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Destruction of helminth eggs (*Ascaris suum*) by ozone: second stage

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Abstract With the purpose of destroying helminth eggs, laboratory tests were carried out using a batch reactor of 1 L with 500 mL of eggs in suspension (1×10^5 eggs/L). In all the tests, a 36.8 mg O_3/L concentration of gas-phase ozone was applied at the bottom of the reactor, with flows of 0.25 and 0.5 L/min to achieve rates of 9.2 and 18.4 mg O_3/min, respectively. The results (first stage) showed that the eggs of *Ascaris suum* were destroyed at a pH of less than 5, with a dose of 18.4 mg O_3/min., giving a 94% success rate; these results were improved when applying a pH 3 (second stage) with 18.4 mg O_3/min for an hour, and 96.7% of the eggs showed damage to their external covers. In comparison with other species, total destruction was achieved in 1 hour and 45 minutes at pH 3 for the case of the *Fasciola hepatica* and the *Strongiloides sp*. The destruction of the *Dypilidium caninum* was completed after 3 hours. However, with regard to the *Toxocara canis*, eggs still remained, but they were observed to be seriously damaged. The degree of COD removal in the tests with the *Ascaris suum* was 75% after 2 hours. In the case of the other species, the COD removal was better since it varied from 85–94% after 2 hours of treatment. It can be concluded that during the process of oxidation in an acid environment, ozone hydrolyzes the amino acids that characterize the proteins of the layers covering the egg, and at the same time it breaks the cover of the egg and disperses the cytoplasm of the eggs into the solution, therefore making it impossible for the eggs to become reactivated.

Keywords Helminth eggs; ozone treatment; pH

Introduction

Some epidemiological studies developed by the World Health Organization (WHO), the Environmental Protection Agency (EPA), and by Latin American countries, have demonstrated that inadequately treated water for re-use purposes constitutes a potential vehicle for the transmission of pathogenic agents (protozoa and helminths) that cause intestinal parasitosis, because these organisms are resistant to disinfection by chlorination as the doses and times of contact usually applied in water disinfection (WHO, 1989; EPA, 1992).

In terms of helminth life cycle, eggs represent the state of development at which dissemination into the environment takes place. When these organisms are found in wastewater they are precisely at the stage of egg dissemination (Rojas and Orta, 2000). In Mexico, the following species of helminth eggs are commonly found in wastewater: *Ascaris lumbricoides*, *Ascaris suum*, *Trichuris trichiura*, *Taenia solium*, *Hymenolepis nana*, *Strongyloides stercoralis*, *Enterobius vermicularis*, *Toxocara canis* and *Dipylidium caninum*; and occasionally, *Fasciola hepatica* (Maya *et al*., 2000). Gastrointestinal infections produced by such eggs rank first in the mortality rate in Mexico (INEGI, 2000). Because of this risk to public health, it is important to destroy such microorganisms (Toze, 1999).

Helminth eggs are highly resistant to dramatic changes in temperature, even ranging from –12°C to 40°C (Brown and Belding, 1985). Not even a dose of 1,000 mg Cl_2/L of chlorine is enough to render them inactive. Eggs are able to reach an embryonic state successfully in 5% formaldehyde solution (Odda and Jiménez-Albarrán, 1987), as well as in

the presence of iodine solution (133 mg I_2/L), alcohol, heavy metals, synthetic detergents, and ammonia compounds. They can also endure transient immersion in solutions of 50% chlorhydric, nitric, acetic, and sulphuric acids (Schmidt and Roberts, 1984). Helminth eggs have been reported to survive for months or years in soil, without losing their virulence, and from several days to years in wastewater and sludge used on agricultural land without losing their noxiousness (Gadomska *et al*., 1991; Ayres *et al*., 1992).

Gadomska *et al*. (1991) applied a 10-mg/L ozone dose, for 120 min., to a tap water sample containing 2×10^7 *Ascaris* eggs, destroying 30.5–54.5% in samples containing around 15% of non-viable eggs. In such reports, the effect of ozone on viable eggs is not clearly explained, and no specifications are made regarding optimum doses, nor time of contact, required for total destruction of the eggs is achieved.

