DP-MS-75-115

CONF-760562--1

# *AEROMONAS* DISTRIBUTION AND SURVIVAL IN A THERMALLY ALTERED LAKE*

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For presentation at the Annual Meeting of the *American Society for Microbiology*, in Atlantic City, NJ on May 2-7, 1976, and for publication in *Applied and Environmental Microbiology*.

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* This paper was prepared in connection with work under Contract No. AT(07-2)-1 with the U. S. Energy Research and Development Administration. 

## DISCLAIMER

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## ABSTRACT

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. Greater population densities of *Aeromonas* occurred below the oxygen chemocline when the lake was stratified. Survival of *A. hydrophila* under *in situ* conditions in both epilimnetic and hypolimnetic waters was determined using 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* below the chemocline when the reactor was in full operation suggests that the fish populations may be exposed to *Aeromonas* for a longer period of time than when the reactor is not operating.

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* The information contained in this article was developed during the course of work under Contract No. AT(07-2)-1 with the U.S. Energy Research and Development Administration, and Contract No. E-(38-1)-900 between Wake Forest University, Winston-Salem, N.C. and the U.S. Energy Research and Development Administration.

## INTRODUCTION

Several species of *Aeromonas* are pathogenic to fish, frogs, and a variety of reptiles (1) as well as man (5, 11). Such wide host infections are termed epizootic and often occur in combination with the peritrichous ciliate, *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 (3). The disease is common in the southeastern U.S. and has reached epidemic proportions on occasion, causing large fish kills (7, 8, 9, 10).

Although *Aeromonas* appears to be an ubiquitous aquatic bacterium (13, 15, 16), previous studies (12) 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. The specific study site was Par Pond, a 1092-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 while 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 Figure 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 multi-probe analyzer (Hydrolab Corp., Austin, Texas). These parameters were recorded during each reactor phase (Tables 1, 2, 3), i.e. when the reactor was operating and releasing thermal effluent and when it was not.

### Culture Studies

Type cultures of *Aeromonas hydrophila* 7966, *A. hydrophila* 19570, *A liquefaciens* 14715, *A. proteolytica* 15338, and

*A. salmonicida* 14174 were obtained from the American Type Culture Collection (ATCC) for use in comparison with 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 R-S medium (14). Organisms utilized in pure culture studies were *A. hydrophila*, while naturally occurring aeromonads isolated from Par Pond on R-S 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 taken at 1.0-meter depth intervals throughout the water column in both the ambient and thermally altered portions of Par Pond. Triplicate water samples from each depth were taken with an alcohol-rinsed Kemmerer bottle. The samples were immediately placed in sterile Whirl-pac bags and returned to the laboratory for processing. Known aliquots were filtered through sterile 0.45-μ Millipore (Bedford, Mass.) filters and placed on sterile pads saturated with R-S medium and incubated for 20 hours at 30°C. The filters were then examined for yellow pigmented colonies which are presumptive *A. hydrophila*. These colonies were then tested for cytochrome oxidase (4), and positive oxidase cultures were taken to be *Aeromonas hydrophila*-like organisms.

## Survival Studies

Type cultures of *A. hydrophila* were checked for purity on R-S medium, transferred, and routinely grown in nutrient broth at 30°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 (6) as modified for deep water studies (Fliermans and Gordon, submitted for publication). 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 5% 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 hours for 48 hours, then once every 24 hours through the remainder of the experiment. Samples, taken aseptically using sterile

plastic syringes, were measured for optical density and tested for diffusion chamber purity by immunofluorescence (Fliermans, unpublished data) and/or plating on a selective medium. Sampling ports in the chambers were flamed with a butane cigarette lighter, a syringe was attached, aspirated five times, and then 1.0-ml samples were removed from each chamber. Samples were placed directly in sterile culture tubes, capped, and returned to the laboratory within 60 minutes for processing. Optical densities were determined at 550 nm using a Beckman Model 25 double beam spectrophotometer. Optical density measurements are 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% and values greater than 100% represented an increase in optical density, while 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 were formed

which interfered with optical density measurements. Thus, solutions inside the control chamber were used as reference samples in double beam spectroscopy for optical density measurements.

## RESULTS

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 1, 2, 3). 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 (14) who previously demonstrated the good selectivity and specificity of the medium. The population densities (expressed as the mean of triplicate samples) are shown in Table 4 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, the depth of thermal stratification, and the 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 Figures 2 through 11.

The data in Figures 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, while Figures 3, 5, 7, 9, and 11 show survival data when the reactor was operating. *A. hydrophila* populations demonstrated initial growth and/or maintenance in all the chambers regardless of the depth. 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 initially increased in optical density followed by a decline after an exposure period at Station 2 (Figures 4 and 5) and at Stations 3 and 4 (Figures 6, 7, 8 and 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, 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 (Figure 10), while a slower decrease in optical density was noted at the same depths when the reactor was operating (Figure 11). Cultures in chambers placed in hypolimnetic waters were 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 (3), 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 suggested that elevated temperatures may be a significant variable in the epizootiology of the disease. Esch *et al.* (3) also demonstrated that the greatest incidence of infection occurred in 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 anaerobe, *Aeromonas*, in aquatic ecosystems, and its role as a fish pathogen in thermally stressed waters is virtually unknown.

