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# Limax Amoebae in Public Swimming Pools of Albany, Schenectady, and Rensselaer Counties, New York: Their Concentration, Correlations, and Significance

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A survey was conducted on 30 halogenated public swimming pools, located in Albany, Schenectady, and Rensselaer counties, to determine their open-water limax amoeba densities. Six were outdoor pools. Other variables measured were the standard plate count, total seston, free residual chlorine or bromine, total alkalinity, total hardness, orthophosphate, total soluble phosphorus, specific conductance, pH, temperature, and several engineering parameters including the rate and type of filtration as well as a saturation index. Amoebae were isolated on agar plates at 37°C using heat-killed bacterial suspensions of *Enterobacter cloacae* or *Escherichia coli*. Most probable number estimates of amoebic densities ranged from not detectable (<0.01) to 110 amoebae per liter. The median concentration of amoebae was 0.9/liter. Eighty percent of the pools examined had less than 5 amoebae per liter. Significant correlations ($P < 0.05$) were found between amoebic densities and the $\log_{10}$ of the standard plate count, orthophosphate, and total soluble phosphorus. No significant difference was found between amoebic densities in outdoor and indoor pools. Preliminary tests for the presence of the human pathogen *Naegleria fowleri* were inconclusive.

Since the documentation of *Naegleria fowleri* as a causative agent of primary amoebic meningoencephalitis (PAM) in humans (9), there has been an upsurge of interest in limax amoebae, both pathogenic and nonpathogenic (11). Investigations have associated swimming or bathing activities with the onset of this disease and have established the presence of limax amoebae within the open water of bathing areas such as pools and recreational lakes (4, 8). Concern over the possible presence of such pathogenic amoebae within pools in New York State prompted the Environmental Health Center, in conjunction with the Bureau of Residential and Recreation Sanitation, to survey 30 halogenated pools between 27 August and 3 October 1974. Our objectives were fourfold: (i) to determine amoebic densities; (ii) to determine whether any of the limax forms isolated were potentially pathogenic; (iii) to note any correlations between amoebic counts and other physical, chemical, and biological conditions in the pools; and (iv) to document the median, range, and variance of each parameter studied.

Limax amoebae are typically small (7 to 50 μm long) and monopodal, and they move like slugs. They are readily found in soils, natural freshwaters (including meltwaters of Antarctica), air (17), amphibians, and reptiles (13). In addition, they have been isolated from human throats and nasal mucosa (6, 28). These amoebae form cysts (5 to 20 μm in diameter) which can endure a wide range of environmental conditions, including chlorine concentrations up to 10 ppm (2). Cysts of these limax amoebae, unlike those of the intestinal parasite *Entamoeba histolytica*, can withstand long periods of desiccation (7). Limax amoebae excyst readily in the presence of *Escherichia coli* or its aqueous extract (26).

## MATERIALS AND METHODS

**Amoebic densities.** Bacterial suspensions were prepared by inoculating either *E. coli* or *Enterobacter cloacae* into 250 ml of lactose broth (3 g of beef extract, 5 g of peptone, and 5 g of lactose per liter) in sterile polyethylene centrifuge bottles. Preliminary laboratory tests showed that both species served equally well as food sources for a variety of free-living limax amoebae. After overnight incubation at 35°C, the bacteria were killed by immersing the bottles in a 60°C water bath for 1 h. The cultures were then centrifuged at 4,200 × *g* and washed three times with sterile water (Millipore Super-Q-filtered,

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then autoclaved) at pH 7.5 to 8.0. The final bacterial suspension was formed by adding the washed bacteria to sterile water until a slight whitish turbidity appeared.

Petri dish bottoms (100 by 15 mm and 60 by 10 mm, Optilux; Becton-Dickinson and Co.) were covered with melted nonnutrient agar (Difco Laboratories) (1.5% in sterile water) to a thickness of 2 to 4 mm. After cooling, the smaller dishes received either 2 or 3 ml of bacterial suspension and the larger plates received 5 ml. All bacterial and dish preparations were conducted in a laminar flow hood (Pure Aire Corp.) under sterile conditions. A set of 63 plates was used for each pool surveyed. To maintain a humid atmosphere, a moist sponge was placed with each set in a plastic container, and the container was then placed in a plastic bag.