The objective of this paper is to demonstrate that ozone treatment is a viable alternative, with encouraging perspectives for eliminating helminth eggs.

Materials and methods

This research was divided into two stages: first, the effect of ozone on *Ascaris suum* eggs was tested (Orta-De Velásquez *et al*., 2001); and secondly, the optimum conditions obtained from the first stage were applied to other species that are commonly found in wastewater.

In the case of *Ascaris suum*, 40 worms were collected in a slaughterhouse. The other species: *Fasciola hepatica* (Trematode), *Dipylidium caninum* (Cestode), and the nematodes (*Hymenolepis nana*, *Enterobius vernicularis* and *Toxocara canis*), were donated by the Laboratory of Parasitology of the Veterinary School at the National Autonomous University of Mexico.

In all the cases, the worms were washed in a saline solution (0.85%) and preserved in formaldehyde (5%) before dissection. Once detached from organic material, eggs were preserved in a saline solution (0.85%) (stock suspension) at around 4°C. From this stock suspension, 4 L of a new suspension, containing approximately 40 eggs for each 40 µL, was prepared. In order to determine both the initial and final number of eggs, 40 µL suspension samples were taken every hour, before and during the tests. The number of eggs in the samples was counted under a Leitz-brand Laborlux-s optical microscope.

In all the tests, the ozone applied was generated by Emergy Trailigaz Labo 76 equipement. 36.8 mg O_3/L concentration of gas-phase ozone was applied, with flows of 0.25 and 0.5 L/min, to achieve rates of 9.2 and 18.4 mg O_3/min., respectively. Gas-phase ozone concentrations were determined through the iodometric method (Birdsall *et al*., 1952), and dissolved ozone was measured by the indigo method (Bader and Hoigne, 1981).

First stage

In the first stage two initial pH (5 and 9), and two ozone rates (9.2 and 18.4 mg O_3/min.) were evaluated (in eggs in stock suspension 1×10^6 eggs/L). Applied ozone was generated by Emergy Trailigaz Labo 76 equipment, using oxygen-enriched air as the input gas (Orta-De Velásquez *et al*., 2001).

Experiments thus executed followed a 2^K factorial design, for $k = 2$, with each test performed randomly. Statistic analyses of the results made it possible to assess the significance of variables involved in the destruction of the eggs, for selected levels of reliability.

Second stage

Another oxidation test at pH 3 and 18.4 mg O_3/min. was included, to monitor the behaviour of the helminth eggs: *Fasciola hepatica*, *Dipylidium caninum*, *Hymenolepis nana*, *Enterobius vernicularis*, *Ascaris suum* and *Toxocara canis*, in an acid pH. The treatment

system consisted of dosing 500 mL of the egg stock suspension (1×10^5 eggs/L) in a closed glass reactor of 1 L capacity. Ozone was fed into the bottom part of reactor by means of a diffuser with pore diameters ranging from 10–15 μm (Figure 1).

As in the first stage, suspensions of eggs were thus ozoned in a batch reactor for 4 hours. Samples were extracted every 15 minutes for 2 hours, and subsequently every hour, to evaluate the amount of destroyed eggs. Another parameter determined for each test for indirect corroboration of the degree of destruction or oxidation of the helminth eggs, was the reduction of chemical oxygen demand (COD). Three replicates of each test were made in order to evaluate experimental errors.

Results and discussion

Results of the first stage

The following results were determined from the first stage regarding *Ascaris suum* eggs (Orta-de Velásquez *et al.*, 2001).

After a period of 1 hour in the test, with initial pH 5, and with 18.4 mg O_3/min. ozone, around 94% of the viable eggs showed destruction of a portion of their external protein cover, even though at pH 9 some undamaged helminth eggs were still found at the end of the experiment. Additionally, it can be observed that, contrary to the pH 9 tests, in the pH 5 tests the concentration of dissolved ozone reaches equilibrium from 4.6–4.7 mg/L, practically within the first hour of sampling, regardless of the ozone rate (Figure 2).

