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Aeromonas Distribution and Survival in a Thermally Altered Lake

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Par Pond is a thermally enriched monomictic southeastern lake which receives heated effluent from a production nuclear reactor. Fish populations in the lake have lesions of epizooty from which *Aeromonas* spp. are readily isolated. Distribution and population densities of *Aeromonas* in the water column were measured along an oxygen and temperature gradient as well as seasonally. Greater population densities of *Aeromonas* occurred below the oxygen chemocline when the lake was stratified. Survival of *Aeromonas hydrophila* under in situ conditions in both epilimnetic and hypolimnetic waters was determined through the use of polycarbonate membrane diffusion chambers during two separate reactor operating conditions. Survival levels of pure cultures of *A. hydrophila* corresponded to the distribution patterns of the naturally occurring *Aeromonas*-like populations. The greater survival of *A. hydrophila* during full reactor operation suggests that the fish populations may be exposed to *Aeromonas* for a longer period of time than when the reactor is not operating.

Several species of *Aeromonas* are pathogenic to fish, frogs, and a variety of reptiles (1) as well as man (4, 9). Infections of epidemic proportions in animals are termed epizootic, and *Aeromonas* infections often occur in combination with the peritrichous *Epistylis*. Fish infections generally result in scale erosion and sloughing, purulent lesions, and bleeding of the fins. The disease, caused by the *Aeromonas-Epistylis* complex, is commonly called "red-sore disease," and under certain conditions this pathology subsequently leads to hemorrhagic septicemia and eventual death (G. W. Esch, T. C. Hazen, and J. W. Gibbons, Abstr. Annu. Meet. Parasitol. 1975). The disease is common in the southeastern United States and has reached epidemic proportions on occasion, causing large fish kills (6-8; W. A. Rogers, Abstr. Am. Conf. Southe. Assoc. Game Fish Comm., 25th, 1971).

Although *Aeromonas* appears to be an ubiquitous aquatic bacterium (11, 13, 14), previous studies (10) have considered neither the survival nor the distribution of this bacterium in situ. This paper describes the survival of *Aeromonas hydrophila* in natural waters altered by thermal effluents discharged from a nuclear production reactor and assesses the natural distribution of *Aeromonas* in these waters.

MATERIALS AND METHODS

Study area. Studies were conducted at the Savannah River Plant, a national environmental research park operated by E. I. du Pont de Nemours & Co., Inc. The specific study site was Par Pond, a 1,092-hectare monomictic lake that is used as a cooling reservoir for a nuclear production reactor. Ambient-temperature waters are used to cool the reactor, and subsequently thermal waters are discharged through a canal into a series of cooling ponds before entering Par Pond. Some areas of the pond are thermally altered, whereas other portions reflect ambient conditions common for other southeastern lakes. Five permanent sampling stations were established throughout the lake at various distances from the thermal discharge into Par Pond. The position of each station is shown in Fig. 1.

Physical and chemical water parameters, including temperature, dissolved oxygen, pH, conductivity, and redox potential, were measured at each of the sampling stations on a weekly basis using a Hydrolab surveyor multiprobe analyzer (Hydrolab Corp., Austin, Tex.). These parameters were recorded during each reactor phase (Tables 2-4), i.e., when the reactor was operating and releasing thermal effluent and when it was not.

Culture studies. Type cultures of *A. hydrophila* ATCC 7966, *A. hydrophila* ATCC 19570, *Aeromonas liquefaciens* ATCC 14715, *Aeromonas proteolytica* ATCC 15338, and *Aeromonas salmonicida* ATCC 14174 were obtained from the American Type Culture Collection (ATCC) for use in comparison with

