ENVIRONMENTAL EPIDEMIOLOGY. II. ECOLOGICAL STUDIES ON *VIBRIO* SP. IN CANAL AND TANK ENVIRONMENTS

W.M. Spira
K.M.S. Aziz
W.F. Verwey

Introduction

Contaminated water sources are strongly associated with the spread of cholera and other diseases transmitted by the fecal — oral route. The environmental transmission of *V. cholerae* in particular seems to be centered on water. Epidemiologic research on cholera has established a clear picture of the spectrum of the disease and has accurately characterized the general nature of endemic environments, both physical and social. It has, however, been unable to supply definitive answers to several questions. Those of particular concern to this study are the seasonality of cholera and the maintenance of *V. cholerae* during inter-epidemic periods. Answers to these questions, especially the first, are very likely to require a knowledge of the specific behaviour of *V. cholerae* in natural waters as well as a broader characterization of seasonal variations in tank and canal ecology.

The approach we have taken in this study is a systematic observation of a small number of water sampling points in an area which has a history of being at high risk for cholera. We have focused mainly on detecting variations in the concentration of *Vibrio* sp. in different habitats as a function of season. In this regard, we have monitored certain chemical, physical and meterological parameters as well. We have also included measure-

ment of total microorganism and plantation concentration, of fecal indicator organisms and of other clinically important organism (pathogens and antibiotic resistant bacteria).

The primary objective is to determine the seasonal variation in Vibrio sp. population and to assess the role of various microenvironments (water, sediment, plants, phyto and zoo-plankton in the maintenance of these organisms. In particular, we are looking for conditions or microenvironments which are conducive to the survival and, perhaps, maintenance of V. cholerae. As a secondary objective, we are attempting to assess whether such seasonal variations are part of a more general seasonal effect on tank or canal ecology. The screening for clinically important bacteria has been included to evaluate their extent in the non-human environment in this area.

Materials and Methods

Sampling was carried out at 28-day intervals at selected tank and canal points in Meharon from March 1976 to the present. A shift to fewer samples and more extensive analysis occurred in October. From March to October, water samples only were collected at weekly intervals and only from near the surface. These were analyzed for Vibrio sp. and some physico-chemical properties.

Water samples were taken from the surface, middle of the water column and from the mud/water interface. Plants (primarily water hyacinth) were collected at each point. A plankton sample was obtained by sieving water through a net. Sediment samples were obtained using a core sampling device. Water samples were also collected from points in Baragaon during the period March - July 1976. Water and plant samples were collected from a number of water sources in the Matlab VTS area during the fall of 1976 as well.

The pH, dissolved oxygen tension, (CO_2) and temperature of water point at 0600, 1200 (sampling time) and 1800 was determined on - site. Weather observations were made daily at the Matlab laboratory.

The sites monitored were a large tank (100 x 200') used for all purposes by a population of 100 - 150 and the canal near the main ghat which is extensively used as a site for bathing and gathering cooking water. Water sample points were established in the center of the tank and along the edge at a point 30 ft. from the main ghat and approximately 10 feet from a latrine area used by 4-6 persons. Sampling points in the canal were established at 30 feet directly out from the main ghat and at the edge of this ghat.

Microbiological examination of samples included total aerobic heterotrophic plate count; coliform, E. coli, fecal streptococci and antibiotic resistant bacteria counts; and enrichment for Salmonella and Shigella sp. Vibrio sp. were detected using a membrane filtration technique in which the cells were allowed to grow on starch agar for 4 h at ambient temperature prior to being transferred to TCBS at 37C. This procedure significantly improved the isolation of vibrios over direct plating on TCBS. Vibrio isolates were characterized by reaction in KIA and MIU, fermentation of sucrose, mannose and arabinose, sensitivity to O/129 vibriostatic compound and by agglutination in O Group I antisera. The plankton biomass in water samples was estimated by microscopic count.

