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Thermal Ecology of *Naegleria fowleri* from a Power Plant Cooling Reservoir

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The pathogenic, free-living amoeba *Naegleria fowleri* is the causative agent of human primary amebic meningoencephalitis. *N. fowleri* has been isolated from thermally elevated aquatic environments worldwide, but temperature factors associated with occurrence of the amoeba remain undefined. In this study, a newly created cooling reservoir (Clinton Lake, Illinois) was surveyed for *Naegleria* spp. before and after thermal additions from a nuclear power plant. Water and sediment samples were collected from heated and unheated arms of the reservoir and analyzed for the presence of thermophilic *Naegleria* spp. and pathogenic *N. fowleri*. Amoebae were identified by morphology, in vitro cultivation, temperature tolerance, mouse pathogenicity assay, and DNA restriction fragment length analysis. *N. fowleri* was isolated from the thermally elevated arm but not from the ambient-temperature arm of the reservoir. The probability of isolating thermophilic *Naegleria* and pathogenic *N. fowleri* increased significantly with temperature. Repetitive DNA restriction fragment profiles of the *N. fowleri* Clinton Lake isolates and a known *N. fowleri* strain of human origin were homogeneous.

The free-living amoeboflagellate *Naegleria fowleri* is the causative agent of primary amoebic meningoencephalitis, a rare but rapidly fatal central nervous system disease of humans who are infected by exposure to heated water (10). *N. fowleri* is ubiquitous and has been isolated from naturally and artificially heated aquatic environments worldwide (4–6, 15–17, 20). Thermal additions from electrical power stations are associated with increased isolation of *N. fowleri* from cooling lakes (15–18).

Although elevated temperature is associated with increased occurrence of *N. fowleri*, the thermal conditions which induce or select for pathogenic strains of the amoeba are incompletely understood. Griffin (7, 8) used an isolation temperature of 45°C to separate mixed populations of thermotolerant and nonthermotolerant amoebae isolated from the environment. Studies suggest that a temperature range of 30 to 40°C is associated with increased occurrence of *N. fowleri* in thermally elevated environments (5, 17, 21). Most amoeba surveys of cooling reservoirs have been conducted following intermittent or long-term thermal additions, and there have been few opportunities to sample the same environment before and after thermal elevation (18). The newly operational Clinton Nuclear Power Station provided a unique study situation which enabled us to conduct a before and after survey to evaluate the following hypothesis: thermal additions select for the amplification of thermophilic and/or pathogenic *Naegleria* species in a newly created cooling reservoir.

MATERIALS AND METHODS

Study area and collection sites. Clinton Lake is a 22-mile-long, 5,000-acre impoundment located near Clinton, Dewitt County, Illinois, that provides cooling water for the Clinton Nuclear Power Station of the Illinois Power Company. The V-shaped Clinton Lake was divided into two study areas: (i) the North Fork of Salt Creek was designated the ambient arm of the lake, providing cooling water to the power station, and (ii) the East Fork of Salt Creek was designated the heated arm, receiving effluent from the power station (Fig. 1). Thermal effluent passes through a 3.1-mile flume into the east arm, causing thermal elevation of sites 2 to 9. Heat is dissipated as water moves down the east arm, and the temperatures of sites 10 to 16 on the west arm are not raised above ambient temperatures (temperature data, Illinois Power Co.). Collection sites were selected on both arms of the lake in order to be representative of environmental conditions suitable for the presence of amoebae and for comparative study of the two arms of the lake.

Amoeba sampling. Two types of samples were taken at each collection site: (i) 1,000 ml of surface water with a sterile, hand-held bottle and (ii) 1,000 ml of bottom sediment with a Ponar bottom dredge. Bottom samples were collected at depths of 1 to 3 m for all sites except site 8, where they were taken at the maximum lake depth of 12.5 m. Surface and bottom water temperature was measured with a calibrated temperature probe (model 54; Yellow Springs Instrument Co.). Monthly water samples were taken to bracket the warmest-temperature summer periods of 1986 before the power station began operation, of 1987 during a period of intermittent reactor testing, and of 1988 when the power station was in full production.