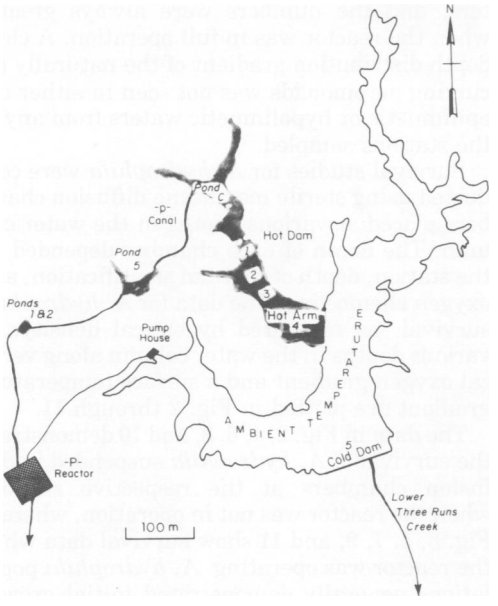

FIG. 1. *Par Pond system showing the ambient and thermal (shaded) temperature regions. Sampling stations are numbered.*

cultures isolated from the natural habitat. Cultures were maintained and routinely grown on nutrient broth, while *A. hydrophila* and presumptive *A. hydrophila* cultures were routinely checked for purity using the selective RS medium (12). Organisms utilized in pure culture studies were *A. hydrophila*, whereas naturally occurring aeromonads isolated from Par Pond on RS medium are referred to as *A. hydrophila*-like.

Isolation and enumeration of *A. hydrophila*-like bacteria were determined by a membrane filter technique modified for *Aeromonas* spp. Water samples were collected at 1.0-m depth intervals throughout the water column in both the ambient and thermally altered portions of Par Pond. Triplicate water samples from each depth were collected with a Kemmerer bottle that was alcohol-rinsed between samples. The samples were immediately placed in sterile Whirl-pac bags (NASCO, Inc.) and returned to the laboratory for processing. Known portions were filtered through sterile 0.45-nm membrane filters (Millipore Corp., Bedford, Mass.), placed on sterile pads saturated with RS medium, and incubated for 20 h at 37°C. The filters were then examined for yellow-pigmented colonies which are presumptive *A. hydrophila*. These colonies were then tested for cytochrome oxidase (3), and positive oxidase cultures were assumed to be *A. hydrophila*-like organisms.

Survival studies. Type cultures of *A. hydrophila* were checked for purity on RS medium, transferred, and routinely grown in nutrient broth at 37°C. Cells were harvested during logarithmic growth phase, washed three times in 0.01 M phosphate-buffered saline (pH 7.2), centrifuged, and resuspended in 40.0 ml of phosphate-buffered saline to a final optical density of 0.150 at 550 nm. The cell suspension was placed into sterile membrane diffusion chambers (5) as modified for deep-water studies (2a). The chambers were immediately suspended from stainless-steel chains and lowered to various depths in the water column. Previous studies with modified chambers indicated that bacterial cultures inside triplicate chambers placed at the same depth in the water column had optical density readings within 10% of each other during a 2-week sampling period. Thus, single chambers were placed at four different depths, with two chambers in the epilimnetic waters and two in the hypolimnetic waters at all five stations. Chambers were never placed at the surface waters, since they were readily attacked by the alligators present in Par Pond.

Chamber sampling. Sampling schedules were intermittent during the 2-week experiments, but, generally, initial samples were taken every 3 h for 48 h and then once every 24 h through the remainder of the experiment. Samples, taken aseptically with sterile plastic syringes, were measured for optical density and tested for diffusion chamber purity by immunofluorescence (C. B. Fliermans, unpublished data) or plating on a selective medium, or both. Sampling ports in the chambers were flamed with a butane cigarette lighter and aspirated five times after a syringe had been attached. Samples of 1.0 ml were then removed from each chamber, placed directly in sterile culture tubes, capped, and returned to the laboratory within 60 min for processing. Optical densities were determined at 550 nm using a Beckman model 25 double-beam spectrophotometer. Optical density measurements were expressed as a percentage of the initial cellular density, since all chambers did not contain *A. hydrophila* populations of exactly 0.150 optical density units. Each chamber was read as 100%; values greater than 100% represented an increase over the initial optical density, whereas those values less than 100% represented a decrease from the initial population densities.