The individual techniques for measuring natural population densities, distribution, and pure culture survival produced

results which permit similar conclusions to be made regarding the growth and survivorship of *A. hydrophila* in Par Pond.

Survival studies of *A. hydrophila* at various temperature and oxygen regimes indicated that the organisms maintained themselves better in the deeper hypolimnetic waters. Thus, 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 hours 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 zero percentage of their initial optical density after 60 hours 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, and even at depths in the water column where the influence of reactor input could not be detected by the parameters measured. It is necessary to emphasize, however, 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 three weeks when survival and distribution experiments were undertaken for full reactor operations. This is reflected in Table 3 by the vertical extension of the chemocline at Station 5, which is closest to the thermal input. Although the presented data are reflective of reactor operations, the described changes in survival of *A. hydrophila* in deeper waters may be confounded in part by the increase of natural stratification.

Facultative anaerobic metabolism may provide a selective advantage to *Aeromonas* for growth in the anaerobic zones of Par Pond, and that advantage is enhanced by the operation of the nuclear reactor, since the natural population densities of aeromonads are greater throughout the water column when the reactor is operating than when it is not. Other southeastern lakes do not have the high population densities of aeromonads as seen in Par Pond (Fliermans, unpublished data). Thus, the large population densities of aeromonads and the high infection levels of the largemouth bass populations in Par Pond may be due to the length of survival of *A. hydrophila* in the water column. Such survival may allow a given bass to be exposed to high levels of aeromonads for a greater 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 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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TABLE 1. *Chemical and physical parameters at Station 1*

| Depth, m | Temperature, °C | pH | Dissolved Oxygen, ppm | Conductivity, μ mho/cm$^2$ | Redox Eh, mV |
|---|---|---|---|---|---|
| | | | Reactor 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 |
| | | | Reactor 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 2. *Chemical and physical parameters at Station 3*

| Depth, m | Temperature, °C | pH | Dissolved Oxygen, ppm | Conductivity, μ mho/cm$^2$ | Redox Eh, mV |
|---|---|---|---|---|---|
| | | | Reactor 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 |
| | | | Reactor 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.

TABLE 3. *Chemical and physical parameters at Station 5*

| Depth, m | Temperature, °C | pH | Dissolved Oxygen, ppm | Conductivity, μ mho/cm$^2$ | Redox Eh, mV |
|---|---|---|---|---|---|
| | | | Reactor 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 |
| | | | Reactor 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.

TABLE 4. *Distribution of* Aeromonas hydrophila-*like bacteria in Par Pond*

| Depth, m | Reactor Operating | | | Reactor Not Operating | | |
|---|---|---|---|---|---|---|
| | *Aeromonas* per liter | | | | | |
| | Station 3 | | | | | |
| 0 | 350 | 400 | ND[a] | 20 | 0 | 0[b] |
| 1 | 340 | 227 | 240 | 0 | 0 | 0 |
| 2 | 20 | 40 | ND | 0 | 5 | 20 |
| 3 ($\bar{v}$ = 280)[d] | 780 | 706 | 260 | ($\bar{v}$ = 4.3)[d] 0 | 8 | 20 |
| 4 | 60 | 220 | 540 | 0 | 0 | 0 |
| 5 | 60 | 74 | 160 | 0 | 0 | 5 |
| 6 | 600 | 724 | 840 | 0 | 2 | 20 |
| 7 | 800 | 867 | 780 | 40 | 8 | 20 |
| 8 ($\bar{v}$ = 596)[d] | 500 | 453 | 600 | ($\bar{v}$ = 226)[d] 500 | 323 | 420 |
| 9 | 740 | 667 | 660 | 380 | 500 | 500 |
| 10 | 200 | 227 | 280 | ND | | |
| | Station 5 | | | | | |
| 0 | 60 | 40 | 40 | 120 | 80 | 100 |
| 1 | 6 | 0 | 0 | 300, | 280 | ND |
| 2 | 0 | 0 | 0 | 400 | 233 | 260 |
| 3 ($\bar{v}$ = 472)[d] | 1200 | 1260 | 1300 | ($\bar{v}$ = 136)[d] 120 | 180 | 120 |
| 4 | 640 | 600 | 492 | 180 | 100 | 100 |
| 5 | 1780 | 1640 | 1860 | 60 | 40 | 60 |
| 6 | 1640 | 1120 | ND | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 420 | 520 | 560 |
| 8 | 0 | 0 | 0 | 120 | 240 | 140 |
| 9 | 0 | 0 | 0 | 1640 | 800 | 960 |
| 10 | 0 | 0 | 0 | 1000 | 1180 | 1040 |
| 11 | 3000 | 3600 | 240 | 740 | 960 | 1120 |
| 12 | >6000 | 4200 | 5000 | 580 | 400 | ND |
| 13 | >6000 | >6000 | >6000 | 260 | 580 | ND |
| 14 ($\bar{v}$ = 3969)[d] | 5200 | 4800 | 2100 | ($\bar{v}$ = 894)[d] 840 | 720 | 840 |
| 15 | >6000 | >6000 | ND | 1400 | 1360 | 1380 |
| 16 | >6000 | >6000 | ND | 1440 | 2000 | 1800 |
| 17 | >6000 | >6000 | ND | ND | | |

[a] Not determined.

[b] Actual numbers are less than 2/liter.