Amoeba isolation. Water and sediment samples were transported to the laboratory and processed within 2 h of collection. Each 1,000-ml surface water sample was thoroughly mixed, and 100 ml was filtered through a cellulose filter (pore size, 1.2 nm; 97-mm Spectrum microfilter). The filter was inverted on a nonnutrient agar plate (15 by 100 mm) seeded with 1 ml of live *Enterobacter cloacae* bacteria grown in a tryptic soy agar broth culture for 24 h at 37°C. Since the concentrated bottom sediment caused the filter to clog, a smaller 25-ml portion was filtered and similarly processed.

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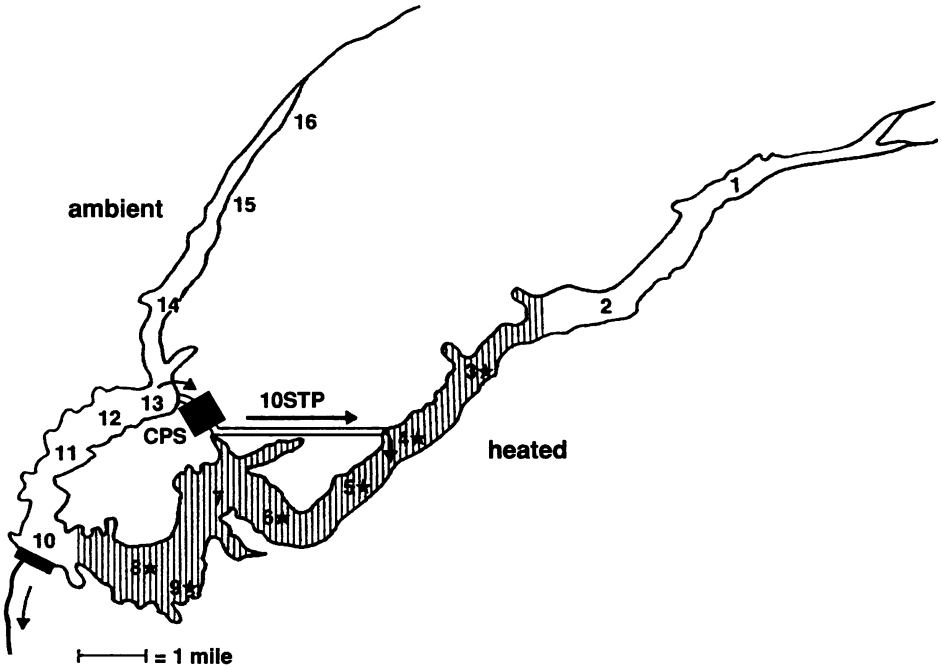

FIG. 1. Map of Clinton Lake showing sample sites 1 to 9 on heated arm (cross-hatched) and sites 10 to 16 on ambient-temperature arm. CPS, Clinton Power Station; arrows indicate cooling loop. *, Site where *N. fowleri* was isolated.

Test plates were incubated at 45°C in a high-humidity, constant-temperature tissue culture incubator and examined at 24 and 48 h and for up to 7 days postisolation for growth of amoeboid plaques. Amoebae isolated at 45°C were considered thermophilic.

In vitro cultivation of amoebae. Amoebae were harvested from agar-*E. cloacae* plates and established in axenic, in vitro culture by using a mixture of Balmuth-Nelson medium in a 50/50 ratio (19), supplemented with 2% fetal calf serum and antibiotics (penicillin, 10,000 U/ml; streptomycin, 10 mg/ml). Amoebae were cultivated to log phase (48 h at 37°C) and harvested for use in identification procedures and mouse pathogenicity tests.

Amoeba identification procedures. Amoeba isolates were identified as *N. fowleri* if they satisfied all of the identification criteria listed as follows and described below: thermophilic growth in culture at 45°C, enflagellation at 37°C, nonagglutination in concanavalin A, DNA homology with known human Lee strain of *N. fowleri* by restriction fragment length analysis, and pathogenicity to mice. Other isolates satisfying all of the above identification criteria, but nonpathogenic to mice, were identified as thermophilic *Naegleria* species unknown. These identification criteria were used throughout the entire study.

Amoebae isolated in culture were studied microscopically and characterized by morphology of trophozoite and cyst stages and type of pseudopodial movement (13). Trophozoites of *Naegleria* spp. were tested for enflagellation by incubation in Page's saline at 37°C for 3 h (1). The concanavalin A agglutination reaction was used to separate *N. fowleri* (nonagglutinating) from other species of *Naegleria* agglutinating in the presence of concanavalin A (9).