The utilization of membrane chambers in deep waters required separate sterile chambers containing only sterile phosphate-buffered saline as controls. These chambers were suspended alongside those containing the test bacterium, and both chambers were sampled simultaneously. Such controls were necessary when measuring optical density of bacteria in deep water, since under anaerobic conditions iron compounds are in a reduced state and remain in solution. During sampling, the chambers were pulled through oxygenated water, and insoluble iron oxides that interfered with optical density measurements were formed. Thus, solutions inside the control chamber were used as reference samples in double-beam spectroscopy for optical density measurements.

RESULTS

Since this particular cooling reservoir is fed by a production nuclear reactor that operates at

full power 80 to 90% of the year, a seasonal distribution of *A. hydrophila*-like organisms could not be obtained for nonoperating reactor conditions. The data in Table 1 demonstrate that a seasonal distribution of *A. hydrophila*-like organisms occurred in Par Pond, with highest numbers occurring throughout the lake during the spring of the year. Each mean was representative of four weekly samples taken each month throughout the water column. Each depth was sampled in triplicate, and the data were expressed as the number of organisms in the water column of a particular station for a particular month.

Initial population densities and survival studies for *A. hydrophila* were made after the reactor had not been operating for 30 days; subsequent measurements were conducted after the reactor had been operating for over 21 days, so that lake temperatures had stabilized (Tables 2–4). All sampling was performed during normal lake stratification, and such stratification remained stable during both phases of reactor operations.

The distribution of *A. hydrophila*-like organisms was measured indirectly using the selective medium of Shotts and Rimler (12), who previously demonstrated the good selectivity and specificity of the medium. The population densities (expressed as the mean of triplicate samples per liter of lake water) are shown in Table 5 for *A. hydrophila*-like bacteria in the water columns at two of the stations. Numbers of aeromonads in the hypolimnetic waters were always greater than those from epilimnetic waters, and the numbers were always greater when the reactor was in full operation. A clear depth distribution gradient of the naturally occurring aeromonads was not seen in either the epilimnetic or hypolimnetic waters from any of the stations sampled.

Survival studies for *A. hydrophila* were conducted using sterile membrane diffusion chambers placed at various depths in the water column. The depth of each chamber depended on the station, depth of thermal stratification, and oxygen chemocline. The data for *A. hydrophila* survival, as measured by optical density, at various depths in the water column along vertical oxygen gradient and a surface temperature gradient are plotted in Fig. 2 through 11.

The data in Fig. 2, 4, 6, 8, and 10 demonstrate the survival of *A. hydrophila* suspended in diffusion chambers at the respective stations when the reactor was not in operation, whereas Fig. 3, 5, 7, 9, and 11 show survival data when the reactor was operating. *A. hydrophila* populations generally demonstrated initial growth and/or maintenance in all the chambers regardless of the depth during the reactor operating experiments. Although contamination usually occurred in at least one of the chambers at each station during the experiments, bacterial cultures placed in the deeper anoxic waters always demonstrated greater survival over organisms placed in epilimnetic waters. Regardless of the depth of the chambers, or the station, *A. hydrophila* survived longer when the reactor was in full operation than when it was not. Epilimnetic cultures experienced an initial in-

TABLE 1. *Seasonal distribution of A. hydrophila-like bacteria at selected stations in Par Pond*

Mo and yr	Station 5			Station 3			Station 1		
	$\bar{X}$[a]	SD[b]	N[c]	$\bar{X}$	SD	N	$\bar{X}$	SD	N
August 1975[d]	0.39	0.54	114	0.24	0.38	72	ND[e]		
September 1975[f]	1.51	1.42	56	3.01	1.06	33	ND		
October 1975	5.22	1.86	57	6.02	1.84	33	ND		
November 1975	3.14	1.93	50	14.62	7.96	39	9.58	3.78	30
December 1975	1.47	1.10	27	1.13	0.65	18	1.21	0.73	18
January 1976	3.55	1.65	15	16.18	8.20	11	17.71	6.36	12
February 1976	2.54	1.07	33	5.20	3.56	26	3.22	2.71	26
March 1976	154.57	176.31	44	141.85	96.78	32	27.37	34.63	30
April 1976	32.83	14.76	18	147.80	71.25	15	99.20	67.74	15
May 1976	18.00	16.25	24	21.86	17.69	14	51.47	50.57	15
June 1976	300.09	147.01	27	20.25	30.42	15	0.44	1.37	27
July 1976	67.04	89.42	47	25.67	43.91	25	41.92	72.82	27
August 1976	7.94	6.19		18.33	8.89	15	12.67	9.96	15