After two hours of ozone application to the egg suspension the level of COD removal in the tests was 75%. According to the variance analysis, for *F*-distribution and a level of reliability of 0.95, pH appears the most relevant variable in the destruction of helminth eggs.

Results of the second stage

pH is an important factor in the reaction of ozone. At pH 3 after the first hour a removal rate of 96.7% of *Ascaris suum* eggs was achieved, improving the results demonstrated at pH 5 (95%). After 2 hours, total destruction was obtained in both cases (Figure 3); however, at pH 9 total destruction was never achieved. The maximum removal of helminth eggs at pH 3 confirms the influence of acid conditions in an increased reactivity of ozone.

This result coincides with the report prepared by Langlais *et al.* (1991), which states that the reactivity of polypeptides and proteins with ozone depends on the nature of the amino acid constituents as well as on pH, considering that the chemical structure of the proteins

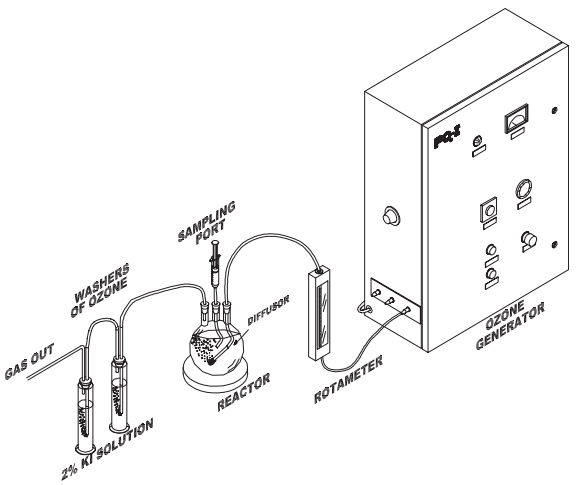

Figure 1 Treatment system with ozone

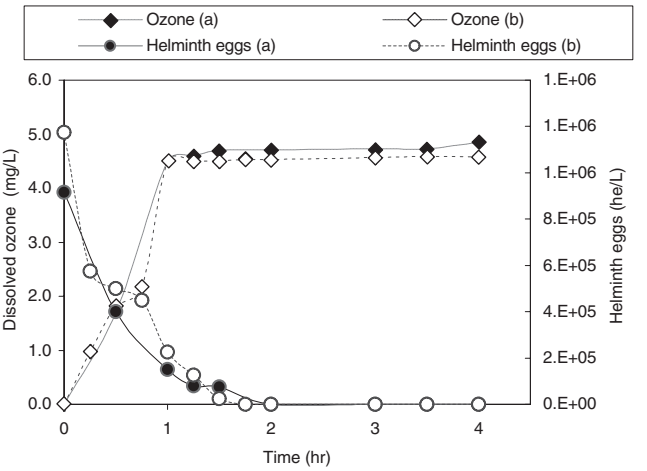

Figure 2 Effect of dissolved ozone concentrations on the destruction of helminth eggs at an initial pH 5. Ozone application rates in a batch reactor: (a) 9.2 mg O_3/min.; (b) 18.4 O_3/min.

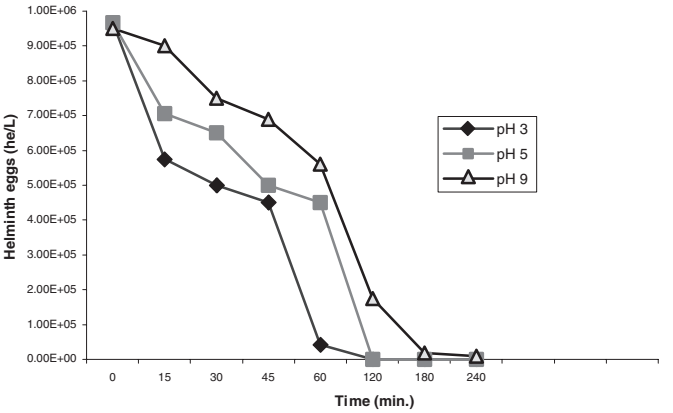

Figure 3 Removal efficiency of helminth eggs at different values of pH and at 18.4 mg O_3/min.

covering helminth eggs contain covalent links of terminal amino acids.