Results and Discussion

The weather normals for the Matlab area are given in Figure 1. These three climatic parameters (rainfall, temperature and sunlight) along with nutrient input tend to be the dominant determinants of natural water ecosystems. It is only in relatively extreme situations, such as the presence of industrial wastes, that other factors become equally important. It is possible to distinguish four seasons from the data in Figure 1: June - September (high rainfall and temperature, low sunlight);

September - December (decreasing rainfall and temperature, increasing sunlight); December - February (low rainfall and temperature, high sunlight); and February - June (increasing rainfall and temperature, decreasing sunlight). The weather monitoring in Matlab as well as observation obtained from the weather station at Narayanganj since November has proven to be fairly close to the 30 - year normals. The noon temperature at 60 cm depth in both tank and canal (Figure 2) closely parallels the maximum in temperature during the warmer months but tends to be closer to the temperature minimum during the colder months. The great difference in mixing between the two water sources had no demonstrable affect on water temperature.

We observed a very strong tendency for a DoT gradient to form in the stationary tank water as the day progressed (Figure 3a). DoT at the tank surface was in excess of equilibrium concentration and, hence, unstable while the DoT at 150 cm and below was too low to measure. The oxygenated zone deepened as the day progressed and this effect became more marked in December - January as a result of increased photosynthetic activity in response to more sunlight. Lower temperatures did not appear to inhibit photosynthetic activity although overall oxygen demand is reduced as indicated by the shift in sunrise DoT profile. The well-mixed canal water column failed to develop a significant DoT gradient (Figure 3b), though an increase in DoT at later sampling times

is apparent. The seasonal influence on DoT is also apparent in this sytem. The thorough mixing, however, prevents the overnight oxygen depletion observed in the tank water column.

A similar pattern exists for pH (Figure 4a-b) which is highly correlated with CO_2 concentration and, thus, strongly linked to the level of photosynthetic activity. The pH of these sampling sites does not appear to be strongly influenced by laundry soaps at the level they are used at present.

Data on the concentration of various life-forms others than Vibrio sp. is available only since November. Analysis is incomplete but data for the seasonal extremes so far will be presented. The concentration of phytoplankton (Table 1) is, in general, higher at the surface than deeper in the water column by about a factor of 10. The concentration is somewhat lower in January than in November and lower in the canal than in the tank. No difference between edge and center of water sources was observed.

The seasonal change in total heterotrophic aerobe concentration (Table 2) shows a similar decrease with colder weather, particularly in the center of water sources. The count at the edges is less affected by season and possibly reflect an increased role of human contamination of these sites in adding to the bacterial population. The concentration is greater at the surface

and mid-water interface than in the center of the water column in the tank. The thorough mixing in the canal prevented this layering to a great extent. Gram negative organisms comprise the bulk of the surface microflora while the mud-water interface population is more than half Gram positive bacteria.

The coliform count (Table 3) shows no layering effect but does show the effect of season. This count is primarily Enterobacteriaceae. The concentration of E. coli varies widely between samples and shows some effect of edge vs center, the edge having a higher concentration. A seasonal effect is not apparent thus far with this data. The fecal coliform/fecal streptococci ratios are consistently in the range of 1-7 indicating that virtually all fecal contamination in our sampling points can be attributed to humans.

Isolations of Vibrio sp. for the past year are shown in Table 4. No V. cholerae biotype El Tor were isolated during this period and Meharon experienced no demonstrated cases of cholera. This "unfortunate" situation points out one difficulty with this type of study. Isolation of the so-called "NAG vibrios" (V. cholerae, NAG) were frequent throughout the study period. Heiberg groups II and V dominated these isolation, though groups I, III and VII were occasionally found as well. A statistically significant decrease in the frequency of isolations occurred in

December - January. Surprisingly, the frequency of isolation during the monsoon period was not lower than during the diarrhea peak periods of Spring and Fall.

The concentration of NAG vibrios in positive water samples (Table 5) shows no significant seasonal trend. It is clear, however, that the frequency and concentration of NAG vibrios in these water sources during the monsoon period are little different than during the peak diarrhea seasons.