[a] $\bar{X}$, Mean values of *A. hydrophila*-like bacteria per milliliter.
[b] SD, Standard deviation.
[c] N, Number of determinations.
[d] Experiments with reactor not operating.
[e] ND, Not determined.
[f] Experiments with reactor operating.

TABLE 2. *Chemical and physical parameters at station 1*

Reactor operating conditions	Depth (m)	Temp (°C)	pH	Dissolved oxygen (mg/liter)	Conductivity (μmho/cm²)	Redox E_h (mV)
Not operating	0	28.6	7.9	7.0	80	ND[b]
	1[a]	28.7	7.9	7.0	65	ND
	2	28.7	7.8	6.8	60	ND
	3	28.3	7.6	6.4	60	ND
	4[a]	27.6	7.4	5.9	60	ND
	5	27.2	7.4	5.4	60	ND
	6[a]	25.2	7.2	2.9	70	ND
	7	24.2	7.2	1.6	70	ND
	8[a]	23.0	7.2	0.3	100	ND
	9	22.0	7.2	0.2	110	ND
Operating	0	34.0	7.2	6.7	55	290
	1[a]	33.8	7.4	6.3	60	290
	2	30.5	7.5	6.5	60	295
	3	30.0	6.9	5.9	60	310
	4[a]	29.3	6.6	4.8	60	320
	5	28.5	6.2	1.8	60	340
	6[a]	27.8	6.0	0.3	60	355
	7	25.8	6.1	0.3	70	360
	8[a]	23.5	6.4	0.2	95	220
	9	21.0	6.6	0.2	110	80
	10	20.8	6.7	0.2	110	50

[a] Denotes chamber location.
[b] ND, Not determined.

TABLE 3. *Chemical and physical parameters at station 3*

Reactor operating conditions	Depth (m)	Temp (°C)	pH	Dissolved oxygen (mg/liter)	Conductivity (μmho/cm²)	Redox E_h (mV)
Not operating	0	32.0	6.7	5.4	60	300
	1[a]	32.0	6.8	5.3	60	310
	2	30.5	6.9	5.6	60	315
	3	29.5	6.9	5.7	50	320
	4	28.0	6.9	5.3	60	325
	5[a]	28.0	6.7	2.8	60	340
	6	27.7	6.5	0.1	60	350
	7[a]	25.5	6.7	0.1	70	355
	8	23.5	6.8	0.1	85	340
	9[a]	22.0	6.9	0.0	100	140
Operating	0	38.0	7.0	6.8	60	330
	1[a]	36.0	7.0	6.4	60	330
	2	31.0	6.9	5.4	60	325
	3	30.0	7.0	6.3	60	330
	4	29.5	7.0	6.2	60	330
	5[a]	29.0	6.8	3.7	60	335
	6	26.5	6.4	0.3	60	350
	7[a]	25.5	6.4	0.2	80	365
	8	23.0	6.6	0.2	95	365
	9[a]	22.5	6.7	0.2	100	190
	10	20.5	6.9	0.2	110	140

[a] Denotes chamber location.

crease in optical density that was followed by a decline after an exposure period at station 2 (Fig. 4 and 5) and at stations 3 and 4 (Fig. 6–9, respectively). On the other hand, optical densities of hypolimnetic cultures decreased more slowly and had greater variability.