[c] Dashed line denotes chemocline position.

[d] $\bar{v}$ = mean of *Aeromonas* determinations for epilimnetic and hypolimnetic waters.

LIST OF FIGURES

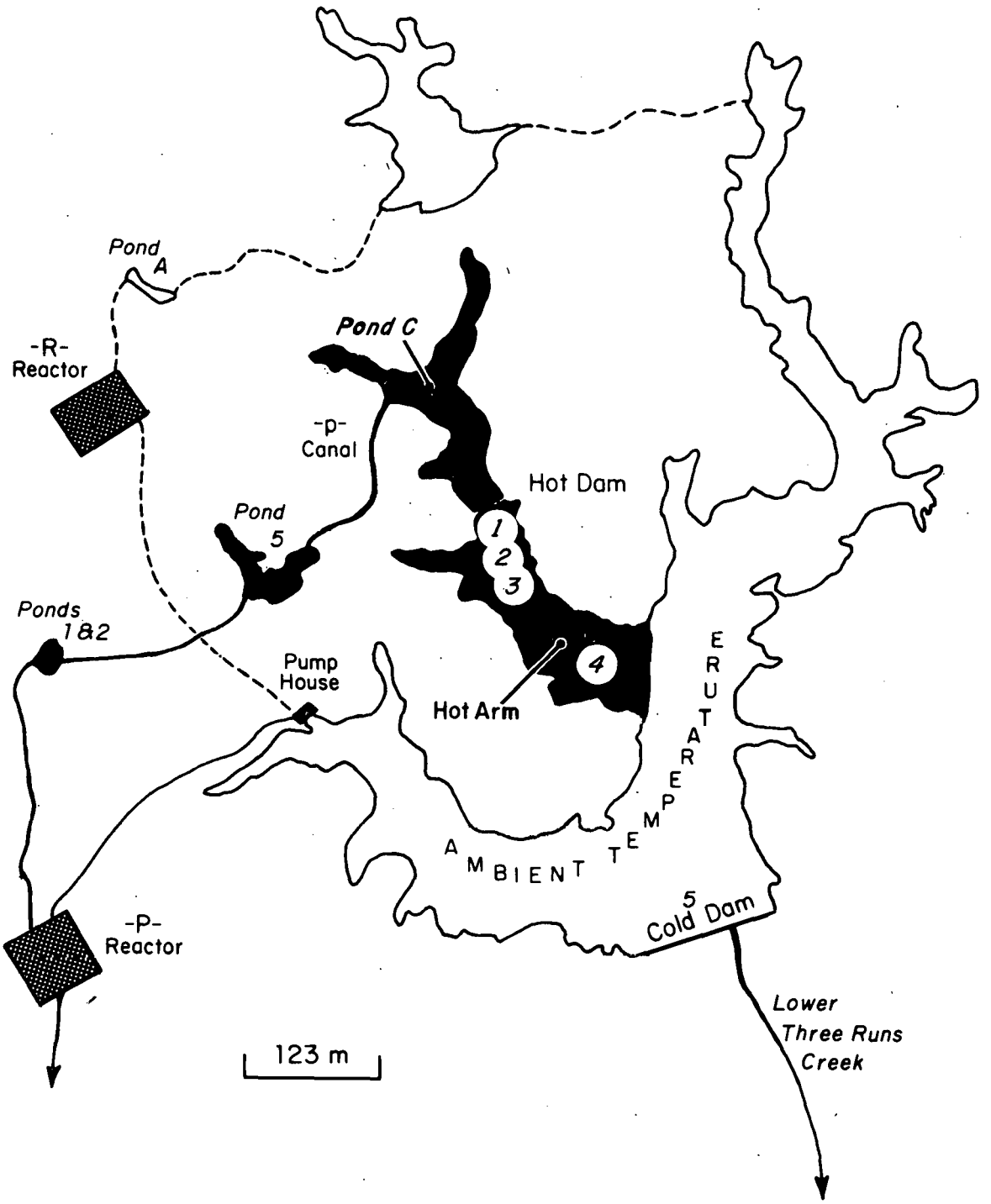


Fig. 1. Par Pond system showing the ambient and thermal (shaded) temperature regions. Sampling stations are numbered.

