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Presence of Pathogenic Amoebae in Power Plant Cooling Waters Final Report for the Period October 15, 1977 to September 30, 1979

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ENVIRONMENTAL SCIENCES DIVISION
Publication No. 1623

Prepared for
Dr. Judith Foulke, Project Representative
Environmental Effects Research Branch
Office of Nuclear Regulatory Research
U.S. Nuclear Regulatory Commission
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PRESENCE OF PATHOGENIC AMOEBAE IN POWER PLANT COOLING WATERS
FINAL REPORT FOR THE PERIOD
OCTOBER 15, 1977 TO SEPTEMBER 30, 1979

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ENVIRONMENTAL SCIENCES DIVISION
Publication No. 1623

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Task: Presence of Pathogenic Amoebae in Power Plant Cooling Waters

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ABSTRACT

TYNDALL, R. L., E. WILLAERT, AND A. R. STEVENS. 1981. Presence of pathogenic amoebae in power plant cooling waters. Final report for the period October 15, 1977 to September 30, 1979. ORNL/NUREG/TM-413 and NUREG/CR-1761. Oak Ridge National Laboratory, Oak Ridge, Tennessee. 56 pp.

Cooling-water-associated algae and sediments from five northern and five southern or western electric power plants were tested for the presence of pathogenic amoebae. In addition, water algae and sediments from five northern and five southern/western sites not associated with power plants were tested. There was a significant correlation at northern power plants between the presence of thermophilic, pathogenic amoebae in cooling waters and thermal additions. Presence of the pathogenic amoebae did not correlate with salinity, pH, conductivity, or a variety of various chemical components of the cooling waters. Selected pathogenic isolates were tested serologically and were classified as Naegleria fowleri. Although thermal additions were shown to be a contributing factor in predisposing cooling waters to the growth of pathogenic amoebae, the data suggest the involvement of other currently undefined parameters associated with the presence of the pathogenic amoebae.

SUMMARY

Cooling-water-associated algae and sediments from five northern and five southern or western electric power plants were tested for the presence of pathogenic amoebae. Water, algae, and sediments from five northern and five southern/western sites not associated with power plants were also tested. More samples from the power plant sites were positive for thermophilic amoebae, for thermophilic Naegleria, and for pathogenic Naegleria. The difference in number of samples positive for thermophilic Naegleria between heated and unheated waters, however, was attributable to the northern sites. Four of five northern cooling water sites yielded pathogenic Naegleria, whereas pathogens were not isolated from southern/western cooling water sites or any control sites. Some of the pathogenic isolates were analyzed serologically and classified as pathogenic Naegleria fowleri. The temperature of northern sites positive for pathogens was generally 30 to 40°C at, or close to, the time of sampling. Southern/western sites ranged in temperature from 21 to 36°C. Salinity, pH, conductivity, and various chemical constituents did not obviously correlate with the presence or absence of the pathogenic Naegleria. Levels of total organic carbon were higher in most of the sites positive for the pathogen. While thermal addition was significantly associated with the presence of pathogenic Naegleria in northern sites ($P = \leq 0.025$) the data implicate other as yet undefined parameters associated with the presence of the pathogens. Until these factors are identified, generalizations cannot be made concerning the effect of thermal impact on the growth of

pathogenic amoebae in a particular cooling system. Thus, each system needs to be judged individually.

Our studies suggest that cooling systems may inadvertently serve as habitats for thermophilic amoebae pathogenic for humans. These results may be significant in assessing public health risks, if any, relating to the proposed construction of new power plants with closed-cycle systems using lakes available for public recreation.

CONCLUSIONS AND RECOMMENDATIONS

As a result of our studies on the association between thermal additions and the presence of thermophilic, pathogenic free-living amoebae in cooling waters of electric power plants, we have concluded that:

(1) Thermal additions enhance the growth and/or persistence of thermophilic, free-living amoebae in cooling waters of electric power plants.

(2) Thermal additions enhance the growth and/or persistence of thermophilic Naegleria in cooling waters of electric power plants.

(3) Thermal additions enhance the growth and/or persistence of pathogenic Naegleria in cooling waters of electric power plants.

(4) Ecological parameters, in addition to thermal input appear to play a role in fostering the growth and/or persistence of thermophilic, pathogenic Naegleria in cooling waters of electric power plants.

Based on our study, we make the following recommendations:

(1) Consideration should be given to managing the public use of cooling lakes shown to contain pathogenic amoebae.

(2) Rudimentary protective measures for plant personnel in close contact with cooling water shown to contain pathogenic amoebae should be considered. Contact of the nasal passages with such waters should be avoided.

(3) Development of more rapid screening assays for the presence of the pathogenic, free-living amoebae should be developed so that monitoring of power plant cooling systems could be more easily and economically effected.

(4) The ecological parameters, other than thermal additions, which contribute to the growth of pathogenic amoebae need further study. Defining of such factors may lead to the methodology for controlling these pathogens.

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I. INTRODUCTION

That some free-living amoebae could be encephalitic for mammals was originally shown by the isolation of an encephalitic Acanthamoeba from cultured monkey kidney cells (Culbertson et al. 1958, 1959). When inoculated intranasally into weanling mice, this species (Acanthamoeba culbertsoni) caused a rapid, fatal encephalitis. The first confirmed case of human encephalitis associated with free-living limax amoeba, however, involved Naegleria rather than Acanthamoeba (Fowler and Carter 1965, and Carter 1968).