Survival measurements at station 5 (ambient control) indicated that similar results occurred

TABLE 4. *Chemical and physical parameters at station 5*

Reactor operating conditions	Depth (m)	Temp (°C)	pH	Dissolved oxygen (mg/liter)	Conductivity (μmho/cm^2)	Redox E_h (mV)
Not operating	0	28.5	6.8	7.6	60	375
	1[a]	28.5	6.9	7.0	60	375
	2	28.5	6.9	6.7	60	375
	3	28.5	7.0	6.5	60	375
	4	28.5	7.0	6.4	60	380
	5	28.5	6.9	5.9	60	380
	6[a]	28.0	6.8	5.2	60	390
	7	26.0	6.6	0.2	65	410
	8[a]	22.5	6.8	0.2	80	420
	9	21.5	6.8	0.2	80	410
	10	20.5	6.8	0.2	80	395
	11	20.0	6.9	0.2	85	170
	12	19.0	7.0	0.2	90	110
	13	18.5	6.7	0.2	90	90
	14	18.5	6.7	0.2	90	60
	15	18.0	6.7	0.2	90	40
	16[a]	18.0	6.8	0.2	95	30
	17	18.0	6.8	0.2	95	20
Operating	0	29.5	6.8	7.7	60	390
	1[a]	29.5	6.8	7.2	60	385
	2	29.5	6.9	7.0	60	385
	3	29.5	6.9	6.9	60	385
	4	29.5	7.0	6.8	60	385
	5	29.5	7.0	6.7	65	385
	6[a]	29.0	6.8	5.9	65	390
	7	26.0	6.4	0.4	70	410
	8[a]	23.5	6.5	0.3	80	415
	9	22.0	6.5	0.2	85	415
	10	21.3	6.5	0.2	95	415
	11	20.0	6.8	0.2	100	200
	12	19.5	6.9	0.2	100	130
	13	19.0	6.9	0.2	100	100
	14	18.0	7.0	0.2	100	70
	15	18.0	7.0	0.2	100	55
	16[a]	18.0	7.0	0.2	105	45
	17	18.0	7.0	0.2	100	20

[a] Denotes chamber location.

but less dramatically than in the stations closer to the thermal effluent. Chambers placed in the epilimnion had a rapid decrease in optical density when the reactor was not operating (Fig. 10), whereas a slower decrease in optical density was noted at the same depths when the reactor was operating (Fig. 11). Cultures in chambers placed in hypolimnetic waters were more variable, but indicated that growth and survival were greater in the anoxic portions of the water column when the reactor was operating.

DISCUSSION

Previous studies on the bass populations of Par Pond (G. W. Esch, T. C. Hazen, and J. W. Gibbons, Abstr. Annu. Meet. Am. Soc. Parasitol. 1975) indicated that the occurrence of "red sore disease" was significantly higher among bass captured in heated waters of Par Pond than among those captured in ambient locations. These investigations suggest that elevated temperatures may be a significant variable in the epizootiology of the disease. Esch et al. (Abstr. Annu. Meet. Am. Soc. Parasitol. 1975) also demonstrated that the greatest incidence of infection occurred in the larger bass. Such a distribution suggests that either the infections were lethal to smaller fish, and thus the smaller fish were not measured, or that the larger and older fish have had a longer exposure to the pathogens. Except for these studies, the distribution and physiological ecology of the facultative anerobe, *Aeromonas*, in aquatic ecosystems and its role as a fish pathogen in

TABLE 5. *Distribution and population densities of A. hydrophila-like bacteria in Par Pond*