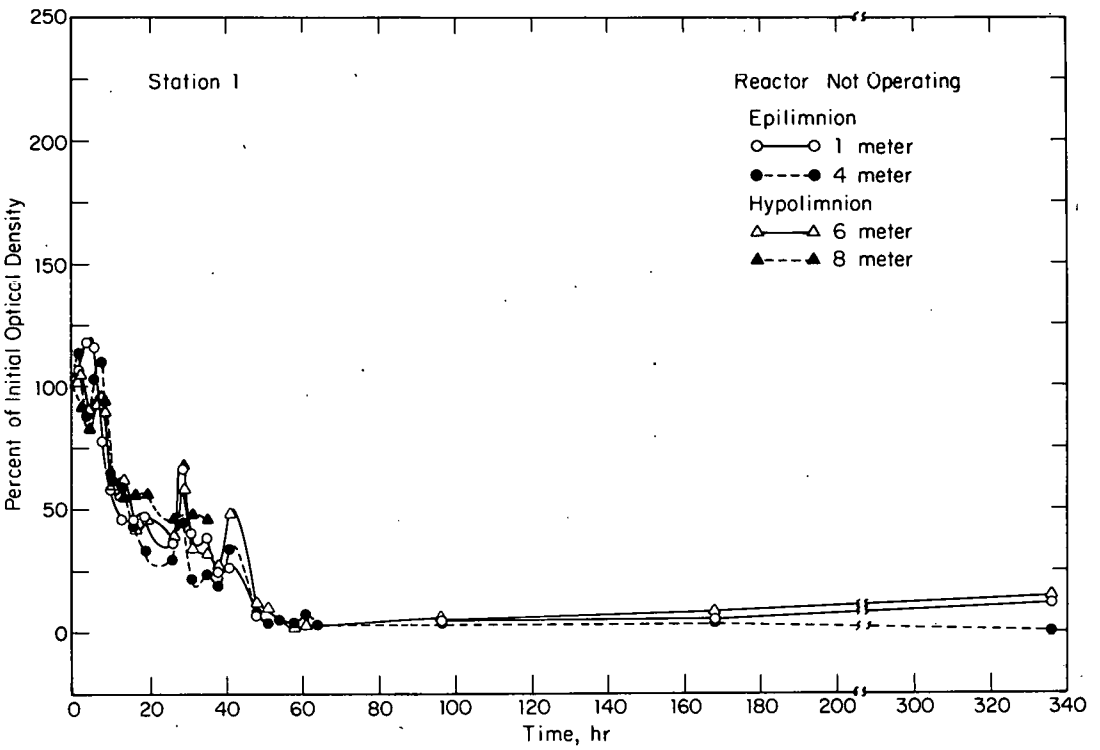


Fig. 2. Survival of *A. hydrophila* at Station 1 when the reactor was not operating.

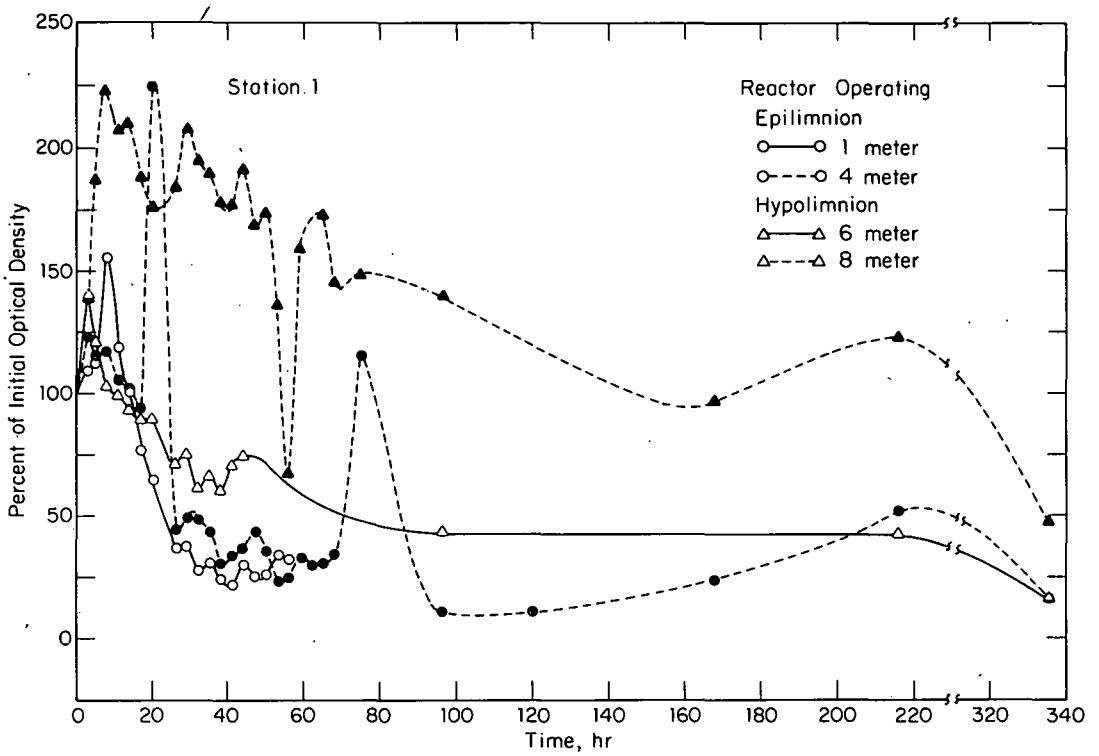


Fig. 3. Survival of *A. hydrophila* at Station 1 when the reactor was operating.