Heiberg groups I and II have traditionally been thought to contain most of the human — associated NAG vibrios while those in group V were usually considered water vibrios. Table 6 shows the concentration of group I and II organisms at the various sampling points. No differences are seen in the tank while the samples taken at the canal ghat are significantly higher than those in the center. Other data collected recently at other points in the tank indicate that the concentration of NAG vibrios directly at the main ghat can be extremely high (10^4/ml) without a noticeable change occuring at the sampling point 30 feet away. Apparently the stratification in this water source is intense both horizontally and vertically. Data for group V organisms show no statistically significant difference between sampling points as would be expected if these were true inhabitants of the water system. This may also be the reason why the group V

organisms managed somewhat better than other groups during the monsoon season (Table 4).

Vibrio sp. were not isolated from sediment, phyto - or zooplankton specimens taken at any time since November. A significant association was found, however, between Group V NAG vibrios and water hyacinths collected at the sampling points (Table 7). This association may be a maintenance factor for these organisms and may act for other vibrios as well. To test this possibility in the case of El Tor vibrios, we collected paired hyacinth and water specimens from tank and canals known to be used by persons with cholera. Initial analysis of this data shows that El Tor vibrios do associate with water hyacinths as do Heiberg Groups I and V of V. cholerae, NAG. Heiberg Group II organisms were not found in association with hyacinths. The behavior of El Tor vibrios and those in groups I and V were identical in our study. The combined results show a significantly greater association of V. cholerae with hyacinths than the water column (Table 8) and a significantly higher concentration in positive specimens (Table 9). The maximum concentration observed for an El Tor isolation was 4800 per gram in a water hyacinth specimen taken from water containing 10 El Tor vibrios/ml. This association with a surface plants may play a role in the dissemination of the cholera vibrio during outbreaks and it may provide a means by which the organism can maintain itself for longer periods than in the water column.

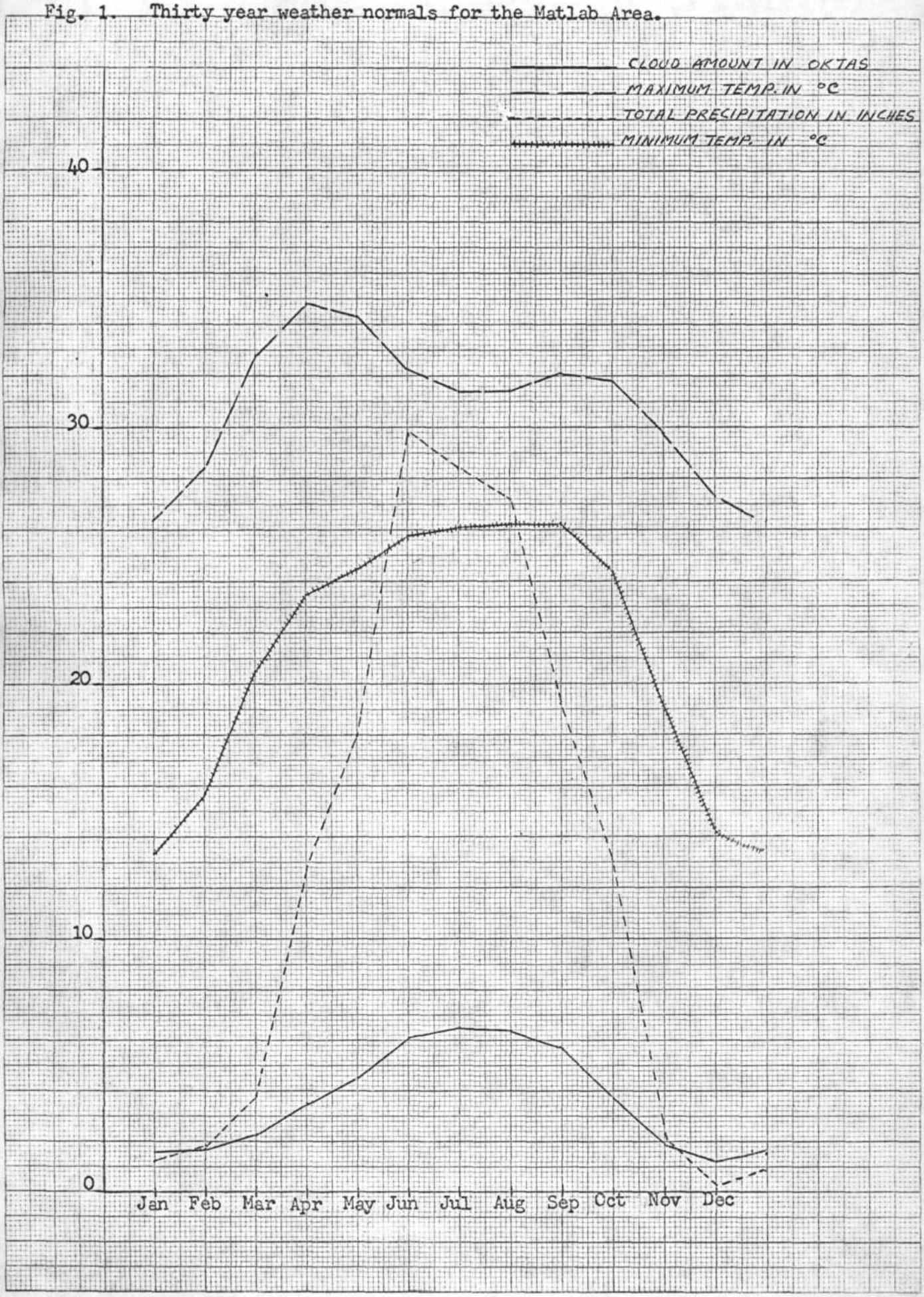
The screen for antibiotic resistance has yielded a high number of resistant isolates. Most are Pseudomonas sp. but about 1-5% of the isolates which appear to be Enterobacteriacea are resistant to three or all four of the screening antibiotics (penicillin, streptomycin, tetracycline, chloramphenicol). These, too, have tended to occur in higher concentration on surface plants than in the water column.

