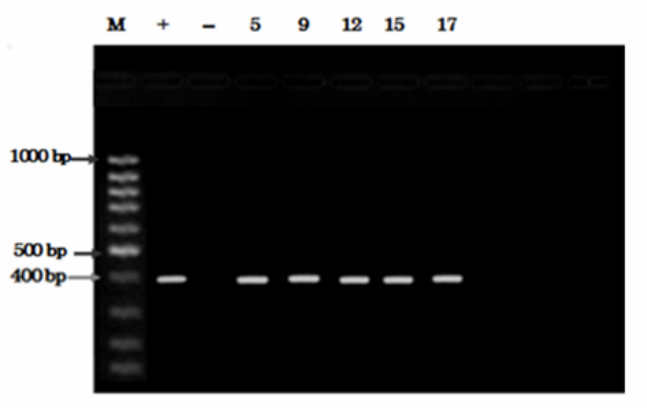

Fig. 2. Ethidium bromide stained 2% agarose showing *Cryptosporidium* semi-nested PCR amplified product of DNA extracted from positive treated wastewater samples, the specific band length is 400bp, M: 100bp DNA ladder, +: positive control, and –: negative control

By using the initial PCR technique, genomic *Giardia* DNA was detected in only one treated wastewater sample having 6 *Giardia* cysts per liter. The other two samples which were microscopically positive samples were found to have only one *Giardia* cyst per liter (Fig. 3). When the initial PCR product of that positive sample was used as a template for the semi-nested PCR technique, the same positive result was obtained (Fig. 4).

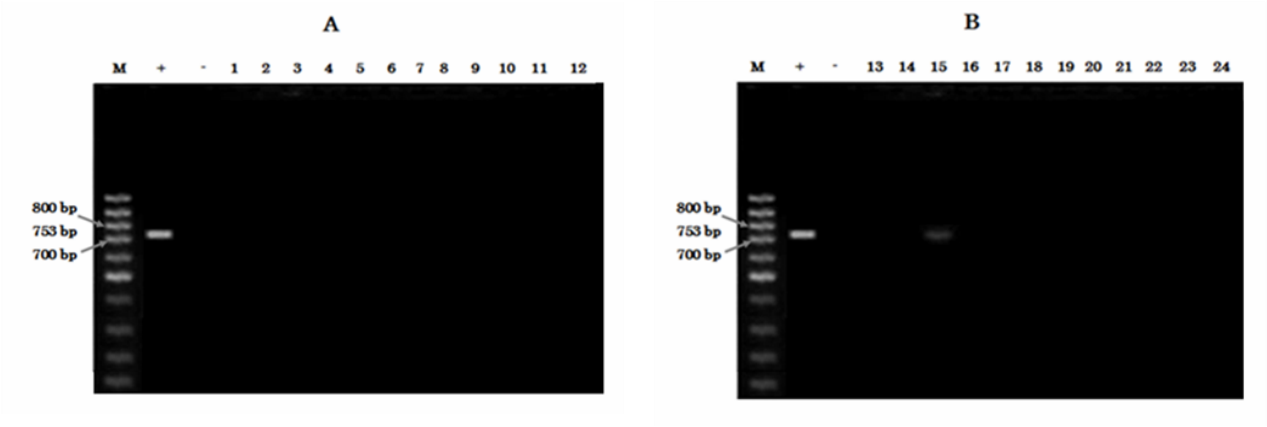

Fig. 3. Ethidium bromide stained 2% agarose showing initial *Giardia* PCR amplified product of DNA extracted from the collected treated wastewater samples, the specific band length is 753bp. (A): Treated wastewater samples from 1 to 12, (B): Treated wastewater samples from 13 to 24, M: 100bp DNA ladder, +: positive control, and –: negative control

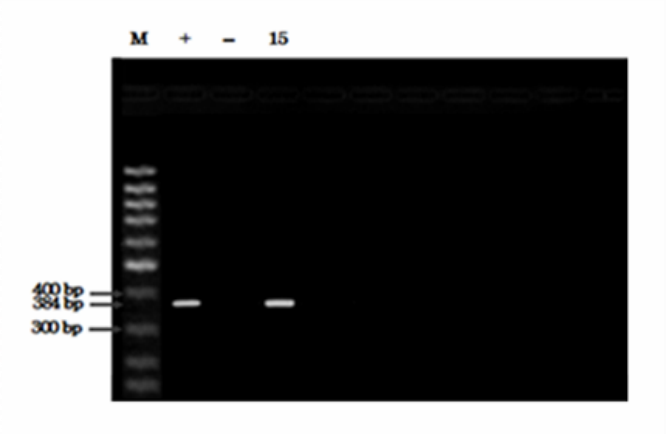

Fig. 4. Ethidium bromide stained 2% agarose showing *Giardia* semi-nested PCR amplified product of DNA extracted from the positive treated wastewater samples, the specific band length is 384bp, M: 100 bp DNA ladder, +: positive control, and –: negative control.