Following these first reported isolations of potentially pathogenic free-living amoebae from monkey kidney tissue cultures (Jahnes et al. 1957; Culbertson et al. 1958, 1959) and association of Naegleria with fatal human encephalitis, free-living amoebae of the genera Naegleria and Acanthamoeba were recognized and confirmed as the etiological agents of primary amoebic meningoencephalitis (PAME) (Carter 1968, 1969; Bhagwandeen et al. 1972; Willaert and Stevens 1976a), chronic meningoencephalitis (Kenny 1971, Jager and Stamm 1972, and Robert and Rorke 1973), pneumonitis (Martinez et al. 1975), intestinal disorders (Jadin et al. 1973), and serious eye infections (Nagington et al. 1974; Warhurst et al. 1976). In the past fifteen years more than 100 cases of PAME, including those that were confirmed and those that were suspected to have been caused by the free-living amoebae, occurred throughout the world (Willaert and Stevens 1976a and b; Willaert 1974; Sotello-Avila et al. 1974; Butt 1964, 1966; Butt et al. 1968; Singh and Das 1972; Gordeeva 1973; De Jonckheere

et al. 1975; Van den Driessche et al. 1973). Most, if not all, of these cases of PAME resulted from individuals swimming or bathing in naturally or artificially heated waters.

We previously reported the isolation of pathogenic strains of N. fowleri from a lake and river in Florida (Willaert and Stevens 1976b) and from a man-made lake in Texas (Stevens et al. 1977b, Tyndall et al. 1978), both of which received electric power plant effluents. This latter lake is currently used for recreational purposes and also supplies drinking water for a surrounding suburban area. Wellings et al. (1977) also reported the isolation of strains of pathogenic N. fowleri in Florida from freshwater lakes, including one which was thermally enriched. The increasing demand for energy production, the concomitant construction of new power plants (some with closed-cycle cooling lakes and towers), and the recent observations that pathogenic Naegleria are associated with warmed water indicated the need for this study to evaluate the extent of this problem. Thus, the objective of this research was to determine, on the basis of screening studies, the extent of distribution of Naegleria fowleri (and possibly other thermophilic amoebae potentially pathogenic for man) in cooling systems of electric power stations. Presence of pathogenic amoebae associated with power plant cooling could greatly affect the power plant impact assessments by various regulatory agencies and thus the siting and design of new power stations.

II. MATERIALS AND METHODS

Test sites were chosen according to both the type of cooling system and the load factor. We concluded it was important to test a variety of cooling system types, even though this meant the majority of test sites were nonnuclear. A brief description of the types of cooling systems tested is as follows:

Dresden (Illinois) is a closed system in which the heated water is discharged into a canal with multiple spray modules. Water is subsequently pumped into a cooling lake from which it is returned to the plant via an intake canal.

Merrimack (New Hampshire) is an open-ended system in which the heated water is discharged into a small bay and then traverses a spray canal before entering the Merrimack River.

Black Dog (Minnesota) is also an open system in which the heated water is discharged into a small bay from which it enters twin cooling lakes before it is eventually discharged into the Minnesota River.

Shawville (Pennsylvania) is an open-cycle river reservoir in which the heated water enters the dammed waters of an arm of the upper Susquehanna River. The water has some overflow into the river *per se*.

Sangchris (Illinois) is a closed system in which the heated water is discharged into a canal before entering a large cooling lake from which it is subsequently returned to the plant intake.

Valmont (Colorado) is a closed-cycle cooling lake in which the water is discharged into a small bay before entering the main cooling lake.

Kyrene (Arizona) is a closed-cycle system composed solely of cooling canals.

Braunig (Texas) and North Anna (Virginia) are closed systems in which the heated effluent is discharged into a canal leading to a large cooling lake before it is returned to the plant intake.

Horseshoe Lake (Oklahoma) is a closed system in which the water enters a small cooling lake via a discharge bay. Supplemental cooling is also providing by cooling towers.

The Dresden and North Anna Power Plants are nuclear whereas the other plants are coal burning. Control waters close to the individual power plants and not receiving thermal additions were also tested for the presence of amoebae.

Samples consisting of 50 to 250 ml of water were filtered through 1.2-μm cellulose membranes at the sampling site. These filters were inverted and placed onto nonnutrient agar plates seeded with a lawn of live or heat-killed Escherichia coli. The plates were incubated at 45 or 37°C until return to the laboratory for analyses of amoebic growth. In addition to water samples, algae, mud, and various sediment samples from the heated and control waters were also plated on coliform-seeded agar plates. Other water samples were filtered through 0.45-μm membrane filters and kept chilled until return to the laboratory for chemical analyses by the Analytical Chemistry Division at Oak Ridge National Laboratory. Water, pH, temperature, salinity, and conductivity were measured at the sampling sites using appropriate meters. Ten milliliters of lake water samples were also analyzed for

number of total aerobic bacteria and coliform bacteria using millipore test kits MT0000025 and MC0000025, respectively.

On return to the laboratory, the agar plates were incubated for several additional days or until growth of amoebae was observed. Amoebae that grew out at 45°C were tested for their ability to flagellate. All amoeba flagellates and some nonflagellate amoebae were tested for pathogenicity by intranasal inoculation into weaning ICR mice (Appendix). Moribund mice were sacrificed and the brain tissues were plated on coliform-seeded agar plates. Pieces of brain were also inoculated directly into axenic medium for growth of amoebae. Some of the pathogenic Naegleria isolates were speciated by serologic analysis (see Appendix).

The chilled, filtered water was analyzed for levels of nitrites, nitrates, sulfates, phosphorus, chlorine, and total organic carbon. Total aerobic bacteria and total coliform bacteria per milliliter of chilled test water were also determined by colony counts of the millipore test plates.