Restriction fragment length polymorphism (RFLP) analysis was used to characterize the DNA of presumptive *N. fowleri* isolates. Amoebae contain repetitive, simple DNA which was extracted and analyzed by using the methods of McLaughlin et al. (11). Pellets of amoebae (7×10^6) were lysed and digested by incubation in 5 ml of 1% sodium dodecyl sulfate–5 mM EDTA–0.3 mg of proteinase K per ml–50 mM Tris (pH 8.0) for 2 h at 37°C. Equivolume phenol saturated with 1 mM EDTA–10 mM Tris (pH 8.0) was added, and samples were thoroughly mixed by gentle inversion for 5 min at room temperature. Samples were chilled for 5 min on ice and then centrifuged at $4,000 \times g$ for 10 min at 4°C. The aqueous phase was removed, and 0.1 volume of 3 M sodium acetate (pH 5.4)–equivolume isopropanol were added, with mixing. Samples were centrifuged at $10,000 \times g$ at 4°C for 20 min. The pellet was washed with 1 ml of 70% ethanol containing 0.1 mM EDTA–1 mM Tris (pH 7.6) and once with 95% ethanol, dried for 10 min at 50°C, and dissolved in 0.5 ml of 1 mM EDTA–10 mM Tris (pH 7.8). DNA samples (1 to 7 μg) were digested for 1 h at 37°C with *Bgl*II, *Eco*RI, and *Hin*dIII endonucleases according to the directions of the manufacturer (International Biotechnologies, Inc.). DNA samples (1 μg) were electrophoresed at 4 V/cm in 0.7% flat-bed agarose gels without ethidium bromide. Gels were stained for 20 min with 1 μg of ethidium bromide per ml, destained for 20 min in water, and photographed under shortwave UV illumination. Repetitive DNA profiles above the genomic smear were compared with known reference strains of *Naegleria gruberi* (ATCC 3087), *Naegleria australiensis* (ATCC 30958), and *N. fowleri* Lee strain (ATCC 30894).

Amoeba pathogenicity test in mice. Amoebae from log-phase cultures were concentrated by gentle centrifugation (500 rpm, 5 min). The cells were counted with a hemacytometer and adjusted with Page saline to a concentration of 10^5 amoebae per 10 μl, which was inoculated intranasally into each of five weanling, 19- to 25-day-old BALB/c mice. Test mice were observed for 2 weeks for signs of encephalitis. Mice showing morbidity were euthanized, and brain tissue was removed aseptically with a Pasteur pipette and trans-