Table 1 and Figures 4 and 5 show the results of COD and of the helminth eggs count after the application of pH 3 and 18.4 mg O_3/min. as a function of the reaction of four species of helminths, in addition to *Ascaris suum*, to the time of ozonation. These species belong to the three major groups of helminths of medical importance detected in wastewater.

As in the case of *Ascaris suum*, the COD (Table 1) and the number of helminth eggs of the different species, decrease with the time of ozone application. In regard to the destruction of helminth eggs (Figure 4), total destruction was registered after 1 hour and 45 minutes in the case of *Fasciola hepatica* as well as in *Strongiloides sp*. In the case of *Dypilidium caninum*, total destruction was achieved after 3 hours. In the case of *Toxocara canis*, even although eggs were still found until the last moment, they were observed to be seriously damaged. According to Janex *et al*. (2000), the efficiency of the disinfection process depends on each microorganism and on the time of contact for their destruction.

With respect to the COD values (Figure 5), better results were obtained in comparison with those of the first stage, because COD removal for the different species tested ranged between 85 and 94% after two hours. This result is confirmed by the diminishing number of eggs after each test.

Table 1 Results of dissolved ozone, COD and helminth egg count at pH 3 and 18.4 mg O_3/min., in terms of ozone application time

Time (min.)	Trematode			Cestode			Nematode								
	Fasiola hepatica (snail–man)			Dypilidium caninum (flea–dog–man)			Ascaris suum (ingestion–pig)			Toxocara canis (dog–man)			Strongiloides sp. (ingestion–man)		
	O_3	COD	He	O_3	COD	He	O_3	COD	He	O_3	COD	He	O_3	COD	He
0	0	364	675,000	0	407	725,000	0	310	775,000	0	359	625,000	0	284	625000
15	1.0	——	550,000	1.3	——	625,000	1.2	——	575,000	1.5	——	475,000	1.9	——	325000
30	1.7	261	125,000	1.5	199	450,000	1.8	275	475,000	2.5	179	375,000	2.4	142	250000
45	2.8	——	75,000	2.0	——	425,000	2.1	——	275,000	2.9	——	400,000	3.2	——	200000
60	4.4	99	25,000	4.4	125	425,000	4.6	112	200,000	3.7	89	300,000	3.6	71	125000
75	4.8	——	25,000	4.4	——	450,000	4.6	——	125,000	4.2	——	200,000	4.7	——	50000
90	4.8	47	25,000	4.4	127	400,000	4.6	60	50,000	4.2	45	225,000	4.7	35	75000
105	4.8	——	0	4.4	——	175,000	4.6	——	25,000	4.2	——	125,000	4.7	——	50000
120	4.9	29	0	4.4	52	75,000	4.6	47	0	4.2	22	50,000	4.7	17	0
180	4.8	14	0	4.4	33	0	4.6	23	0	4.2	11	50,000	4.7	8.9	0
240	4.9	13	0	4.4	33	0	4.6	17	0	4.2	11	25,000	4.7	8.0	0

O_3 = dissolved ozone (mg/L); COD (mg/L); He = helminth eggs (he/L); —— not determined

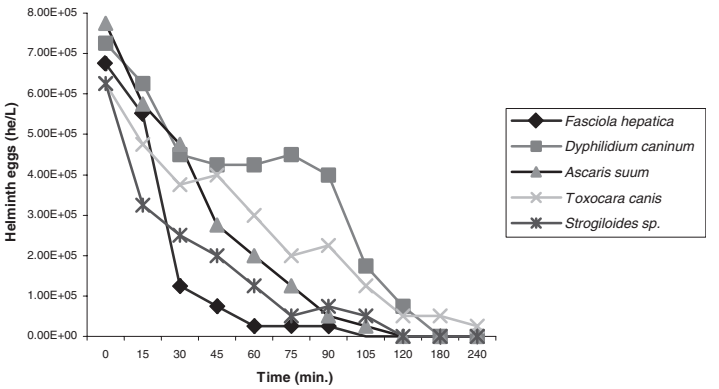

Figure 4 Removal efficiency of five species of helminth eggs at pH 3 and 18.4 mg O_3/min.