III. RESULTS

There was a prevalence of pathogenic Naegleria in power plant cooling waters relative to control unheated waters (Tables 1-4 and Appendix Tables A-1 to A-14). Four of five northern cooling systems were positive for pathogenic amoebae. The pathogenic isolate from the Black Dog cooling lake was obtained from sediment in the discharge bay, whereas the isolate from the Dresden system came from aquatic vegetation in the canal water returning from the cooling lake to the plant. Two of the three pathogenic isolates from the Shawville reservoir were obtained from 50- and 250-ml water samples downstream from the discharge area. The other isolates were from sediment in the discharge area. The pathogenic Naegleria isolates at the Lake Sangchris site were found in the discharge area per se or in the canal leading from the discharge bay to the cooling lake. All mice injected with the pathogenic isolates from the various test sites developed acute encephalitis and either died or were sacrificed when moribund within 10 d after infection. The pathogenic amoebae were reisolated from the brain tissue. Most of the isolates were axenized. Some of the isolates were subsequently analyzed serologically and identified as pathogenic Naegleri fowleri (Table 5).

The open-cycle cooling canal of the Merrimack plant in New Hampshire and the five southern/western power plant cooling systems were negative for pathogenic amoebae (Table 1). Pathogenic amoebae were not detected in samples from any of the unheated control waters (Table 2).

Table 1. Prevalence of thermophilic amoebae in power plant cooling systems

Site (plant)	Cooling system type	Temperature range[a] (°C)	Number of samples				
			Taken[b]	Incubated 45°C	Positive for amoebae at 45°C	Positive for flagellate amoebae of 45°C	Positive for pathogenic isolates[c]
Dresden (IL)	Spray canal-lake-closed	31-38	17	17	17	14	1
Black Dog (MN)	Lake-open	25.5	12	12	8	5	1
Merrimack (NH)	Spray canal-open	20-23.5	21	21	12	4	0
Shawville (PA)	River-reservoir	27-30	16	16	14	5	3
Sangchris (IL)	Lake-closed	32-34	12	12	8	7	5
Valmont (CO)	Lake-closed	23-30	21	14	1	0	0
Kyrene (AZ)	Canal	25-26	18	11	1	0	0
Braunig (TX)	Lake-closed	21-26.5	33	21	8	1	0
North Anna (VA)	Lake-closed	22	16	16	0	0	0
Horseshoe (OK)	Lake-closed	28-36	22	22	10	0	0

[a]Range in temperature from different sites samples, including inlet, outlet, and sites several hundred meters away.

[b]Samples were incubated at both 45 and 37°C. Results reported are only for samples incubated at 45°C.

[c]All amoebic flagellate growing at 45°C were tested for pathogenicity.

Table 2. Prevalence of thermophilic amoebae in unheated control waters

Site[a]	Temperature (°C)	Number of samples				
		Taken[b]	Incubated at 45°C	Positive for amoebae at 45°C	Positive for flagellate amoebae at 45°C	Positive for pathogenic isolates[c]
Strip Mine Lake (Dresden)	30	8	8	1	1[d]	NG[e]
Lake Nokomis (Black Dog)	24	9	9	3	1[d]	NG[e]
Merrimack River (Merrimack)	20	7	7	4	0	NT[e]
Susquehanna River (Shawville)	23	7	7	3	1[d]	NG[e]
Lake Lou Yeager (Sangchris)	25	5	5	0	0	0
Boulder Reservoir (Valmont)	24	10	6	0	0	NT[e]
Lake Saguaro (Kyrene)	31	5	3	3	3	0
Canyon Lake (Kyrene)	29.5	7	4	1	1	0
Canyon Lake (Braunig)	17.5	7	4	0	0	NT[e]
Lake Louise (North Anna)	21	5	5	1	0	0
Lake Thunderbird (Horseshoe)	30	7	7	5	1	0

[a]The power plant in the vicinity of the control lake is given in ( ). State in which plant and control sites are located is indicated in Table 1.

[b]Samples were incubated at both 45 and 37°C. Results reported are only for samples incubated at 45°C.

[c]All amoebic flagellates growing at 45°C were tested for pathogenicity.

[d]A few flagellates seen in the original sparse outgrowth. These amoebae could not be subcultured at 45°C and thus cannot be considered as true thermophiles.

[e]NG = No growth when subcultured at 45°C from original 45°C outgrowth; NT = not tested.

Table 3. Chemical and bacteriological profiles of power plant cooling waters

Site	Chemicals (μg/ml)						Bacteria (colonies/ml)	
	NO_3-N	NO_2-N	Pa	Cl	SO_4	TOC[b]	Total	Coliform
Dresden	2.50	0.03	0.063	29.0	127.0	17.1	63	2
Black Dog	0.63	0.03	0.14	41.0	110.0	17.0	50	0
Shawville	0.23	0.02	0.006	14.3	9.3	13.1	40	6
Sangchris	0.26	0.02	<0.001	28.0	143.0	14.0	30	3
Merrimack	0.19	0.003	0.010	14.1	0.6	11.7	100	0
Valmont	0.009	0.001	0.012	102.0	304.0	5.42	228	0
Kyrene	4.51	<0.001	0.007	388.0	175.0	3.6	196	23
Braunig	0.16	0.108	0.06	230.0	188.0	9.0	500	0
North Anna	0.009	<0.001	0.075	3.50	8.7	5.3	>500	0
Horseshoe	0.024	0.006	0.026	64.0	580.0	22.0	340	0

[a]Orthophosphate.