Future work

The longitudinal surveillance of water sampling points in Meharon will continue though the monsoon season in order to complete one year's observation of seasonal changes in the fundamental parameters affecting these water sources' ecology. Since there is no likelihood of gathering any direct data on the behavior of El Tor vibrios at these sampling sites, the surveillance will be, of necessity, somewhat limited in scope. We will, however, be able to follow seasonal variation in the NAG vibrios.

The association between vibrios and water hyacinth will be examined very thoroughly. In particular we will attempt to determine the mechanism involved in adherence, the range of surface plants in which the phenomenon occurs, and its role in the maintenance and transmission of El Tor vibrios during outbreaks.

Fig. 1. Thirty year weather normals for the Matlab Area.



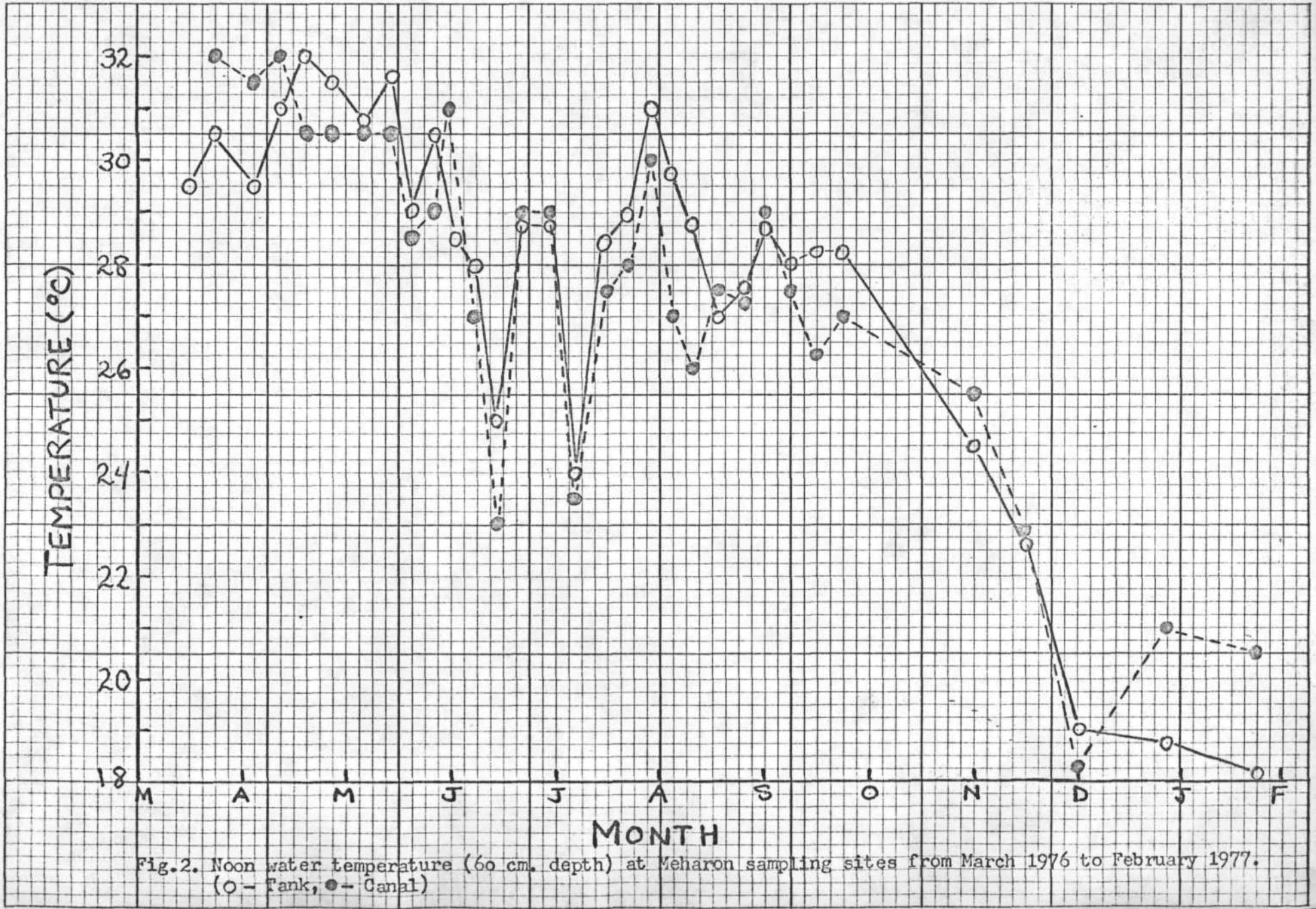
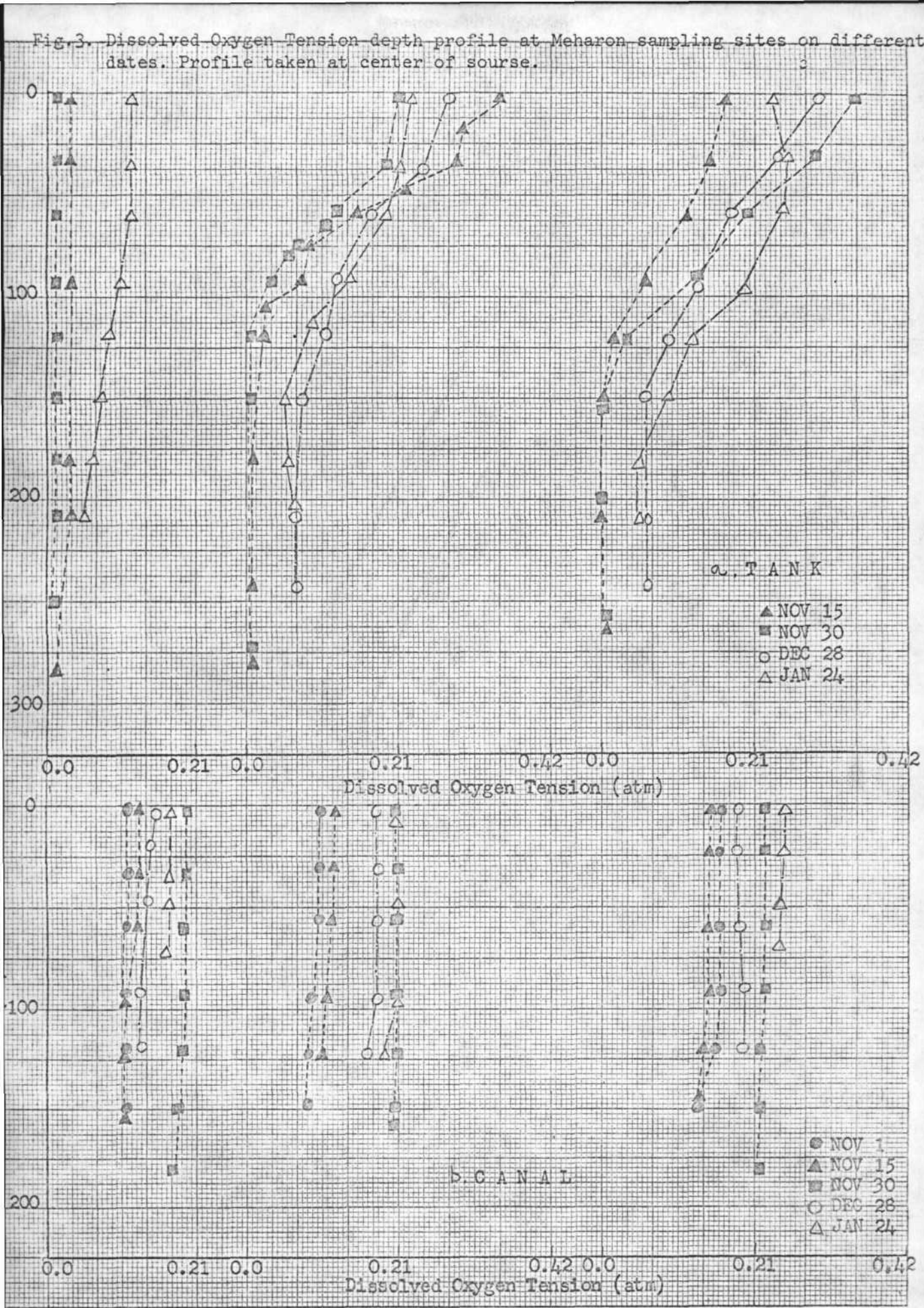