These results were following those of a Tunisian study conducted by **Khouja *et al.* (2010)**, who assessed the effectiveness of 6 wastewater treatment plants (using different processes) for the removal of parasites. They used the modified Bailenger method, immunomagnetic separation followed by immunofluorescent assay microscopy, and PCR technique for the detection of parasitic stages in wastewater samples. *Ascaris* eggs were detected in 1 out of 8 treated wastewater samples; *Giardia* cysts were detected

in 4 out of 8 treated wastewater samples, and *Cryptosporidium* oocysts were detected in 1 out of the same 8 treated wastewater samples.

Another Tunisian study was carried out by **Ben Ayed *et al*. (2009)** for the enumeration of *Ascaris* eggs and *Giardia* cysts in treated wastewater samples to provide quantitative data for developing regulations for wastewater quality that are currently lacking. Treated wastewater samples were collected from 17 plants in Tunisia during 2006–2007 and analyzed for parasites using the modified Bailenger method. *Ascaris* eggs were detected in 7 out of 14 treated wastewater samples, while *Giardia* cysts were detected in 13 out of 15 treated wastewater samples.

On the other hand, an Egyptian study was conducted by **Ashmawy *et al*. (2005)**, who assessed the sludge treatment processes applied at the 6th October wastewater treatment plant in Giza. The parasitological evaluation showed that the system was able to remove 100% of *Ascaris lumbricoides* eggs and *Giardia* cysts. While only 69.3% of *Cryptosporidium* oocysts were removed; these results agree with that of the present investigation.

Ramo *et al*. (2017) investigated the presence and removal efficiency of *Cryptosporidium* and *Giardia* in wastewater treatment plants at the 20 most populated towns in Aragón (North-eastern Spain). Samples of the influent and effluent wastewater were seasonally collected from 23 plants and processed according to USEPA method 1623. *Cryptosporidium* oocysts were identified in most samples from both raw (85/92) and treated (78/92) wastewater samples.

In another study, influent and effluent wastewater samples from three wastewater treatment plants in Sweden were collected over nearly one year and assessed for the prevalence of parasitic protozoa. Results revealed that the mean removal efficiencies of *Giardia intestinalis*, *Entamoeba dispar*, and *Dientamoeba fragilis* varied between 67-87, 37-75, and 20-34%, respectively **(Berglund *et al*., 2017)**.

In the south-east of Algiers, **Hamaidi-Chergui *et al*. (2019)** determined the efficiency of Médéa wastewater treatment plant (conventional activated sludge) in the removal of protozoan cysts (*Giardia intestinalis*, *Entamoeba coli*, and *Entamoeba histolytica*) and parasitic eggs (*Ascaris*, *Trichuris*, *Hymenolepis*, and *Toxocara*). This study was carried out during four months, and samples were collected at weekly intervals from influent and effluent of the wastewater plant. Samples were analyzed according to the modified Bailenger method. The wastewater treatment plant of Médéa has removed 88.9 -100% of parasitic eggs and about 95% of protozoan cysts.

CONCLUSION

It can be concluded from the present findings that the removal of helminth eggs from treated wastewater does not reliably indicate the removal of other protozoan parasitic

stages. Thus, to assess the suitability of treated wastewater for agricultural use, it is essential to evaluate both intestinal helminth eggs and protozoan parasitic stages.

REFERENCES

Ashmawy, M.A.; Al-Herrawy, A.Z.; Kamel, M.M. and El-Senousy, W.M. (2005). Microbiological assessment of sludge treatment at the 6th October wastewater treatment plant, Giza, Egypt. Egypt. J. Microbiol., 40: 81-93.

Ayres, R. M. and Mara, D. (1996). Analysis of wastewater for use in agriculture - a laboratory manual of parasitological and bacteriological techniques. World Health Organization, Geneva.

Ben Ayed, L.; Schijven, J.; Alouini, Z.; Jemli, M. and Sabbahi, S. (2009). Presence of parasitic protozoa and helminth in sewage and efficiency of sewage treatment in Tunisia. Parasitol. Res. 105(2), 393–406. https://doi.org/10.1007/s00436-009-1396-y

Berglund, B.; Dienus, O.; Sokolova, E.; Berglind, E.; Matussek, A.; Pettersson, T. and Lindgren, P. E. (2017). Occurrence and removal efficiency of parasitic protozoa in Swedish wastewater treatment plants. Sci. Tot. Environ. 598, 821–827. https://doi.org/10.1016/j.scitotenv.2017.04.015

Blumenthal, U. J.; Duncan Mara, D.; Peasey, A.; Ruiz-Palacios, G. and Stott, R. (2000). Guidelines for the microbiological quality of treated wastewater used in agriculture: recommendations for revising WHO guidelines.