[b]Total organic carbon.

Table 4. Chemical and bacteriological profiles of unheated control waters

Site[a]	Chemicals (μg/ml)						Bacteria (colonies/ml)	
	NO_3-N	NO_2-N	Pb	Cl	SO_4	TOC[c]	Total	Coliform
Strip Mine Lake (Dresden)	0.25	0.008	0.002	10.0	756.0	20.1	300	0
Lake Nokomis (Black Dog)	0.10	0.03	0.140	52.0	11.8	16.0	300	0
Merrimack River (Merrimack)	0.68	0.02	0.01	10.8	155.0	14.5	100	0
Susquehanna River (Shawville)	0.23	0.02	0.01	14.3	9.3	13.1	40	6
Lake Lou Yeager (Sangchris)	1.47	0.058	0.006	19.0	41.0	17.9	300	0
Boulder Reservoir (Valmont)	0.012	< 0.001	0.026	2.75	109.0	4.33	140	3
Canyon Lake (Kyrene)	0.024	< 0.001	0.012	169.0	56.3	8.3	< 500	0
Lake Saguaro (Kyrene)	0.001	0.001	0.012	210.0	52.0	6.6	5	0
Canyon Lake (Braunig)	0.46	0.001	0.008	14.7	16.0	24.0	42	0
Lake Louise (North Anna)	0.003	< 0.001	0.038	1.9	4.0	8.1	88	7
Lake Thunderbird (Horseshoe)	0.014	0.004	0.016	28.0	9.8	7.0	214	0

[a]The power plant in the vicinity of the control lake is given ( ).

[b]Ortophosphate.

[c]Total organic carbon.

Table 5. Indirect immunofluorescent analysis of pathogenic Naegleria isolated from cooling waters of northern electric power plants

Isolate code	Plant site	Antiserum[a]			
		N. fowleri (Morgan)	N. jadini (ITMAP 400)	N. gruberi (CCAP 1518)	N. fowleri (nonpathogenic)
Dr-18	Dresden	1:1024	NT[b]	NT	1:256
BD-6	Black Dog	1:1024	NT	NT	1:256
Sh-20	Shawville	1:512	1:16	1:32	NT
San-11	Sangchris	1:512	NT	NT	1:128

[a]End point titers are given relative to titers for N. fowleri (Morgan) isolated from a human case of PAME.

[b]NT = not tested.

Thermophilic nonpathogenic amoebae were more widely distributed. Of the 162 samples from ten cooling systems incubated at 45°C, 79 showed growth of amoebae (48%). In contrast, only 21 of 65 samples from unheated waters incubated at 45°C showed growth of amoebae (32%). Of the 36 thermophilic isolates identified as Naegleria by the flagellation test and from morphologic criteria, only 10 of these were pathogenic for mice. The remaining isolates are probably the nonpathogenic variants of N. fowleri, which have been invariably isolated in association with pathogenic N. fowleri in previous investigations of heated waters by us and others. Their relation to pathogenic N. fowleri has not yet been clearly established.

It is of interest to point out the very high percentage of thermophilic amoebae, other than amoeboflagellates, that we obtained from the majority of the cooling systems (Table 1). Of the 79 thermophilic amoeba isolates from the power plant cooling systems, 43 (54%) were nonflagellates. Although we have not specifically identified these amoebae, morphologically they represent species of the genera Acanthamoeba, Valkampfia, Mayorella, and Hartmannella. Some Acanthamoeba species are known to be pathogenic. Until these studies, only one genus of amoebae, i.e., Naegleria, was known to be capable of growth at 45°C.

The samples incubated at 37°C were of little value. Too many species of amoebae were capable of growing at this temperature and they tended to overgrow the Naegleria. Newer methods have to be developed to select for growth of pathogenic amoebae at this temperature.

In contrast to the cooling waters, only 21 of 65 samples (i.e., ~32%) from unheated waters incubated at 45°C showed growth of amoebae and only 8% were flagellates. Although several of these amoebae were flagellates, they were not pathogenic (Table 2). It is conceivable that the four isolates from unheated Lake Saguaro and Canyon Lake are nonpathogenic variants of N. fowleri. However, the failure of the flagellates obtained from unheated waters of Strip Mine Lake, Lake Nokomis, and the Susquehanna River to grow at 45°C after initial subculture makes it unlikely that these are nonpathogenic variants of N. fowleri (Table 2). We and others showed previously that the nonpathogenic variants are capable of continued growth at 45°C.

Tables 3 and 4 give the chemical and bacteriological profiles of the cooling systems and unheated control waters. Generally, the total bacteria counts were lower than might be expected in some of the cooling waters. This might be related to the fact that many of the samples, especially those of the northern lakes, were taken close to the discharge area. Such areas might contain some biocidal effluent from the plant. Some samples yielding pathogenic Naegleria had a low bacterial count and a high total organic carbon (TOC). This is of interest since thermophilic Naegleria can grow on either enterobacteriaceae or an organic material. Generally, however, in our experience and that of others, the pathogenic N. fowleri can outgrow the nonpathogenic form in organic media devoid of bacteria, whereas the nonpathogen will outgrow the pathogen when feeding on coliform bacteria (De Jonckheere 1977).

Tables 6 and 7 provide information on several additional properties of the cooling and unheated control waters. The southwestern cooling systems in Arizona, Colorado, and Texas generally had a higher pH than did the northern lakes. The majority of samples from these lakes were obtained in areas where the pH was 8 or above. High pH in itself, however, is not a deterrent to the presence of thermophilic Naegleria; various thermophilic Naegleria were isolated by DeJonkheere et al. (1975) from waters in excess of pH 8.0.