Fig.2. Noon water temperature (6o cm. depth) at Meharon sampling sites from March 1976 to February 1977. (O - Tank, ● - Canal)

Fig.3. Dissolved Oxygen Tension-depth profile at Meharon sampling sites on different dates. Profile taken at center of sourse.



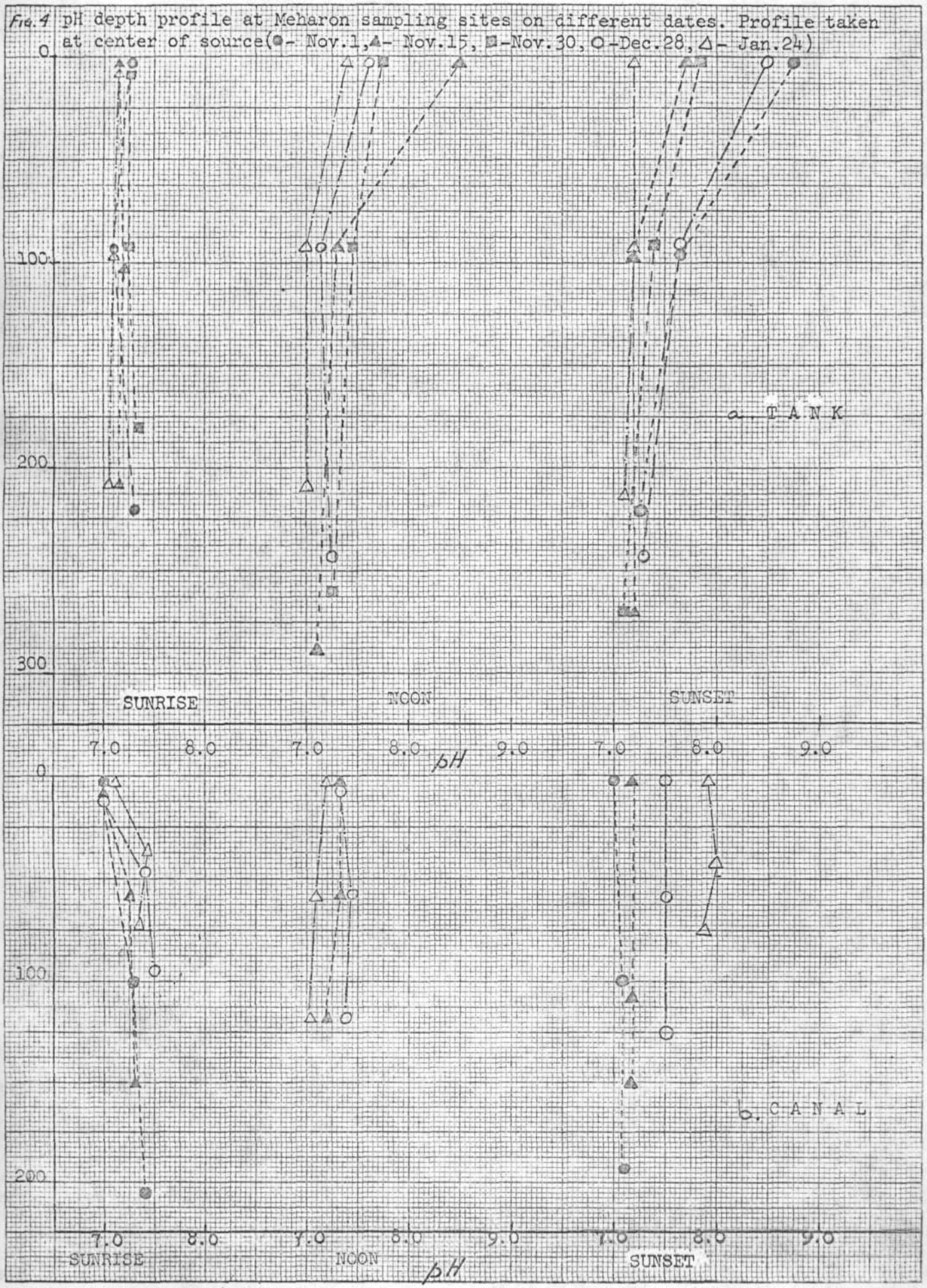


Fig. 4 pH depth profile at Meharon sampling sites on different dates. Profile taken at center of source(● - Nov.1, ▲ - Nov.15, ■ - Nov.30, ○ - Dec.28, △ - Jan.24)
0
100
200
300
a. TANK
SUNRISE
NOON
SUNSET
7.0
8.0
9.0
pH
0
100
200
b. CANAL
7.0
8.0
9.0
SUNRISE
NOON
pH
SUNSET

Table 1

Seasonal Variation in Concentration of Phytoplankton at Different Sampling Points

		Log No./ml on	
Sampling Point	Depth	November 15	January 24
Canal - Edge	Surface	2.1	1.6
	Interface	1.3	1.2
Center	Surface	2.2	1.5
	60 cm	1.4	1.3
	Interface	1.4	1.1