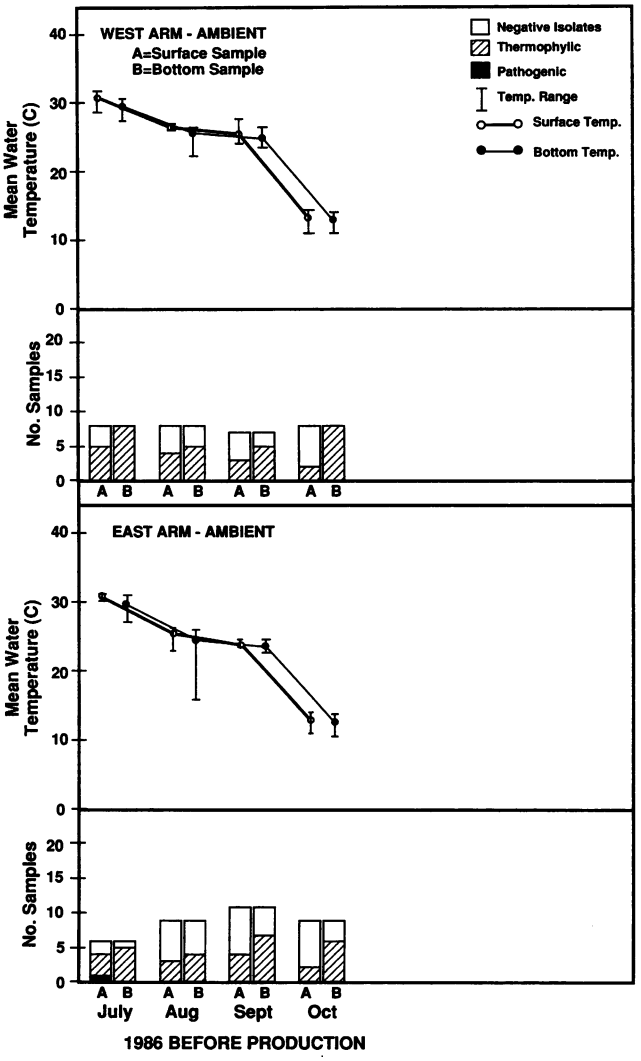

FIG. 2. Isolation of thermophilic *Naegleria* spp. and *N. fowleri* from Clinton Lake before Clinton Power Station began production, 1986.

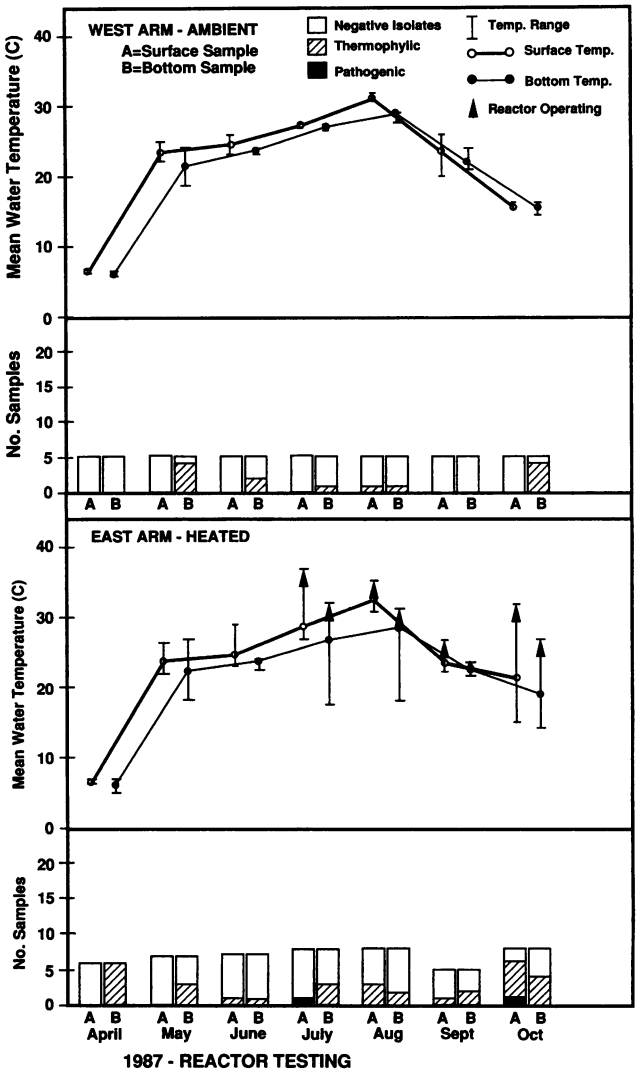

FIG. 3. Isolation of thermophilic *Naegleria* spp. and *N. fowleri* during intermittent testing of Clinton Power Station, 1987.

ferred to *E. cloacae*-agar media and Balmuth-Nelson media. The growth of amoeboid plaques confirmed the causative agent to be pathogenic *N. fowleri*. Negative control mice were inoculated intranasally with Page saline and processed as described for experimental mice.

Statistical analysis. The probability of isolating *Naegleria* spp. in 1988 samples was analyzed by logistic regression of the discrete variables, thermophilic *Naegleria* (+ or −) and pathogenic *N. fowleri* (+ or −) versus water temperature (12, 14). The effects of sample level (surface or bottom) and sample station were also tested. Significant level or station effects indicate that given identical temperatures, there are differences in the probability of isolating thermophils or pathogens between levels or stations. A significant temperature effect indicates that the probability of isolating thermophiles or pathogens is related to temperature.