Table 6. Properties of power plant cooling water

Site	pH range	Salinity (parts per thousand)	Conductivity range (μS/cm)
Dresden (IL)	7.8-8.5	0.5-1.5	1900-4000
Black Dog (MN)	7.5	0.01	820
Merrimack (NH)	6.3-6.7	0	90-115
Shawville (PA)	6.8	0	435-465
Sangchris (IL)	6.9	0.02	700
Valmont (CO)	8.15-8.5	0.5	800-900
Kyrene (AZ)	7.5-8.2	0	650-900
Braunig (TX)	7.6-8.0	0.5	1250-1420
North Anna (VA)	6.9-7.9	0	55-60
Horseshoe (OK)	7.8-8.5	0.5-1.5	1900-4000

Table 7. Properties of unheated control waters

Site[a]	pH range	Salinity (parts per thousand)	Conductivity range (μS/cm)
Strip Mine Lake (Dresden)	LA[b]	0.1	1800
Lake Nokomis (Black Dog)	7.4	0.01	420
Merrimack River (Merrimack)	6.5	0	113
Susquehanna River (Shawville)	6.8	0	405
Lake Lou Yeager (Sangchris)	7.5	0	295
Boulder Reservoir (Valmont)	7.8	0	320
Canyon Lake (Kyrene)	8.8	0.1	1000
Lake Saguaro (Kyrene)	8.6	0	725
Canyon Lake (Braunig)	7.8	0	320
Lake Louise (North Anna)	6.9	0	32
Lake Thunderbird (Horseshoe)	8.1	0	415

[a]The power plant in the vicinity of the control lake is given in ( ).

[b]LA = laboratory accident; data not available.

IV. DISCUSSION

Analysis of data from the ten test sites supports the tentative conclusion of our previous report (Tyndall et al. 1979b) on the association between thermophilic amoebae and artificially heated water. While heated waters had higher proportions of thermophilic amoebae in general, and Naegleria in particular, relative to unheated water, the difference is most striking if data from northern versus southern/western power plant cooling systems are compared to their respective unheated controls. In analysis of northern systems, outgrowth of thermophilic amoebae, presence of thermophilic Naegleria, and isolation of pathogenic Naegleria in heated versus unheated systems are significant at < 0.05, < 0.05, and < 0.05 levels, respectively, by chi-square analysis. Conversely, analysis of southern and western systems shows no significant differences with regard to presence of thermophilic Naegleria or isolation of pathogenic Naegleria in heated versus unheated waters. Indeed, encephalitic Naegleria were not isolated from either control or heated sites in southern/western waters. It should be emphasized at this point, however, that the inability to isolate pathogenic amoebae from either heated or control waters may indicate a lower concentration of these amoebae rather than their absence. Testing of larger volumes of both heated and unheated waters could conceivably yield a higher percentage of samples positive for pathogenic amoebae. Quantitiative studies are clearly desirable.

The lower percentage of samples positive for amoebae capable of growth at 45°C and the "apparent" lack of pathogenic amoebae in the

western cooling lakes are of interest because the temperatures of these systems were close to ambient and did not generally reflect the thermal additions they receive. Southern/western systems were generally below 30°C, with the exception of Horseshoe Lake. The absence of marked temperature increase in such cooling waters may be related to the very low humidity in these locales and the resultant high levels of evaporation and cooling. The lack of temperature elevation in these cooling lakes, however, seems to correlate with the low level of thermophilic amoebae and once again suggests the association of these amoebae with elevated temperatures. Water temperatures of northern cooling systems that were positive for pathogenic Naegleria, however, generally ranged from 30 to 40°C at, or close to, the time of sampling.

In our experience with this and other related studies (Fliermans et al. 1980, Tyndall et al. 1978, Tyndall et al. 1979a and b), water in excess of 40°C generally yields thermophilic nonpathogenic Naegleria and water never heated above 28 to 30°C yields few thermophilic Naegleria, while pathogenic Naegleria thus far have often been associated with waters in the 30 to 40°C range. These findings suggest a thermal niche as one factor favorable for the emergence of pathogenic Naegleria.

While the association of thermal additions and presence of pathogenic Naegleria is significant, particularly in northern waters, it is difficult to generalize until the other factors enhancing the growth of these pathogens are defined. For instance, the pathogenic Naegleria were not isolated from the Horseshoe Lake site which had sufficient thermal additions nor, in related studies, from some other

northern sites which had characteristics similar to those sites in this study which yielded the pathogen.

Thus, while thermal impact may predispose a cooling system to the presence of pathogenic amoeba, other undefined factors may mitigate this predisposition. Therefore, each cooling system needs to be judged individually.

It is of interest that many of the pathogens came from sites high in total organic carbon and low in coliforms. As mentioned previously, pathogenic Naegleria generally grow more readily on dissolved organic media devoid of bacteria than do their nonpathogenic counterparts. The pathogenic isolates in this study were no exception as many were also readily axenized. The number of sites yielding pathogens is too small, however, to draw significant conclusions relative to any correlation between high organic content and presence of pathogenic Naegleria.

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APPENDIX

I. METHODOLOGY

A. Identification and Pathogenicity of Amoebae

Pathogenic Naegleria were identified in the samples by (1) their ability to grow at a temperature of 45°C, (2) their ability to flagellate, (3) their trophozoite and cyst morphology, and (4) their capacity to produce acute PAME in mice. Final species identification of cloned, mouse brain reisolates was accomplished by the indirect fluorescent technique (IFAT) using specific antisera.