Tank - Edge	Surface	3.2	2.5
	Interface	2.9	2.5
Center	Surface	3.6	2.5
	60 cm	3.2	2.5
	Interface	2.5	2.0

Table 2

Seasonal Variation in Concentration of Heterotrophic Aerobes at Different Sampling Points

		Log No./ml on	
Sampling Point	Depth	November 15	January 4
Canal - Edge	Surface	4.0	4.0
	Interface	4.1	4.2
Canal - Center	Surface	4.8	3.0
	60 cm	4.3	3.0
	Interface	4.3	3.2
Tank - Edge	Surface	5.2	4.0
	Interface	4.8	4.2
Tank - Center	Surface	5.3	3.4
	60 cm	3.8	2.4
	Interface	4.2	3.5

Table 3

Seasonal Variation in Coliform Concentration at Different Sampling Points

		Log No./ml on	
Sampling Point	Depth	November 15	January 24
Canal - Edge	Surface	2.6	1.5
	Interface	3.2	1.4
Canal - Center	Surface	3.5	1.3
	60 cm	3.6	1.4
	Interface	3.6	1.3
Tank - Edge	Surface	2.2	2.0
	Interface	2.0	2.0
Tank - Center	Surface	2.3	1.5
	60 cm	2.0	1.7
	Interface	2.5	2.5