RESULTS

An amoeba survey of Clinton Lake was conducted in 1986, 1987, and 1988, before and after the Clinton Power Station began production and thermal enrichment of the lake. Thermophilic *Naegleria* spp. were isolated from both the ambient and heated arms of the lake during all sample periods (Fig. 1, 2, 3, and 4). The probability of isolating thermophilic *Naegleria* spp. increased significantly with temperature (Fig. 5; $P = 0.0312$). In addition, probability of isolating thermophilic *Naegleria* spp. was greater for bottom samples than for surface samples (Fig. 5; $P < 0.0001$). There were also significant differences among stations ($P = 0.0002$). Three stations (no. 3, 4, and 5) nearest the heated outfall had conspicuously higher predicted probabilities of yielding a thermophilic isolate, even if temperature was held constant at 30°C (Fig. 6).

Water samples from the source flume (Fig. 1; site 10STP) yielded 11 of 16 thermophilic *Naegleria* spp. but no *N. fowleri* during the sample period of August, September, and October, 1988, when effluent temperatures ranged from 26.3 to 46.2°C (mean 35.3).

Pathogenic *N. fowleri* was isolated from sites 3 to 9 in the heated arm of the lake, but none was isolated from sites 10 to 15 in the ambient-temperature arm of the lake during the

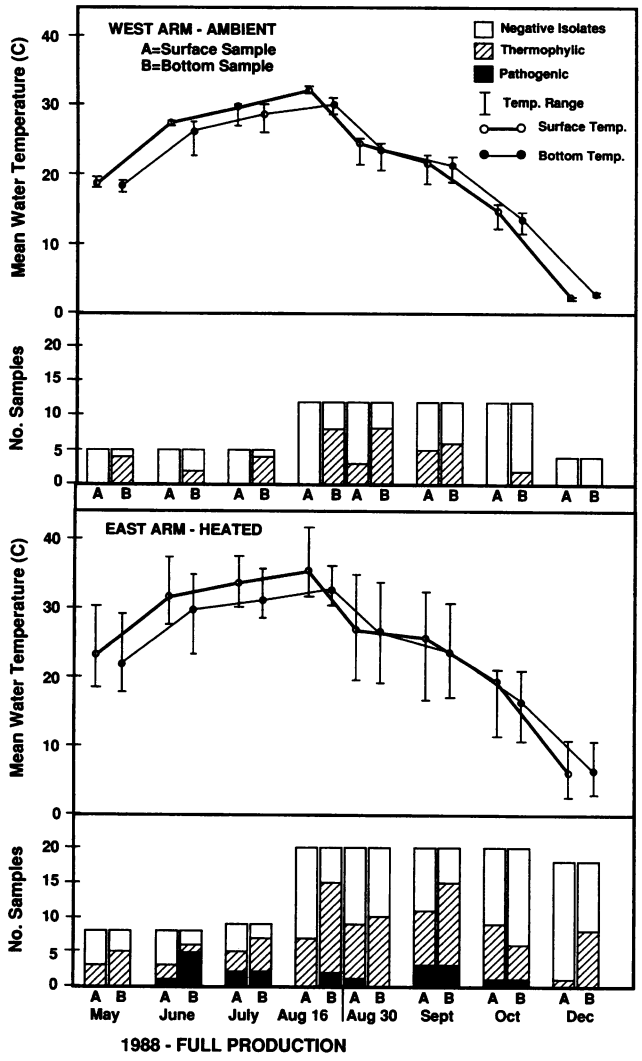

FIG. 4. Isolation of thermophilic *Naegleria* spp. and *N. fowleri* during full production of Clinton Power Station, 1988.

entire 1986 to 1988 survey (Fig. 1, 2, 3, and 4). However, *N. fowleri* was isolated from site 13 of the ambient-temperature arm during a separate 1989 survey (data not reported). A single pathogenic isolate of *N. fowleri* was collected from

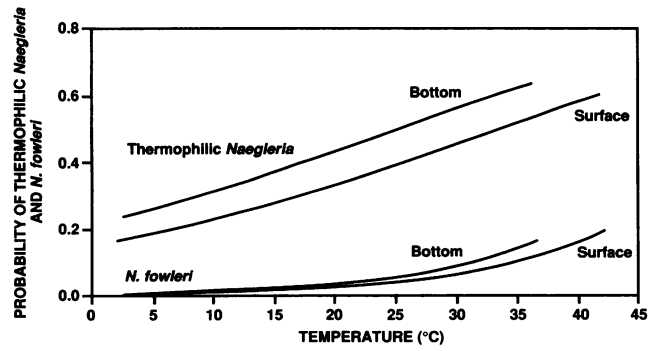

FIG. 5. Logistic regression of probability of isolating thermophilic *Naegleria* and *N. fowleri* versus water temperature for surface and bottom samples.