Castro-Hermida, J. A.; García-Presedo, I.; Almeida, A.; González-Warleta, M.; Correia Da Costa, J. M. and Mezo, M. (2009). Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in surface water: A health risk for humans and animals. Wat. Res. 43(17), 4133–4142. https://doi.org/10.1016/j.watres.2009.06.020

Dufour, A.; Snozzi, M.; Koster, W.; Bartram, J.; Ronchi, E.; Fewtrell, L.; Bailey, K.; Chilton, J.; Dahi, E.; Lennon, M.; Jackson, P.; Fawell, J.; Bartram, J.; Cotruvo, J.; Exner, M.; Fricker, C.; Glasmacher, A. and Cotruvo, J. (2003). Pathogenic Mycobacteria in Water: A Guide to Public Health Consequences, Monitoring and Management. www.iwapublishing.com

Feenstra, S.; Hussain, R. and van der Hoek, W. (2000). Health risks of irrigation with untreated urban wastewater in the southern Punjab, Pakistan. Institute of Public Health, Lahore Pakistan Program International Water Management Institute, Lahore.

Grimason, A.M.; Wiandt, S.; Baleux, B.; Bontoux, W.N. and Smith, H.V. (1996). Occurrence and removal of *Giardia* sp. cysts by Kenyan and French waste stabilization pond systems. Wat. Sci. Technol. 33, 81-89.

Hamaidi-Chergui, F.; Errahmani, M. B. and Ouahchia, C. (2019). Occurrence and removal of protozoan cysts and helminth eggs in the Médéa sewage treatment plant (south-east of Algiers). Ann. Parasitol. 65(2), 139–144. https://doi.org/10.17420/ap6502.193.

Hashimoto, A.; Sugimoto, H.; Morita, S. and Hirata, T. (2006). Genotyping of single *Cryptosporidium* oocysts in sewage by semi-nested PCR and direct sequencing. Wat. Res. 40(13), 2527–2532. https://doi.org/10.1016/j.watres.2006.04.038

Helling, R. B.; Goodman, H. M. and Boyer, H. W. (1974). Analysis of R. EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. 14, 1235-38. American Society for Microbiology. http://jvi.asm.org/

Idrissi, Y. A.; Mansouri, D.; El Yemli, A.; Kherrati1, I.; Berrid, N.; Ghachoui, E.; Hussein, A. K.; El Kharrim, K. and Belghyti1, D. (2020). Parasitological assessment of the purifying performance of the wastewater station by natural lagoon of Azilal, Morocco. Egyptian Journal of Aquatic Biology & Fisheries, 24(3): 299 – 309

Khouja, L. B. A.; Cama, V. and Xiao, L. (2010). Parasitic contamination in wastewater and sludge samples in Tunisia using three different detection techniques. Parasitol. Res. 107(1), 109–116. https://doi.org/10.1007/s00436-010-1844-8

Nichols, R.A.B.; Moore, J.E. and Smith, H.V. (2006). A rapid method for extracting oocyst DNA from Cryptosporidium-positive human faeces for outbreak investigations. *J. Microbiol. Meth.* 65: 512-524.

Nikaeen, M.; Mesdaghinia, A.R.; Tehrani, M.J.; Rezaian, M. and Vaezi, F. (2003). Sensitive detection of *Giardia* cysts by polymerase chain reaction (PCR). Iranian J. Publ. Health. 32(1),15-18. http://journals.tums.ac.ir/

Ramo, A.; del Cacho, E.; Sánchez-Acedo, C. and Quílez, J. (2017). Occurrence and genetic diversity of *Cryptosporidium* and *Giardia* in urban wastewater treatment plants in north-eastern Spain. Sci. Tot. Environ. 598, 628–638. https://doi.org/10.1016/j.scitotenv.2017.04.097

Risebro, H. L.; Doria, M. F.; Andersson, Y.; Medema, G.; Osborn, K.; Schlosser, O. and Hunter, P. R. (2007). Fault tree analysis of the causes of waterborne outbreaks. J. Wat. Health. 5(1), 1–18. https://doi.org/10.2166/wh.2006.002

Robertson, L.J.; Hermansen, L.; Gjerde, B.K.; Strand, E.; Alvsvåg, J.O. and Langeland, N. (2006). Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl. Environ. Microbiol. *72*(3), 2212–2217. https://doi.org/10.1128/AEM.72.3.2212-2217.2006