Station	Depth (m)	*Aeromonas*/liter							
		Reactor operating				Reactor not operating			
		Sample 1	Sample 2	Sample 3	$\bar{v}$[a]	Sample 1	Sample 2	Sample 3	$\bar{v}$
No. 3	0	350	400	ND[b]		20	0	0[c]	
	1	340	227	240		0	0	0	
	2	20	40	ND	280	0	5	20	4.3
	3	780	706	260		0	8	20	
	4	60	220	540		0	0	0	
	5	60	74	160[d]		0	0	5	
	6	600	724	840		0	2	20	
	7	800	867	780		40	8	20	
	8	500	453	600	596	500	323	420	226
	9	740	667	660		380	500	500	
	10	200	227	280		ND	ND	ND	
	0	60	40	40		120	80	100	
	1	6	0	0		300	280	ND	
	2	0	0	0		400	233	260	
No. 5	3	1,200	1,260	1,300		120	180	120	136
	4	640	600	492	472	180	100	100	
	5	1,780	1,640	1,860		60	40	60	
	6	1,640	1,120	ND		0	0	0	
	7	0	0	0		420	520	560	
	8	0	0	0		120	240	140	
	9	0	0	0		1,640	800	960	
	10	0	0	0		1,000	1,180	1,040	
	11	3,000	3,600	240		740	960	1,120	
	12	>6,000	4,200	5,000		580	400	ND	894
	13	>6,000	>6,000	>6,000	3,969	260	580	ND	
	14	5,200	4,800	2,100		840	720	840	
	15	>6,000	>6,000	ND		1,400	1,360	1,380	
	16	>6,000	>6,000	ND		1,440	2,000	1,800	
	17	>6,000	>6,000	ND		ND	ND	ND	

[a] $\bar{v}$, Mean of *Aeromonas* determinations for epilimnetic and hypolimnetic.
[b] ND, Not determined.
[c] Actual numbers are less than 2/liter.
[d] Dashed line denotes chemocline position.

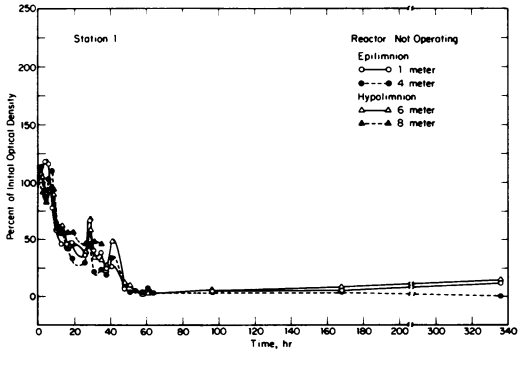

FIG. 2. *Survival of A. hydrophila at station 1 when the reactor was not operating.*

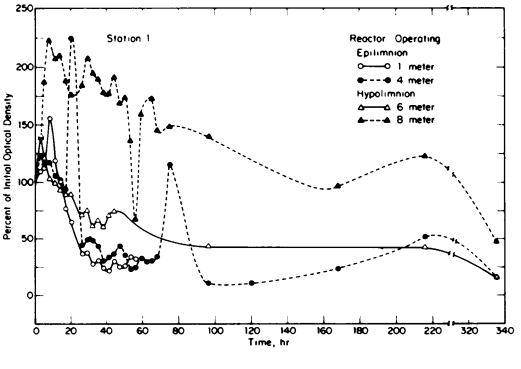

FIG. 3. *Survival of A. hydrophila at station 1 when the reactor was operating.*

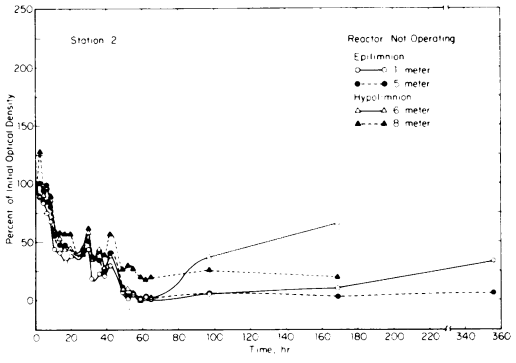

FIG. 4. *Survival of A. hydrophila at station 2 when the reactor was not operating.*