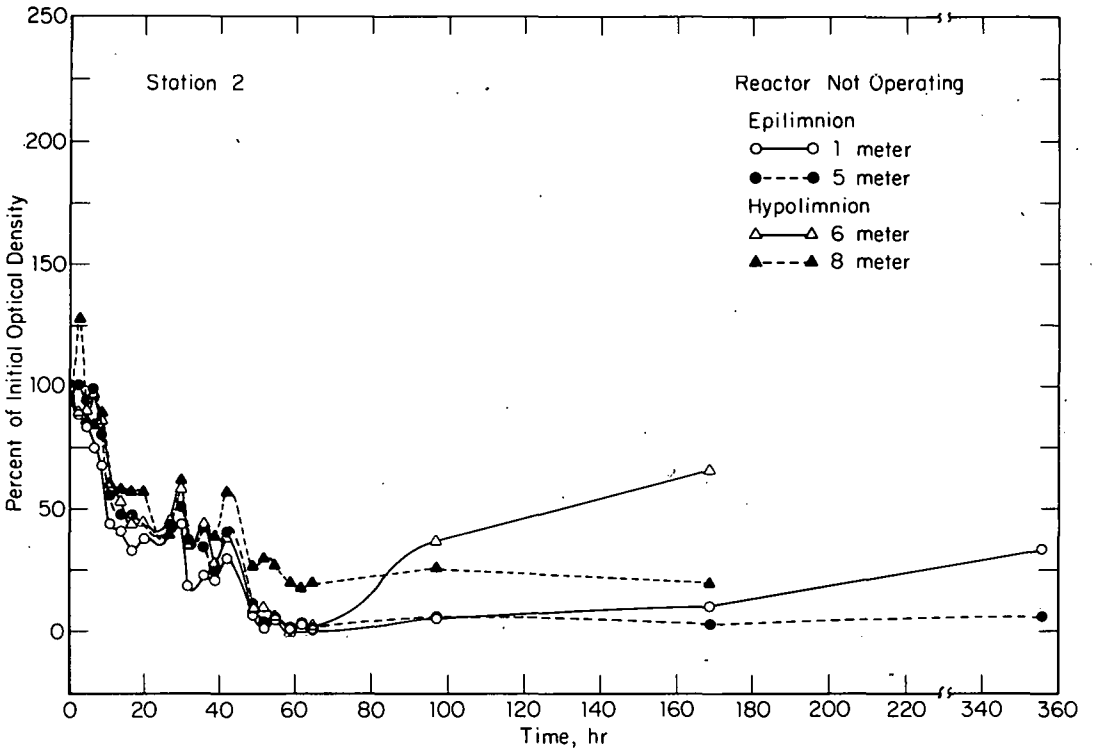


Fig. 4. Survival of *A. hydrophila* at Station 2 when the reactor was not operating.

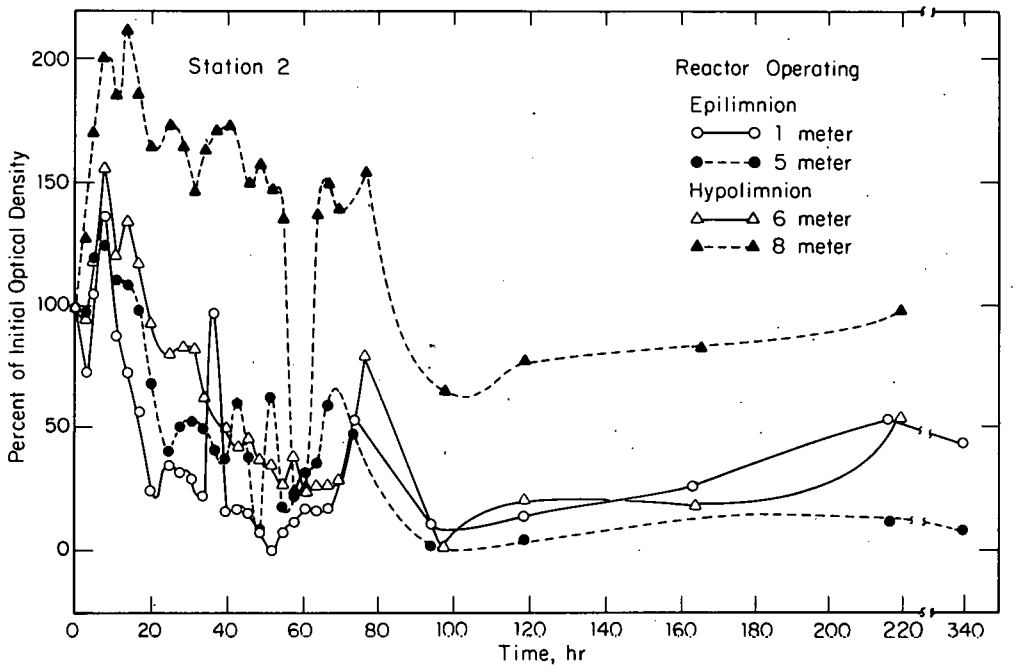


Fig. 5. Survival of *A. hydrophila* at Station 2 when the reactor was operating.