The flagellation tests were performed at 45°C. After harvesting, a drop of trophozoites suspended in sterile distilled water was placed on a coverslip and inverted on a cavity slide (hanging-drop technique). The hanging drops were examined under a phase-contrast microscope for the presence of flagellates after 30 min, and 1, 2, and 3 h. Morphologic characteristics typical of the Naegleria trophozoite and cyst (i.e., eruptive-like formation of the pseudopodia and smooth cyst wall, respectively) were taken into consideration for identifying amoebae belonging to the genus Naegleria.

To determine pathogenicity, amoebae growing at 45°C were harvested from the plates, washed, suspended in sterile distilled water, and intranasally instilled in weanling mice.

B. Indirect Immunofluorescent Analysis (IFAT)

Antigen. Amoebae, obtained from 4-d-old axenic, exponentially growing cultures, were concentrated by centrifugation and washed three times in a phosphate-buffered saline solution (1.5 M NaCl, 0.25 NaH_2PO_4, pH 7.2). The cells were then fixed for 30 min in a

2% (v/v) formalin solution prepared in the buffer, rewashed three times, and diluted with buffer to provide approximately 25 amoebae per microscope field (obj. 25X, ocular 10X). Drops of this suspension were placed on immunofluorescent slides which allow for 10 dilutions of antisera per slide. After drying, the slides were stored at -25°C until use. Sections of formalin-fixed brain tissue, prepared by routine histological procedures, were used after deparaffinization for IFAT analyses.

Antisera. Two methods were used to prepare the antisera against the various amoeba species. Rabbits were immunized by intravenous inoculation of 1×10^6 whole, living cells (Willaert 1976) or by intradermal inoculation with purified plasma membrances (100 to 300 μg of membrane protein) (Stevens et al. 1977b). The rabbits are bled weekly before and after immunization. The antisera giving the highest titers and the least cross reactions are used for the IFAT.

IFAT Procedure. The indirect fluorescent antibody method of Weller and Coons (1954) was used. Fluorescein-conjugated goat antirabbit Ig was obtained and used at a dilution of 1:100 on the culture antigens and at a dilution of 1:50 on tissue sections. All dilutions of the antisera, washes, etc., were prepared with the phosphate saline buffer. After the procedure, the slides were immediately examined with a microscope equipped for fluorescence.

The degree of fluorescence was estimated on a subjective scale ranging from 1+ to 4+, with 4+ denoting the highest fluorescence. The end-point titer was considered as the dilution of antisera producing a 2+ or higher fluorescence.

II. PROFILE OF AMOEBIC OUTGROWTH IN INDIVIDUAL TEST AND CONTROL SITES

Table A-1. Prevalence of thermophilic Naegleria in Dresden, Illinois, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Discharge canal	38	H_2O-250 ml	+	+	-
	38	H_2O-250 ml	+	+	-
	38	H_2O-250 ml	+	+	-
	38	Sediment	+	+	-
	38	Sediment	+	-	NT
	38	Sediment	+	+	NG
Lake	37	H_2O-50 ml	+	+	-
	37	H_2O-50 ml	+	+	NG
	37	H_2O-50 ml	+	+	-
	37	Algae	+	+	-
	37	Algae	+	-	NT
	37	Sediment	+	-	NT
	37	H_2O-250 ml	+	+	-
Intake canal	31	H_2O-250 ml	+	+	-
	31	H_2O-250 ml	+	+	-
	31	H_2-250 ml	+	+	-
	31	grass	+	+	+

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NG = No growth when transplanted at 45°C from original 45°C outgrowth; NT = not tested.

Table A-2. Prevalence of thermophilic Naegleria in Black Dog, Minnesota, cooling system

Site	Temperature[a] (°C)	Sample type/vol	Outgrowth[b] at 45°C	Flagella	Pathogenicity
Discharge bay	25.5	H_2O-250 ml	-	NA[c]	NA
	25.5	H_2O-250 ml	-	NA	NA
	25.5	H_2O-250 ml	-	NA	NA
	25.5	H_2O-250 ml	-	NA	NA
	25.5	Grass	+	-	NT[d]
	25.5	Sediment	+	+	+
	25.5	Wood	+	-	NT
	25.5	Grass	+	+	-
	25.5	Sediment	+	+	-
	25.5	Sediment	+	+	NG[e]
	25.5	Sediment	+	-	NT
	25.5	Sediment	+	+	-

[a]Power plant was operating at only 25 MW for about one week prior to testing.

[b]+ (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[c]NA = not applicable.

[d]NT = not tested.

[e]NG = no growth when transplanted at 45°C from original 45°C outgrowth.

Table A-3. Prevalence of thermophilic Naegleria in Merrimack, New Hampshire, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Discharge bay	23.5	H_2O-250 ml	+	+	-
	23.5	H_2O-250 ml	+	+	-
	23.5	H_2O-50 ml	-	NA[c]	NA
	23.5	Algae	-	NA	NA
	23.5	Wood	+	-	NT
	23.5	Grass	-	NA	NA
	23.5	Sediment	+	+	-
Spray canal	20.5	H_2O-250 ml		-	NT
	20.5	H_2O-50 ml	-	NA	NA
	20.5	Sediment	+	-	NT
	20.5	Sediment	-	NA	NA
	20.5	Grass	+	-	NT
	20.5	Grass	+	-	NT
Canal discharge	20.0	H_2O-250 ml	+	-	NT
	20.0	H_2O-250 ml		-	NT
	20.0	H_2O-50 ml		-	NT
	20.0	H_2O-50 ml	-	NA	NA
	20.0	Algae-sediment	+	+	-
	20.0	Algae	+	-	NT
	20.0	Grass	+	-	NT
	20.0	Grass	+	-	NT
	20.0	Grass	+	-	NT

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NT = not tested.