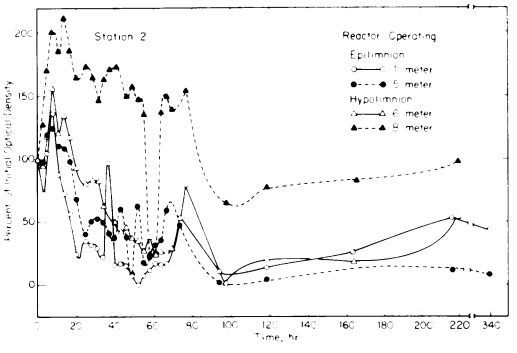

FIG. 5. *Survival of A. hydrophila at station 2 when the reactor was operating.*

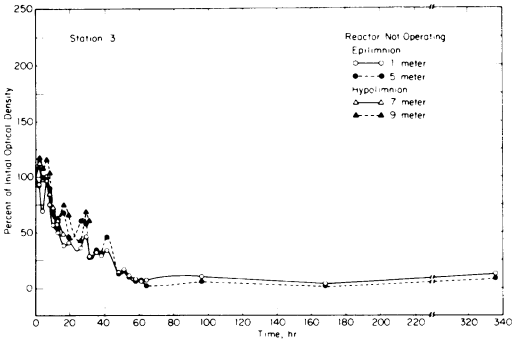

FIG. 6. *Survival of A. hydrophila at station 3 when the reactor was not operating.*

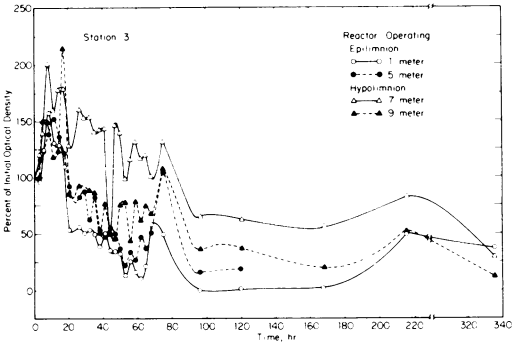

FIG. 7. *Survival of A. hydrophila at station 3 when the reactor was operating.*

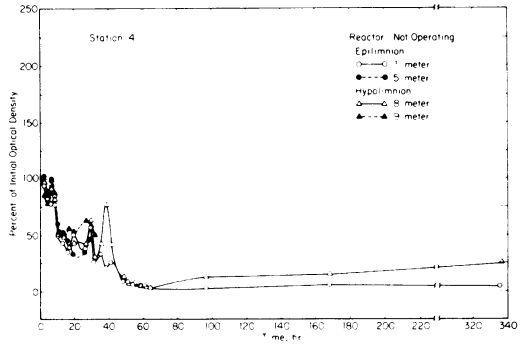

FIG. 8. *Survival of A. hydrophila at station 4 when the reactor was not operating.*

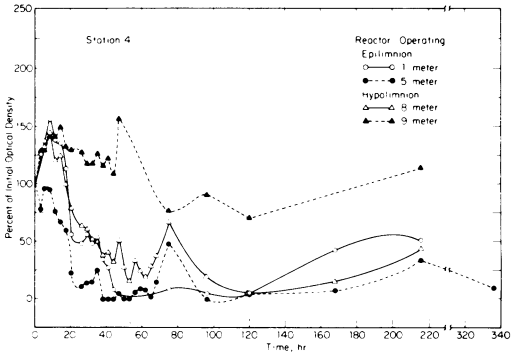

FIG. 9. *Survival of A. hydrophila at station 4 when the reactor was operating.*

thermally stressed waters are virtually unknown.

Bacteria obtained from ATCC were chosen preferentially over Par Pond-isolated aeromonads for the following reasons. (i) Biochemical and physiological parameters of over 200 isolates were virtually identical to the ATCC strain with regard to the API 20E (Analy Tab, Plainview, N.Y.) testing system (15). (ii) Although serologically the isolates, as measured by immunofluorescence, seem to be of three types (C. B. Fliermans and T. C. Hazen, un-

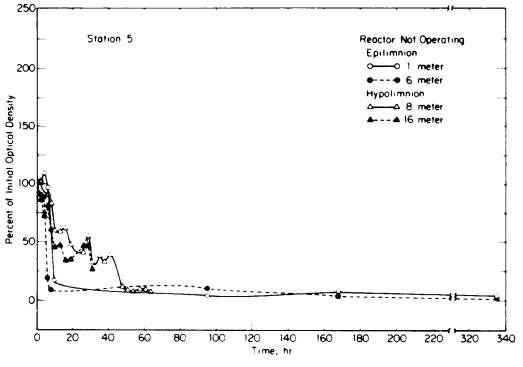

FIG. 10. *Survival of A. hydrophila at station 5 when the reactor was not operating.*