Samples were collected by thoroughly rinsing a clean polyethylene pail with pool water, inverting it, pressing it until submerged, and then turning it slowly upright. The water was taken at the deeper end of each pool, approximately 45 cm from the wall. Samples were immediately processed at poolside, except at three pools, where water was collected in sterile 9-liter glass bottles and returned to the laboratory, where it was processed within 6 h of collection.

Processing of all samples entailed either concentration by filtration or dilution with sterile water of the suspended matter within the pool. Filtration was through sterile membrane filters (HAWP, Millipore Corp.) of 0.45-$\mu$m pore size, either 90, 47, or 25 mm in diameter, under a vacuum equivalent to 250 to 380 cm of water. Appropriate filtration equipment was autoclaved daily.

Filters (47 or 25 mm in diameter) containing pool seston (the total particulate matter suspended in the water) were inverted and placed in the smaller petri dishes holding 3 ml of bacterial suspension. The 90-mm-diameter filters were placed in the larger dishes. One-milliliter pool samples and dilutions were pipetted directly into the dishes containing 2 ml of bacterial suspension. Replication for the subsamples of pool water was as follows: 9 liters (3 dishes); 1 liter, 100 ml, 10 ml, 1 ml, 0.1 ml, and 0.01 ml (10 dishes each).

After incubation at 35°C for 1 week, the plates were examined with an inverted microscope at ×190 and ×375 magnification. Positive plates contained amoebic cysts, trophozoites, or both, and a reduced level of bacteria. Examinations began with the 9-liter subsamples and continued to a set of replicate subsamples which yielded all negative plates.

In lieu of tables, most probable number (MPN) values of amoebae per liter were computed directly, using a Wang 720C programmable calculator, according to a probability curve (16). The number of positive and negative plates within each of the three lower concentration levels immediately following the lowest level yielding all positive plates was used in calculation of MPN.

The works of Singh (24), Singh and Das (25), and Page (21, 22) were used as guides to preliminary identification of amoebic isolates.

**Amoebic pathogenicity.** *N. fowleri* grows at elevated temperatures (>42°C), whereas nonpathogenic limax strains, with few exceptions, cannot tolerate temperatures above 40°C (15). Accordingly, mixed isolates of amoebic cysts and trophozoites from pools positive for amoebae were removed from selected dishes and washed thrice with sterile water. A few drops of concentrated amoebic suspension were reintroduced into small petri dishes containing 3 ml of bacterial suspension (*E. cloacae* on nonnutrient agar). Each dish was then placed in a Whirl-Pak (Scientific Products) polyethylene bag and submerged for 1 week in a water bath held at 45 ± 0.2°C. The presence or absence of amoebae was then determined in duplicate tests, which were identical except that in one the bacterial suspension was heat-killed (60°C for 1 h), and in the other it was not. Growth response of *N. fowleri* to samples of these live and heat-killed bacterial suspensions was tested by the Environmental Protection Agency, Cincinnati, Ohio.

**Other biological measurements.** Seston was estimated as follows. Eighty milliliters of unpreserved pool water was centrifuged for 20 min at 480 × *g*, and all but 10 ml of the supernatant was decanted. The sediment was resuspended and then allowed to settle in glass-bottomed dishes (2.5 cm in diameter) overnight. Qualitative and quantitative analysis was done with an inverted microscope (Wild Heerbrugg, M-40) (18). For a volume estimate of total seston, area coverage (mean percent coverage of 10 fields at ×188) was multiplied by a depth estimate. Apparent particle depth was estimated by measuring the vertical movement of the microscope stage as the focus passed from top to bottom. All volume estimates were obtained by the same individual to a precision of ±25%. Microscopic samples were also qualitatively scanned for the presence of other organisms.

Standard 24-h plate counts and total coliform (membrane filtration) counts were done according to *Standard Methods for the Examination of Water and Wastewater* (1) by the Sanitary Bacteriology Laboratory of the Environmental Health Center. All samples were processed within 20 h of collection.

**Chemical and physical measurements.** Temperature and specific conductance were measured using a YSI model 33 S-C-T meter (Yellow Springs Instrument Co.).