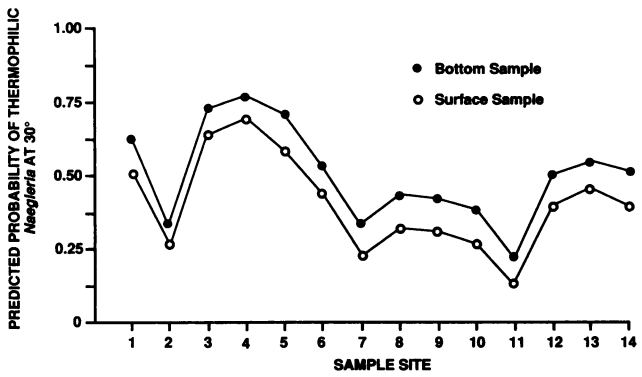

FIG. 6. Predicted probabilities of isolating thermophilic *Naegleria* spp. from sample sites 1 to 14 with temperature held constant at 30°C.

site 9 during July, 1986, before reactor production, when the reservoir was at ambient temperature. This demonstrates the presence of the amoeba in a northern temperate environment before thermal alteration. During July and October, 1987, two pathogenic isolates were collected at site 4, when the reactor was undergoing intermittent testing and heated water was being discharged (Fig. 1 and 3). In 1988 when the reactor was in full production, 21 *N. fowleri* isolates were collected from sites 3, 4, 5, 6, 8, and 9 during a period of sustained elevated water temperature (Fig. 1 and 4). The probability of isolating *N. fowleri* increased significantly with water temperature (Fig. 5; $P < 0.0038$). The low frequency of *N. fowleri* made it impossible to include sample sites in this analysis. There were no significant differences in the probability of isolating *N. fowleri* between surface and bottom samples (Fig. 5; $P < 0.1337$). The majority of *N. fowleri* isolates (17 of 21) were collected at water temperatures in excess of 25°C (Fig. 2, 3, and 4). Exceptions were *N. fowleri* isolates in June, September, and October, 1988, when the plant was in production but water temperatures were seasonally reduced.

Environmental temperatures during the summers of 1987 and 1988 were elevated because of prolonged warm weather and extreme drought conditions. This caused atypically high ambient water temperatures in excess of 30°C (Fig. 2 and 3). However, *N. fowleri* was not isolated from the ambient-temperature west arm of the lake during this period.

Pathogenic isolates of *N. fowleri* from Clinton Lake were identical in biological characteristics to the *N. fowleri* Lee strain. All pathogenic isolates from Clinton Lake were virulent and caused acute meningoencephalitis and death of mice in 5 to 7 days. The RFLP analysis of repetitive DNA restriction fragment banding patterns after *Bgl*II, *Eco*RI, and *Hin*dIII digestion showed identity between *N. fowleri* Clinton Lake isolates and the *N. fowleri* Lee strain but major differences between the patterns of *N. gruberi* and *N. australiensis*. Typical repetitive DNA profiles are shown (Fig. 7). RFLP analysis of thermophilic *Naegleria* isolates revealed a previously uncharacterized fragment pattern (data not shown).

DISCUSSION

The frequency of isolation of thermophilic *Naegleria* spp. and pathogenic *N. fowleri* increased following thermal additions to a newly created power plant cooling reservoir. *N. fowleri* was isolated under ambient-temperature conditions