All helminth eggs are morphologically similar, they are also of similar size and chemical constitution. The egg shell consists of three basic layers that are secreted by the egg itself: an inner lipid layer, a middle chitinous layer, and an outer vitelline layer. The chemical composition of the layers that cover helminth eggs is *N*-terminal amino acid, *o*-quinone, lysyl, tyrosyl, biphenyl, cysteinyl and cystine (Smyth and McManus, 1989). Their variation mainly depends on the number of amino acids incorporated into the covers, a result that agrees with the similarity of results obtained when ozone is applied under acid conditions.

During the oxidation process, ozone breaks the wall or cover of helminth eggs. The acid medium causes hydrolysis of proteins, with amino acids as terminal products (Elmghari-Tabib, 1981). The biphenyls and the quinones are characterized by the presence of the OH donor group on the aromatic nucleus (Langlais *et al*., 1991). This donor group is strongly reactive to ozone. Doré (1989) reported velocity constants for ozonation of cystein at pH 2, and cystine at pH 1.8, as 3×10^4 and $5{,}5 \times 10^2$ $M^{-1}s^{-1}$, respectively, concluding that sulphur amino acids are highly reactive to ozone. Such observations agree with the results obtained in this research using pH 3.

Conclusions

According to variance analysis for *F*-distribution, and a reliability level of 0.95, pH was found to be the most relevant variable in the destruction of helminth eggs. pH 9 (alkaline)

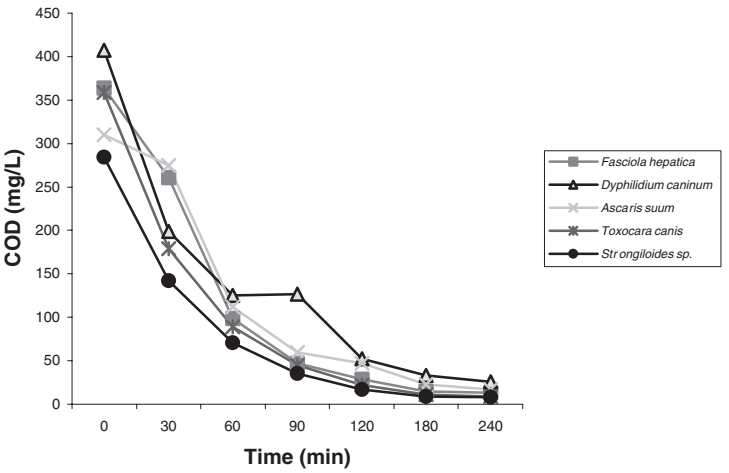

Figure 5 Removal efficiency of COD with a stock solution for five different species of helminth eggs at pH 3 and 18.4 mg O_3/min.

with 18.4 O_3/min. fails to destroy helminth eggs; results are improved with pH 5, however the best results were obtained with pH 3 (acid). These results are evident in the data shown, where total destruction of *Ascaris suum* was observed at pH 3, after 2 hours of ozone application. Results obtained here in an acid environment are consistent with the great reactivity of amino acids formed by the hydrolysis, these aminoacids, being the characteristic protein covering of helminth eggs. With respect to COD, at pH 3 and 18.4 O_3/min. a removal of 85–94% was obtained after 2 hours of treatment, and of 94–97.5% after 4 hours.

The time of contact and the efficiency of the disinfection process varies according to each of the microorganisms. Nevertheless, based on the results obtained in this research it can be concluded that, under the optimum conditions tried in this experiment, most of the species of medical interest commonly found in wastewater (*Fasciola hepatica*, *Strongiloides sp*. *Dypilidium caninum*, *Toxocara canis* and *Ascaris suum*) can be eliminated after a 3 hour contact time with ozone.

Experimental results show that ozone treatment is a viable and promising alternative for the elimination of helminth eggs. These results could become the basis for considering other treatment methods in Mexican legislation, to guarantee the elimination of highly resistant species, in addition to coliform bacteria.