In the field, samples for phosphorus analysis were passed through boiled filters (Millipore Corp., type HA, 0.45 $\mu$m pore size) and placed in 150-ml polyethylene bacteriological bottles, which had been washed with 1:1 nitric acid and rinsed twice with distilled water. These samples were refrigerated (4°C) until arrival at the laboratory, where they were frozen. The maximum time any one sample was frozen prior to analysis was 6 weeks. Orthophosphate was determined by formation of a duction with ascorbic acid (20). Determination of total soluble phosphorus required acid persulfate digestion for 30 min at approximately 100°C (14) for conversion to orthophosphate. Absorbance was read at 882 nm in a Bausch and Lomb Spectronic 100

spectrophotometer. The phosphorus determinations have a precision of ±0.5 μg/liter in the range from 1.5 to 100 μg.

Tests of total alkalinity, total hardness, pH, and free residual chlorine or bromine were performed by the Bureau of Residential and Recreation Sanitation using LaMott-Palin chemical testing kits (LaMott Chemical Products Co.).

**Engineering aspects.** The operational characteristics recorded for each pool included size and volume, type and rate of filtration, type of surface skimming, and number of inlets. Saturation index (SI) values were calculated according to the following formula (10): SI = pH + temperature factor + alkalinity factor + calcium factor − 12.1. Increases or decreases in SI are directly proportional to the increased probability of scale formation or of corrosive activity by the pool water, respectively.

## RESULTS

Of 30 pools, six were outdoor. Chlorination was the most prevalent method of disinfection; only 3 pools were brominated. For filters, 9 pools used high-rate sand (650 to 815 liters/min per $m^2$), 9 used slow-rate sand (about 130 liters/min per $m^2$), and 12 used diatomaceous earth (42 to 81 liters/min per $m^2$). Twelve of the pools were in Albany County, 12 were in Rensselaer County, and 6 were in Schenectady County. Table 1 summarizes the data. Total coliform counts were <1/0.1 liter in all pools.

Limax amoebae were detected in all but three pools. Over half of the pools examined in this study contained <1 amoeba per liter; one pool in five had a count >5. Although 110/liter was the highest count obtained in the present study, 2,900/liter were detected in a brominated swimming pool during a preliminary study.

TABLE 1. *Range, median, and 95% confidence interval of parameters measured in halogenated swimming pools, September 1974*

| Parameter | Range | Median | 95% Confidence interval[a] |
|---|---|---|---|
| Amoebae (no./liter) | <0.01–110 | .85 | 0.2–2.9 |
| Standard plate count (no./ml) | <1–>20,000 | 1 | <1–53 |
| Total seston ($mm^3$/liter) | 0.2–6.6 | 1.2 | 0.8–1.5 |
| Free residual chlorine (mg/liter) | <0.1–>2.0 | 0.8 | 0.6–1.0 |
| Total alkalinity (mg/liter) | 25–>250 | 110 | 60–160 |
| Total hardness (mg/liter) | 80–360 | 150 | 100–160 |
| pH | 7.2–8.4 | 7.9 | 7.6–8.0 |
| Orthophosphate (μg of P/liter) | 8–4,100 | 94 | 28–170 |
| Total soluble phosphorus (μg of P/liter) | 36–5,700 | 300 | 120–380 |
| Temp (°C) | 17–27 | 25 | 22–26 |
| Specific conductance at 25°C (μS/cm) | 158–4,480 | 1,464 | 848–2,200 |

[a] Based on ordered statistics (27).

Significant positive correlations were found between amoebic counts and the $\log_{10}$ of the standard plate counts, orthophosphate, and total soluble phosphorus ($P < 0.05$). No significant correlation was noted between amoebic counts and other parameter values.

Limax amoebae concentrations did not differ significantly among counties or between outdoor and indoor pools.

The most common isolates fell within the *Hartmannella-Acanthamoeba* group. The general absence of flagellate stages of amoebae indicates a scarcity of either *N. gruberi* or *Didasculus thorntoni* in the samples. No amoebic isolates grew when incubated at 45°C with either living or heat-killed *E. cloacae*.

Microscopic examinations, over several weeks, of a sand-filter backwash showed numerous nematodes (nonsegmented worms), rotifers, ciliates, zooflagellates, and amoebic trophozoites and cysts, as well as bacterial masses. Amoeba densities within this backwash ranged from 2,800 to >10,000/liter. All the biota appeared robust despite bromination just prior to filtration. In addition, a sample of yellowish slime taken from a pool wall consisted of a bacterial matrix filled with amoeboid cysts, while one taken from a device used to clean the pool bottom contained numerous aquatic mites.