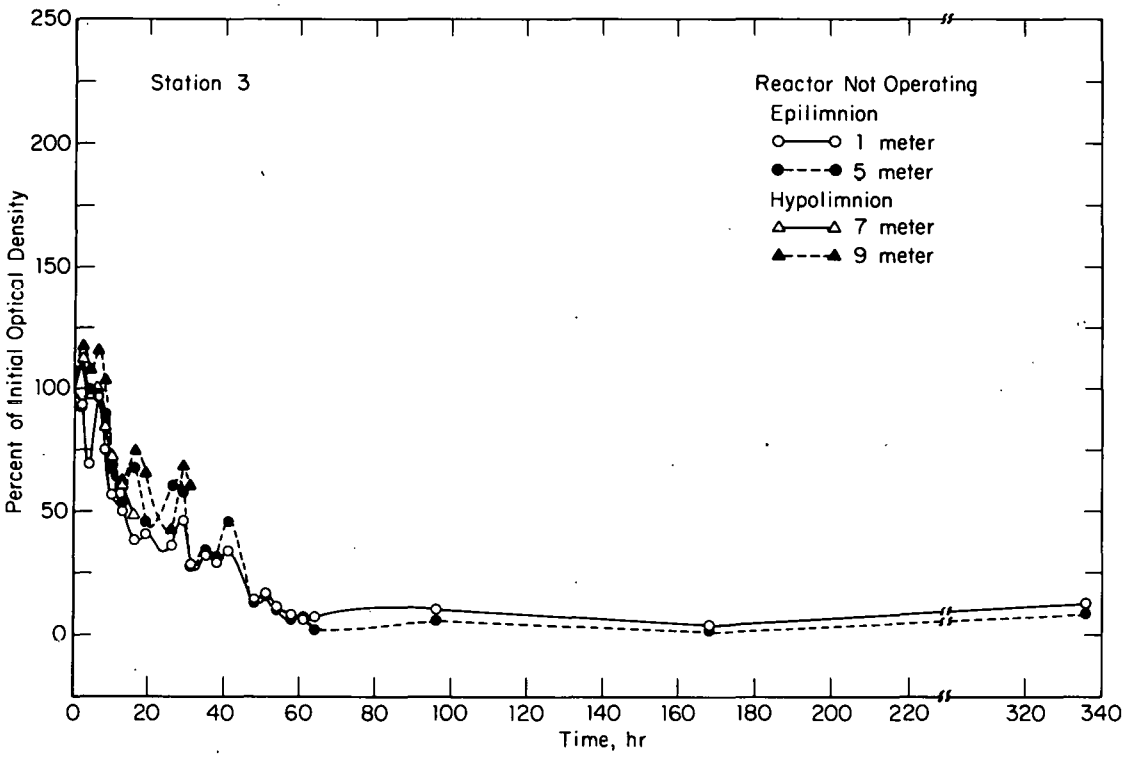


Fig. 6. Survival of *A. hydrophila* at Station 3 when the reactor was not operating.

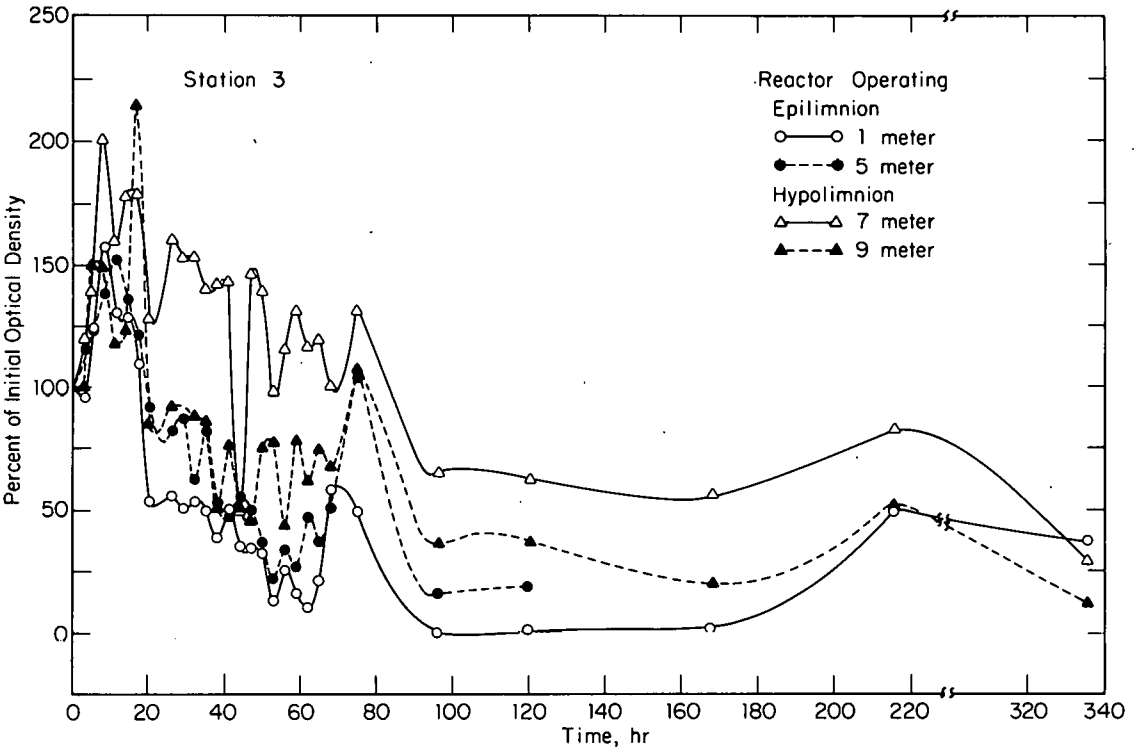


Fig. 7. Survival of *A. hydrophila* at Station 3 when the reactor was operating.