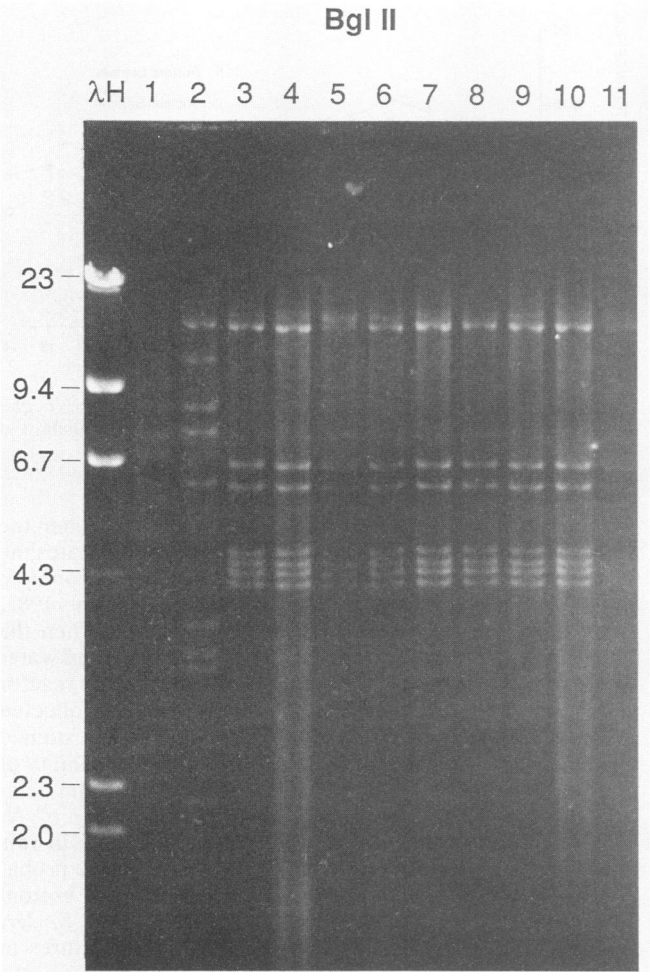

FIG. 7. Agarose gel profiles of *Bgl*II digestions of genomic DNA of *N. australiensis* (lane 1), *N. gruberi* (lane 2), *N. fowleri* Lee strain (lane 3), *N. fowleri* Clinton Lake isolates (lanes 4 through 11), stained with ethidium bromide to visualize repetitive DNA. Lambda *Hin*dIII DNA kilobase standards shown on left.

in 1986 before thermal additions. In 1988, when the power plant was in continuous production, the frequency of isolation of thermophilic *Naegleria* spp. and pathogenic *N. fowleri* increased significantly in the heated arm of the reservoir. *N. fowleri* was isolated from surface and bottom samples and from a deep-water site, suggesting a wide distribution of the amoeba throughout the heated environment. Tyndall et al. (18) were first to quantitate an increased density of thermophilic *Naegleria* spp. and *N. fowleri* in heated water and sediment samples following thermal additions to a newly created cooling lake at the Savannah River Project. They found a small number of samples positive for pathogenic *N. fowleri* and did not isolate *N. fowleri* from source or discharge samples.

Pathogenic isolates of *N. fowleri* from Clinton Lake were identical in biological characteristics to the *N. fowleri* Lee strain. Five species of amoebae in the genus *Naegleria* are known from aquatic habitats, and it is necessary that pathogenic and nonpathogenic species be accurately identified in environmental surveys (2). Because of the lack of distinguishing morphological criteria and antigenic cross-reactivity between species, the biochemical characterization of *Naegleria* spp. by the use of restriction endonuclease digestion of whole-cell DNA has proven to be a reliable method (2, 11). *N. fowleri* isolated from Clinton Lake showed DNA restriction fragment length profiles that were homogeneous with *N. fowleri* Lee strain isolated from a human case of meningoencephalitis acquired in West Virginia. This provides strong evidence for genetic homogeneity of *N. fowleri* isolated from southern and northern areas of the United States. DeJonckheere (3) also determined the restriction fragment profiles of *N. fowleri* isolated from Europe, Australia, India, and the United States to be homogeneous. The RFLP assay provides a useful method to support biological and morphological identifications of environmentally isolated *N. fowleri*.

Pathogenic *N. fowleri* has been isolated most consistently from waters intermittently heated to 30 to 40°C (5, 17, 21). Although the precise temperature conditions associated with amplification of *N. fowleri* in Clinton Lake were not determined, the majority of *N. fowleri* isolates were collected at water temperatures in excess of 25°C. Since temperatures were recorded at the time of sample collection, it was not possible to determine whether previous temperature conditions were responsible for selection of thermophilic amoebae. Selection of *N. fowleri* may depend on the maximum temperature attained and the duration of that temperature. We have studies in progress using continuous water temperature recordings and the RFLP analysis for amoeba identification to develop a predictive temperature model for the occurrence of *N. fowleri* and *Naegleria* species in Clinton Lake.

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