[c] NA = not applicable.

Table A-4. Prevalence of thermophilic Naegleria in Shawville, Pennsylvania, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Discharge area	30	H_2O-250 ml	+	+	-
	30	H_2O-150 ml	+	-	NT
	30	H_2O-50 ml	-	NA[c]	NA
	30	Grass	+	+	-
	30	Grass	+	-	NT
	30	Wood-sediment	+	-	NT
	30	Sediment	+	-	NT
	30	Sediment	+	+	+
Downstream	27	H_2O-250 ml	+	-	NT
	27	H_2O-250 ml	+	+	+
	27	H_2O-50 ml	+	+	+
	27	Grass	+	-	NT
	27	Grass	+	-	NT
	27	Sediment	+	-	NT
	27	Sediment	+	-	NT
	27	Mud	+	-	NT

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NT = not tested.

[c] NA = not applicable.

Table A.5. Prevalence of thermophilic Naegleria in Sangchris, Illinois, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Discharge bay	34	H_2O-200 ml	+	+	-
	34	H_2O-200 ml	+	+	+
	34	H_2O-50 ml	+	-	NT
	34	H_2O-50 ml	-	NA[c]	NA
	34	H_2O-50 ml	-	NA	NA
Discharge canal	32	H_2O-250 ml	+	+	+
	32	H_2O-200 ml	+	+	+
	32	H_2O-50 ml	-	NA	NA
	32	H_2O-50 ml	-	NA	NA
	32	Algae	+	+	+
	32	Sediment	+	+	+
	32	Sediment	+	+	-

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NT = not tested.

[c] NA = not applicable.

Table A-6. Prevalence of thermophilic Naegleria in Kyrene, Arizona cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity
Inlet	25	H_2O-250 ml	-	NA[b]	NA
	25	H_2O-50 ml	-	NA	NA
	25	H_2O-50 ml	-	NA	NA
Outlet	26	H_2O-250 ml	-	NA	NA
	26	H_2O-125 ml	-	NA	NA
	26	H_2O-50 ml	-	NA	NA
Canal	26.5	H_2O-250 ml	-	NA	NA
	26.5	H_2O-50 ml	-	NA	NA
	26.5	Algae	+	-	NA
	25.5	H_2O-250 ml	-	NA	NA
	25.5	H_2O-50 ml	-	NA	NA

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NA = not applicable.

Table A-7. Prevalence of thermophilic Naegleria in Valmont, Colorado, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity
Inlet	23.5	H_2O-250 ml	-	NA[b]	NA
	23.5	H_2O-250 ml	-	NA	NA
	23.5	H_2O-50 ml	-	NA	NA
	23.5	Mud	-	NA	NA
Outlet	27	H_2O-250 ml	-	NA	NA
	27	H_2O-250 ml	-	NA	NA
	27	H_2O-50 ml	-	NA	NA
	27	Grass	-	NA	NA
	30	H_2O-250 ml	+	-	NA
	30	H_2O-50 ml	-	NA	NA
	30	Algae	-	NA	NA
Lake	23.5	H_2O-250 ml	-	NA	NA
	23.5	H_2O-50 ml	-	NA	NA
	23.5	Mud	-	NA	NA

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NA = not applicable.

Table A-8. Prevalence of thermophilic Naegleria in Braunig, Texas, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity
Inlet	21	H_2O-250 ml	-	NA[b]	NA
	21	H_2O-50 ml	-	NA	NA
Outlet	24.5	H_2O-250 ml	+	-	NA
	24.5	H_2O-250 ml	+	-	NA
	24.5	H_2O-50 ml	+	-	NA
	24.5	H_2O-50 ml	+	-	NA
	24.5	H_2O-50 ml	+	-	NA
	24.5	Mud	-	NA	NA
	24.5	Grass	+	-	NA
	24.5	H_2O-250 ml	-	NA	NA
	26.5	H_2O-250 ml	-	NA	NA
	26.5	H_2O-50 ml	-	NA	NA
	26.5	H_2O-50 ml	-	NA	NA
	26.5	H_2O-50 ml	-	NA	NA
	26.5	Mud	-	NA	NA
	26.5	Mud	+	+	Neg
Lake	26.5	H_2O-250 ml	-	NA	NA
	23.5	H_2O-250 ml	+	-	NA
	23.5	H_2O 50 ml		NA	NA
	23.5	Algae	-	NA	NA
	23.5	Mud	-	NA	NA

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NA = not applicable.

Table A-9. Prevalence of thermophilic Naegleria in North Anna, Virginia, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity
Discharge canal	22	H_2O-50 ml	-	NA[b]	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
Lake	22	H_2O-50 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-50 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
Intake	22	H_2O-50 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA
	22	H_2O-250 ml	-	NA	NA

[a] - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NA = not applicable.