Table 4

V. cholerae, NAG Isolations from Water Column Samples Taken in Meharon During March 76 - January 77 - by Heiberg Group

Time Period	n	I	II	III	V	VII
Mar - May	44	3	22	2	25	0
Jun - Sep	68	4	23	1	32	1
Oct - Nov	20	4	10	0	5	0
Dec - Jan	30	0	5	0	5	0

Two factor ANOVA without replication :
Ho : Frequency of isolation is the same for all groups
$\hat{F} = 8.808$ $F_{0.005}(1)\ 5,10 = 3.33$ Ho is rejected
$P(F \geq 8.81) < 0.0025$

Ho : Frequency of isolation is the same at all time periods
$\hat{F} = 1.557$ $F_{0.005}(1)\ 4,10 = 3.48$ Ho is accepted

Scheffés S Test
Frequency I = Freq III = Freq VII Freq II = Freq V
$P(S \geq 4.76) < 0.025$
Overall Freq in Dec - Jan Overall Freq in all other time period
$P(S \geq 8.08) < 0.0025$

Table 5

Concentration of V. cholerae, NAG in Positive Tank and Canal Water Column Samples Taken in Meharon During Mar 76 - Jan 77
Colony Forming Units/ml

Time Period	I			II			V		
	n	X	S.D	n	X	S.D	n	X	S.D
Mar - May	3	12.	16.	20.	15.	14.	24.	18.	27.
Jun - Sep	3	2.7	7.0	35.	12.	20.	38.	26.	41.
Oct - Nov	4	3.8	1.3	10.	9.0	8.4	5.	530.	410.
Dec - Jan	0	-		5.	31.	32.	5.	20.	17.

Table 6

Concentration of Heiberg Group (I) and (II) - V. cholerae, NAG in Water Samples Taken in Meharon During Nov 76 - Jan 77

Sampling Point	n	$\bar{X}$	range
Tank edge	6	5.2	0 - 20.
Tank center - surface	6	4.3	0 - 24.
Tank center - interface	6	7.7	0 - 14.
Canal edge	6	14.5	0 - 80.
Canal center - surface	6	2.7	0 - 8.
Canal center - interface	6	0.15	0 - 1.

Ho : Group I + II organisms occur in the same concentration in all 6 sampling points

Kruskal - Wallis test $H_c = 4.87$

$X^2_{0.05,5} = 11.07$ Ho is accepted.

Table 7

Concentration of Heiberg Group V *V. cholerae*, NAG in Water Hyacinths, Sediment and Water Column Samples in Meharon During Nov 76 - Jan 77

Source	n	No. Positive	$\bar{X}'$	SD
Water	50	10	12.	15.
Hyacinth	25	4	250.	100.
Sediment	12	0	-	

$\bar{X}$ = mean CFU/g in positive samples

Table 8

Isolation of V. cholerae from Water Hyacinths and Paired Water Samples Taken from Water Sources Associated with Cholera Outbreaks During Dec 76 and Jan 77 (n = 152)

		Water	
		Negative	Positive
Plant	Negative	135	4
	Positive	9	4

Ho : Association of V. cholerae with plants is not greater than that with surrounding water column.

By Fishers exact test - P = 0.002 Ho is rejected.

Table 9

Concentration of *V. cholerae* in Positive Water and Hyacinth Samples

Sources	No Positive by Enrichment	Direct Plating	Range of Cfu/g	Mann-Whitney Test Ranks
Water	7	1	0.16	7, 18 (7x)
Plant	7	6	20-4800	1, 3.5(2x), 4, 5.5(2x), 8, 11.5 (6x).

Ho : Concentration of *V. cholerae* in plant samples is no greater than that in water samples.

Mann-Whitney Test $U^1 = 105$

U 0.01 (1), 8, 13 = 84; Ho is rejected

$P(U \geq 105) < .005$