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References

Ayres, R.M., Stott, R., Mara, D.D. and Lee, D.L. (1992). Wastewater reuse in agriculture and the risk of intestinal nematode infection. *Parasitology Today*, **8**(1), 32–35.

Bader, H. and Hoigne, J. (1981). Determination of ozone by the indigo method. *Wat. Res.*, **15**(4), 449.

Birdsall, C., Jenkins, A.C. and Spandinger, E. (1952). Iodometric determination of ozone. *Anal. Chem.*, **24**(4).

Brown, H.W. and Belding, L.D. (1985). *Parasitología Clinical*. Segunda Edición. Editorial Interamericana, S.A., 121–155.

Doré, M. (1989). *Chimie des Oxydants et Traitement des Eaux*. Lavosier, Technique et Documentation, Paris, France, p. 332.

Elmghari-Tabib, M. (1981). *Analyse de la Micropollution Azotée des Eaux en Cours de Potabilisation. Action de l'ozone sur quelques constituants*. These de Docteur, L´Universite de Rennes, France.

Gadomska, K., Maleszewska, J., Krogulska and Wichrowska, B. (1991). Analysis of survival rate of *Ascaris suum* (Goeze, 1782) eggs and of *Escherichia coli* in ozone-disinfected water *Bull. Pol. Acad. Sci.*, *Biol.*, **39**(3), 347–352.

INEGI (2000). Área Metropolitana del la Ciudad de México – Síntesis de Resultados. Instituto Nacional Estadística Geografía y Informática. Censo General de Población y Vivienda 2000. México, D. F.

Janex, M.L., Savoye, P., Roustan, M., Do-Quang, Z. Lainé, J.M. and Lazarova, V. (2000). Wastewater disinfection by ozone: influence of water quality and kinetics modelling. *Ozo. Sci. Eng.*, **22**, 113–121.

Langlais, B., Reckhow, D.A. and Brink, D.R. (1991). *Ozone in Water Treatment: Application and Engineering*. Cooperative Research Report, Lewis Publisher, Inc., USA, p. 43.

Maya, R.C., Salgado, V.G. and Jiménez, C. (2000). Frecuencia y variación estacional de los géneros de huevos de helmintos más comúnmente encontrados en aguas residuales de México. *XII Congreso Nacional Ciencia y Conciencia*, Marzo, No. 1, 704–713.

Minchew, E.P., Gould, J.P. and Saunders, M.F. (1987). Multistage descomposition kinetics of ozone in dilute aqueous solutions. *Ozone Sci. Eng.*, **9**, 165–177.

Odda, R. and Jiménez-Albarran, M. (1987). Nota Parisotológica. Viabilidad de los huevos de Ascaris lumbricoides. *Rev. Ibér. Parasitol.*, **47**(2), 159–160.

Orta-de Velásquez, T., Rojas, V.N., Martínez, J.L. and Monje, R.I. (2001). Destruction of Helminth Eggs (*Ascaris suum*) by Ozone. *Accepted to be presented in oral form in the 15th World Congress*, 10–15 September, London.

Rojas, V.N. and Orta, L.T. (2000). Resistencia de los huevos de helmintos a la desinfección con ozono y luz ultra violeta. *Rev. Tláloc AMH*, Abril–Junio, No. 18, 23–24.

Schmidt, D.G. and Roberts, L.S. (1984). *Fundamentos De parasitología*. Ed. Continental, S. A. de C. V., 439–498.

Smyth, J.D. and McManus, D.P. (1989). *The Physiology and Biochemistry of Cestodes* Cambrige University Press, New York, 156–194.

Toze, S. (1999). PCR and the detection of microbial pathogens in water and wastewater. *Wat. Res.*, **33**(17), pp. 3545–3556.

EPA (1992). *Environmental Regulations and Technology Control of Pathogens and Vector Attraction in Sewage Sludge. Under 40 CFR part 503. Appendix I Analytical Method for Viable Helminth Ova*. Environmental Protection Agency, Washington, D.C. 1540.

WHO (1989). *Health Guidelines for the Use of Wastewater in Agriculture and Aquaculture*. World Health Organization Tech. Rep. Series 778, OMS, Geneva, Switzerland.