Table A-10. Prevalence of thermophilic Naegleria in Horseshoe, Oklahoma, cooling system

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Discharge canal	36	H_2O-50 ml	+	-	-
	36	H_2O-250 ml	+	-	-
	36	H_2O-250 ml	+	-	-
	36	H_2O-250 ml	+	-	-
	36	Sediment	+	-	-
	36	Sediment	+	-	-
Lake	32-35	H_2O-50 ml	-	NA[c]	NA
	32-35	H_2O-50 ml	-	NA	NA
	32-35	H_2O-250 ml	-	NA	NA
	32-35	H_2O-250 ml	+	-	NT
	32-35	H_2O-250 ml	-	NA	NA
	32-35	H_2O-250 ml	-	NA	NA
	32-35	H_2O-250 ml	-	NA	NA
	32-35	Sediment	+	-	NT
Settling basin	28	H_2O-50 ml	-	NA	NA
	28	H_2O-250 ml	-	NA	NA
	28	Sediment	-	NA	NA
Intake	32	H_2O-50 ml	+	-	-
	32	H_2O-250 ml	+	-	-
	32	H_2O-250 ml	-	NA	NA

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NT = not tested.

[c] NA = not applicable.

Table A-11. Prevalence of thermophilic Naegleria in control, unheated northern waters: Strip Mine Lake, Illinois; Lake Nokomis, Minnesota; and Merrimack River, New Hampshire

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Strip Mine Lake	30	H_2O-250 ml	-	NA[c]	NA
	30	H_2O-250 ml	-	NA	NA
	30	H_2O-50 ml	-	NA	NA
	30	H_2O-50 ml	-	NA	NA
	30	Sediment	-	NA	NA
	30	Mud	+	+	NG
	30	Mud	-	NA	NA
	30	Mud	-	NA	NA
Lake Nokomis	24	H_2O-250 ml	-	NA	NA
	24	H_2O-250 ml	+	-	NT
	24	H_2O-50 ml	-	NA	NA
	24	H_2O-50 ml	-	NA	NA
	24	Algae	+	-	NT
	24	Algae	-	NA	NA
	24	Grass	+	+	NG
	24	Grass	-	NA	NA
	24	Sediment	+	-	NT
Merrimack River	20	H_2O-250 ml	-	NA	NA
	20	H_2O-250 ml	-	NA	NA
	20	H_2O-250 ml	-	NA	NA
	20	Grass	+	-	NT
	20	Grass	+	-	NG
	20	Leaf	+	-	NT
	20	Sediment	+	-	NT

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NG = No growth when transplanted at 45°C from original 45°C outgrowth. NT = not tested.

[c] NA = not applicable.

Table A-12. Prevalence of thermophilic Naegleria in control unheated northern waters of the Susquehanna River, Pennsylvania, and Lake Lou Yeager, Illinois

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity[b]
Susquehanna River	23	H_2O-250 ml	-	NA	NA
	23	H_2O-250 ml	-	NA	NA
	23	H_2O-50 ml	-	NA	NA
	23	H_2O-50 ml	-	NA	NA
	23	Grass	+	+	NG
	23	Grass	+	-	NT
	23	Sediment	-	NA	NA
Lake Lou Yeager	25	H_2O-50 ml	-	NA	NA
	25	H_2O-50 ml	-	NA	NA
	25	H_2O-50 ml	-	NA	NA
	25	H_2O-150 ml	-	NA	NA
	25	Sediment	-	NA	NA
	25	Algae	-	NA	NA

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NG = No growth when transplanted at 45°C from original 45°C outgrowth. NT = not tested; NA = not applicable.

Table A-13. Prevalence of thermophilic Naegleria in unheated southwestern control waters: Boulder Reservoir, Colorado; Canyon Lake, Arizona; Saguaro Lake, Arizona; and Canyon Lake, Texas

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity
Boulder Reservoir	24	H_2O-200 ml	-	NA[b]	NA
	24	H_2O-50 ml	-	NA	NA
	23.5	H_2O-250 ml	-	NA	NA
	23.5	H_2O-250 ml	-	NA	NA
	23.5	H_2O-50 ml	-	NA	NA
	23.5	Algae	-	NA	NA
Canyon Lake, Arizona	17.5	H_2O-250 ml	-	NA	NA
	17.5	H_2O-50 ml	-	NA	NA
	17.5	H_2O-50 ml	-	NA	NA
	17.5	H_2O-50 ml	-	NA	NA
Lake Saguaro	31	H_2O-250 ml	+	+	Neg
	31	H_2O-125 ml	+	+	Neg
	31	H_2O-50 ml	+	+	Neg
Canyon Lake, Texas	29.5	H_2O-250 ml	-	NA	NA
	29.5	H_2O-250 ml	+	+	Neg
	29.5	H_2O-50 ml	-	NA	NA
	29.5	Algae	-	NA	NA

[a]+ (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b]NA = not applicable.

Table A-14. Prevalence of thermophilic Naegleria in unheated southern/western control waters of Lake Louise, Virginia, and Lake Thunderbird, Oklahoma

Site	Temperature (°C)	Sample type/vol	Outgrowth[a] at 45°C	Flagella	Pathogenicity
Lake Louise	21	H_2O-50 ml	-	NA[b]	NA
	21	H_2O-250 ml	-	NA	NA
	21	H_2O-250 ml	+	-	-
	21	H_2O-250 ml	-	NA	NA
	21	H_2O-250 ml	-	NA	NA
Lake Thunderbird	21	H_2O-50 ml	+	-	-
	21	H_2O-250 ml	+	-	-
	21	H_2O-250 ml	-	NA	NA
	21	H_2O-250 ml	+	+	-
	21	H_2O-250 ml	+	-	-
	21	Sediment	-	NA	NA
	21	Sediment	+	-	-

[a] + (positive) or - (negative) for outgrowth of amoebae at 45°C and for presence of flagellate amoebae.

[b] NA = not applicable.

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