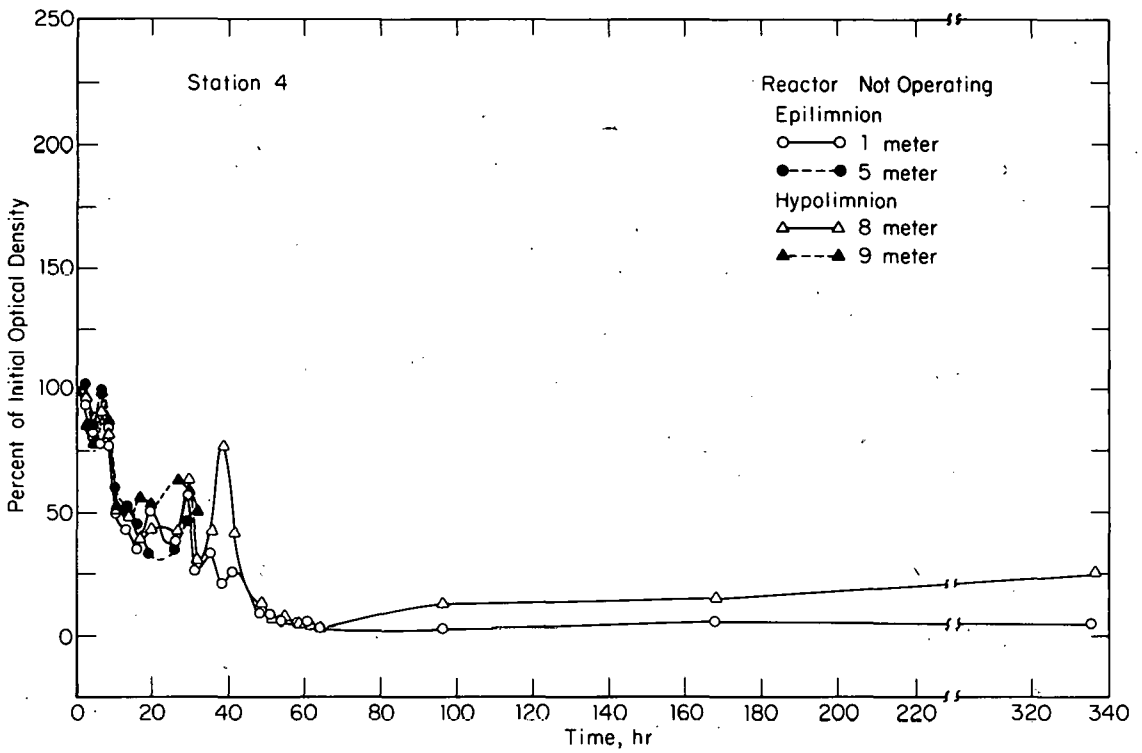


Fig. 8. Survival of *A. hydrophila* at Station 4 when the reactor was not operating.

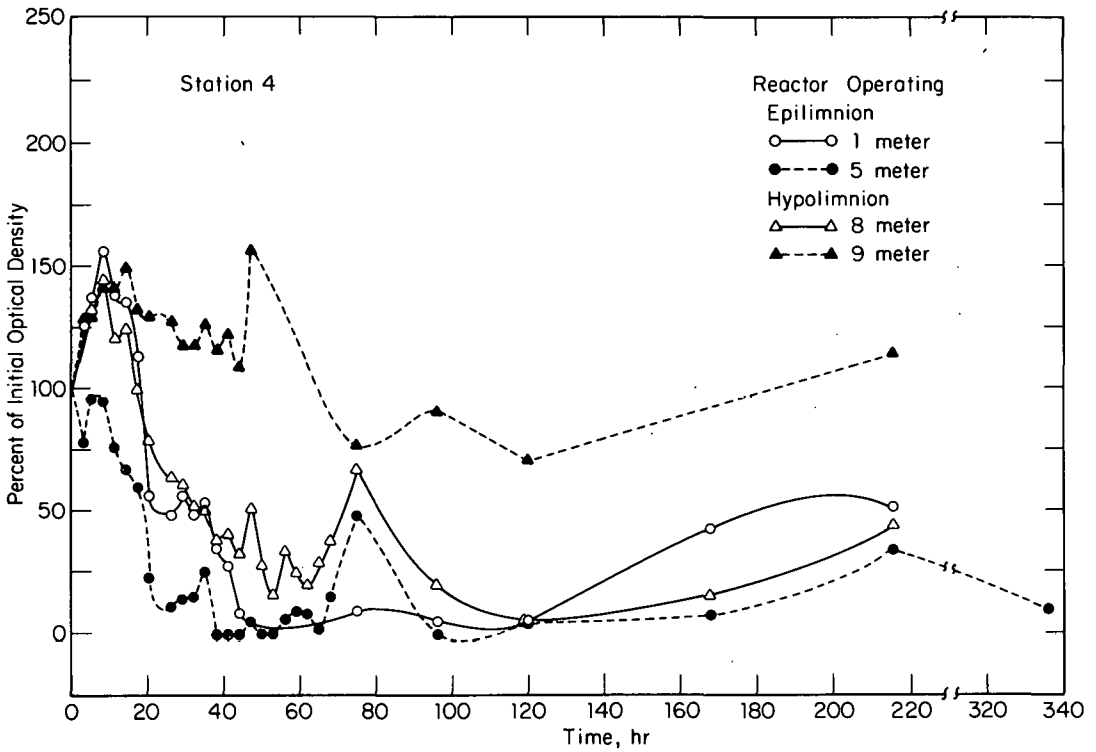


Fig. 9. Survival of *A. hydrophila* at Station 4 when the reactor was operating.