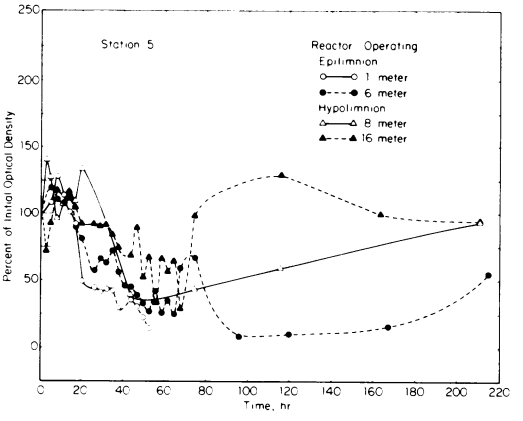

FIG. 11. *Survival of A. hydrophila at station 5 when the reactor was operating.*

published data), Par Pond isolates appear more virulent to the large mouth bass than are the ATCC strains. Because of this virulence, it was thought unwise to subject the Par Pond fish population to potentially large doses of highly virulent *Aeromonas* strains if one of the chambers were ruptured by an alligator attack.

The individual techniques for measuring natural population densities, distribution, and pure culture survival produced results that permit similar conclusions regarding the growth and survival of *A. hydrophila* in Par Pond to be made.

Survival studies of *A. hydrophila* at various temperature and oxygen regimes indicated that the organisms maintained themselves better in the deeper hypolimnetic waters. In hypolimnetic oxygen-depleted waters, at each station in the thermal and ambient portions of the reservoir, *A. hydrophila* survived longer and increased in density to a greater extent than in epilimnetic waters at the same station.

Comparisons among stations demonstrated that when the reactor was not in operation the percentage of initial optical density approached zero after 60 h of in situ incubation in every chamber at all stations. However, when the reactor was operating, only chambers in the epilimnion of station 4 were near 0% of their initial optical density after 60 h of incubation. All other chambers, regardless of depth or station, had greater survival of *A. hydrophila*. The data are clear when a comparison is made of survival at the same station with two different reactor operations, in that survival and growth were always better when the reactor was in operation. This was true regardless of the depth, even at depths in the water column where the influence of reactor input could not be detected by the parameters measured. It is necessary, however, to emphasize that the sampling time between the reactor operations, and thus the experiments, was 21 days. Therefore, the natural stratification of Par Pond had advanced by 3 weeks when survival and distribution experiments were undertaken for full reactor operations. This is reflected in Table 2 by the vertical extension of the chemocline at station 1, which is closest to the thermal input.

Although the population densities of *A. hydrophila*-like bacteria in Par Pond did not increase from August to September, the time of the two experiments, the lowest seasonal densities occurred in late summer and late winter (Table 1). Thus, the experiments were conducted when the least flux in *A. hydrophila*-like densities occurred. Although small changes in the stratification patterns and seasonal distribution densities occurred between studies, the observed differences in survival of *A. hydrophila* were probably not seasonal phenomena but reactor operations phenomena.

Other southeastern lakes and streams do not have the high population densities of aeromonads as seen in Par Pond (C. B. Fliermans, unpublished data). Thus, the large population densities of aeromonads and the high infection levels of the large mouth bass populations in Par Pond may be due to the length of survival of *A. hydrophila* in the water column. Such survival may expose a given bass to high levels of aeromonads for a large portion of its life.

Although it is unclear whether the survival of aeromonads in Par Pond is due directly to thermal inputs or is indirectly related to flow rates and/or nutrient distributions caused by reactor operations, it is clear the aeromonads are ubiquitous throughout Par Pond and that the bass populations are heavily infected by the etiological agent(s) of red sore disease.

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