Tabulations of the biological and chemical values, ancillary pool characteristics, and frequency distributions for each parameter are available on request.

## DISCUSSION

Červa (4), in an intensive study of a single Czechoslovakian pool associated with 16 cases of PAM, found amoebic counts ranging from 20 to 5,000/liter. Červa and Huldt (5) noted counts ranging from 10 to 700 amoebae per liter in an investigation of five pools in Stockholm. Chang (8), in an investigation of three Cincinnati pools, detected only 3, 4, and 130 amoebae/liter. Our results are in general agreement with those of Chang.

Amoebae can be carried to the pool by the local water supply, air, and pool users. Assuming a settling rate of 0.28 cysts per min per $m^2$ (17), 7.5 million amoebae infiltrate an outdoor pool (23 by 9 m) from the atmosphere over a 3-month period. Amoebic input by swimmers can also be significant, especially in pools with adjacent dirt or grass areas. Singh (23) found from 2,900 to 116,000 amoebae per g of soil. His minimum value suggests that tracking 2.6 kg

(about 5 lb) of soil into a 23- by 9-m pool is roughly equivalent to 3 months of cyst sedimentation from the air. If this pool had a mean depth of 2.5 m, one would expect a concentration of at least 29 amoebae per liter by summer's end. The low counts (<3/liter) in the six outdoor pools tested indicate a rate of cyst removal equivalent to natural input.

Removal of viable amoebic cysts and/or trophozoites from the open water of pools occurs by natural accretion of the organisms to pool walls, adequate filtration, effective disinfection, and/or predation. The clean, slime-free walls of the outdoor pools examined in this study, the relatively high cyst resistance to chlorination, and the absence of any potential predators in pool seston suggest that filtration was a major factor in controlling the amoeba population. Five of the outdoor pools examined had diatomaceous earth filtration systems; the sixth utilized a high-rate sand filter. Pool filters, however, can provide an excellent medium for amoeba growth and thus require conscientious maintenance.

**Correlations.** Although the correlation of amoebic count to the $\log_{10}$ of the standard plate count, orthophosphate, and total soluble phosphorus is significant ($P < 0.05$), the low positive correlation coefficients (0.48, 0.45, and 0.46, respectively) preclude prediction of any of these parameters from amoeba density. At best, there is a tendency for increases in amoeba count to be associated with increases in standard plate count and the two soluble phosphorus fractions.

**Public health significance.** De Jonckheere and van de Voorde (12) have shown that an initial concentration of 0.5 mg of free chlorine per liter destroys $10^3$ *N. fowleri* cysts per ml within 1 h. The relative lack of *Naegleria* sp. in the pools tested during this study and in that tested by Červa (4) tends to support their finding. However, caution is advised when extrapolating results from batch-type assays to conditions found in swimming pools. Anderson and Jamieson (2) failed to eliminate *Naegleria* from a swimming pool using superchlorination of up to 10 ppm. It may be that amoebae cysts buried in the bacterial slime found on some pool walls are protected from the killing effects of chlorine.

The methods used to isolate limax amoebae in this survey were similar to Červa's (4) and were thus more selective for nonpathogenic amoebae than pathogenic *Naegleria*. In addition, the method used to detect *N. fowleri* was not conducive to vigorous growth of the pathogen (S. L. Chang, personal communication). Confirmation of the presence or absence of pathogenic *Naegleria*, then, requires further testing.

De Jonckheere and van de Voorde (12) also found that *Acanthamoeba* spp. survived the cysticidal effect of much higher concentrations of chlorine than those normally recommended for swimming pools. This disclosure gains in significance in light of investigations (3, 19) relating PAM to the invasion by amoebae apparently belonging to the *Hartmannella-Acanthamoeba* group.

The results of this study do not warrant any changes in the current procedures for insuring the safety of swimming pools with respect to amoebae and public health. The low amoebic densities (<1/liter) in the majority of the pools surveyed illustrate that these organisms and their cysts can be adequately controlled by proper maintenance. However, the potential health hazards of amoebae, nematodes, flagellates, mites, and ciliates in swimming pools are relatively unknown. The presence of amoebae in human throats has been linked with milder illnesses such as headaches and rhinitis. Every possible effort should be taken by pool operators to discourage and control the growth of these organisms via continual pool surveillance and adequate maintenance.

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