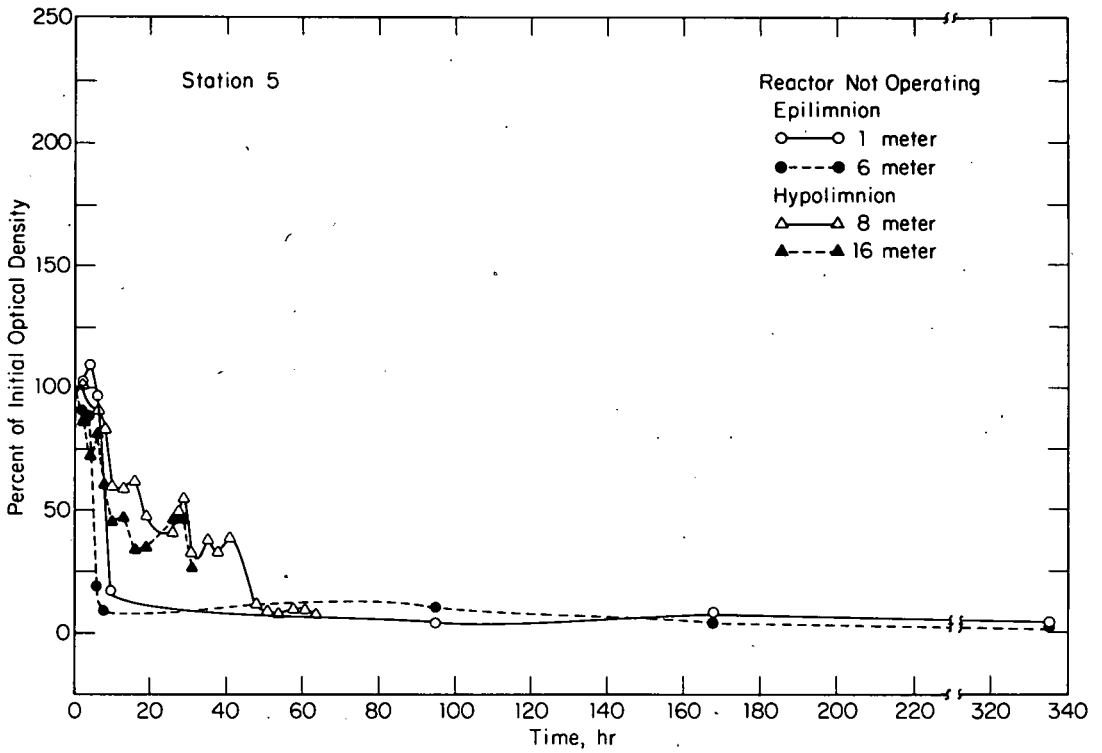


Fig. 10. Survival of *A. hydrophila* at Station 5 when the reactor was not operating.

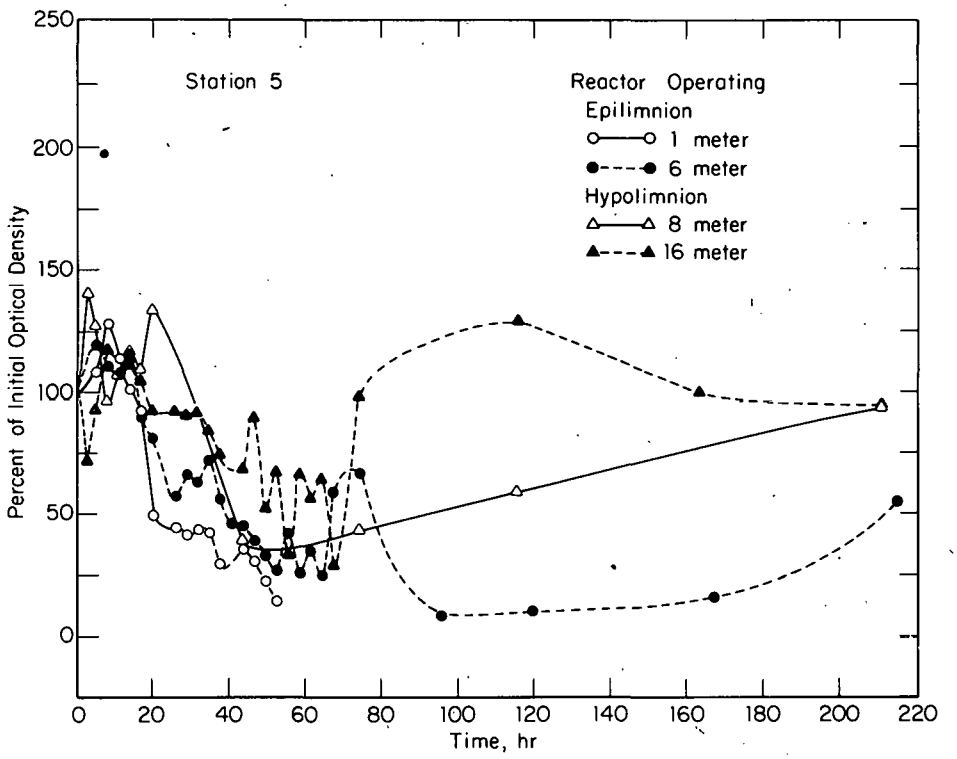


Fig. 11. Survival of *A. hydrophila* at Station 5 when the reactor was operating.