February, 1976

Semiannual Progress Report No. 7
July 1, 1975 – December 31, 1975

RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

(NASA-CR-146666) RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS Semiannual Progress Report, 1 Jul. - 31 Dec. 1975 (Hardin-Simmons Univ., Abilene, Tex.) 42 p HC $4.00 CSCL 06M G3/51

N76-20799

Unclas
23360

Submitted by

Terry L. Foster, Ph. D.
Science Research Center

Hardin-Simmons University
Abilene, Texas

RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

Semiannual Progress Report No. 7 of
Planetary Quarantine Activities
July 1, 1975 - December 1, 1975

Supported by
NASA Grant NGR 44-095-001

Submitted to the
National Aeronautics and Space Administration
Washington, D.C.

by the

Science Research Center
Hardin-Simmons University
Abilene, Texas 79601

Contributors:

T. L. Foster
S. J. S. Helms
L. E. Kirschner
W. C. Stevens
L. K. Talley
L. Winans, Jr.

Report Prepared and Submitted by:

Terry L. Foster, Ph.D.
Principal Investigator

Luther Winans, Jr.
Assistant Professor of Research

February, 1976

FOREWORD

This seventh semiannual progress report summarizes work performed for the National Aeronautics and Space Administration by the Science Research Center at Hardin-Simmons University supported by NASA Grant NGR 44-095-001, and covers the period July 1, 1975 - December 31, 1975.

This report includes results of a new investigation concerning the anaerobic ilization of phosphite or phosphine and the significance of this conversion to potential contamination of Jupiter. At present a sporeforming organism has been isolated from Cape Canaveral soil which anaerobically converts hypophosphite to phosphate. Data is presented which demonstrates that this conversion coincides with an increase in turbidity of the culture and with phosphate accumulation in the medium.

Also included in this report are results of continued investigations of omnitherms (organisms which grow over a broad temperature range, i.e. 3°-55°C). The cellular morphology of 28 of these isolates has been investigated, and all have been demonstrated to be sporeformers. Biochemical characterizations of these are also presented.

In performing microbial population profiles it is necessary to make hundreds of colony transfers. New procedures for replicate plating have been evaluated, and those results are also presented. The procedures for different replicate-plating techniques are presented, and these are evaluated on the basis of reproducibility, percentage of viable transfer, and ease of use.

Standardized procedures for the enumeration of microbial populations from ocean-dredge samples from Cape Canaveral have been determined. These procedures are presented and evaluated in this report.

The NASA Technical Officer for this grant is Richard S. Young, NASA Planetary Programs, Code SBL, Washington, D.C.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ANAEROBIC UTILIZAT. ,F PHOSPHITE

In our previous progress report (No. 6) a new topic was briefly mentioned as a future activity. This investigation was initiated in an attempt to isolate microorganisms capable of utilizing phosphite or phosphine as a sole source of phosphorous under anaerobic conditions. The consensus is that Jupiter possesses an anaerobic atmosphere. One study suggests that quarantine considerations for Jupiter could also be applied to Saturn, Uranus, and Neptune, but this present report will limit its discussions to Jupiter.

In discussions concerning mineral requirements for microorganisms, phosphorous has been mentioned as a potential limiting factor. In the reducing atmosphere of Jupiter, it is anticipated that phosphorous will not be available as phosphate and will have to be converted from its available form to phosphate in order for terrestrial contamination of Jupiter to occur. One report of an advisory committee states that phosphorous will be available as phosphite. They conclude that if it can be demonstrated that microorganisms cannot convert phosphite to phosphate anaerobically, it would make Jupiter uninhabitable for terrestrial organisms. Others feel that available phosphorous will be in the form of phosphine, and the same argument can be made for reducing the probability of contamination of Jupiter if microorganisms fail to convert phosphine to phosphate anaerobically.

At the request of the Planetary Quarantine Office, we began investigations to determine if microorganisms from Cape Canaveral soil can convert phosphite or phosphine to phosphate anaerobically. Much time has been spent selecting and standardizing test procedures and examining the various culture medium ingredients to determine the levels of phosphate contamination. At present, the bulk of this investigation has been concerned with the utilization of phosphite or hypophosphite. Work with phosphine will be begun in the immediate future.

The studies of Casida and co-workers and Malacinski and co-workers have shown that certain aerobic microorganisms can utilize phosphorus (inorganic) in a form more reduced than phosphate. Several different aerobic organisms were shown to grow in media when phosphorus was provided only as phosphite. No such work, however, has been done with anaerobic organisms.

A preliminary study with soil samples has indicated that a facultative organism (or group of organisms) can grow anaerobically in media where phosphorus is supplied only as hypophosphite. Hypophosphite was used initially because it is a more reduced form than phosphite. Similar experiments will be performed with phosphite. The purpose of this work is to determine whether a measurable uptake of hypophosphite can be observed when a phosphate-free basal media is innoculated with an organism apparently capable of growing in such a medium.

Since detection and determination of very low concentrations of inorganic phosphate were to be attempted, all glassware used in this work was washed in hot 6M hydrochloric acid and rinsed with distilled water to remove phosphate that could be adsorbed on glassware surfaces. All solutions and reagents were checked for phosphate contamination using stannous chloride reduction of molybdophosphoric acid, which was determined colorimetrically (*Standard Methods for the Examination of Water* *and Wastewater*, 13th Ed.) using a Beckman DB spectrophotometer. This method permitted the measurement of phosphate concentrations down to .005 ppm phosphorus. This procedure was standardized with solutions of known phosphate concentrations.

Most materials commonly used in media preparation showed high levels of inorganic phosphate, often in excess of 100 ppm phosphorus. Therefore, a basal medium was prepared according to the following formulation:

0.5% glucose

0.2% NH_4Cl

0.002% Na_2SO_4

0.05% $MgSO_4 \cdot 7H_2O$

1.2% Tris Buffer

Adjusted to pH 7.0

Analysis showed 0.01-0.03 ppm phosphorous as phosphate

Mineral supplement

0.01 ppm B as H_3BO_3

0.04 ppm Cu as $CuSO_4 \cdot 5H_2O$

0.2 ppm Fe as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$

0.02 ppm Mn as $MnSO_4$

0.02 ppm Mo as $(NH_4)_6Mo_7O_{24}$

0.18 ppm Zn as $ZnSO_4 \cdot 7H_2O$

Phosphite source

175 ppm $NaH_2PO_2 \cdot H_2O$

Adjusted to pH 7.0

Analysis showed 0.04 ppm phosphorus as phosphate

The organisms to be used in the work were obtained from soil samples in the following manner. Replicate flasks of basal media with sodium hypophosphite as the only source of phosphorus were inoculated with a mixed soil sample from Cape Canaveral. These were incubated for 7-30 days at room temperature in Brewer Anaerobe Jars with Gas-Paks. As soon as growth under anaerobic conditions was indicated by turbidity, 1.0 ml of the solution was transferred to a total of 100 ml of media. When growth was again shown by turbidity, 0.1 ml of the solution was transferred to a total of 100 ml of media. The 0.1 ml to 100 ml transfer was repeated twice more and in successive transfers considerably more time was required for the appearance of turbidity. The sample obtained by the fourth transfer was assumed to be an anaerobic organism capable of utilizing hypophosphite as a source of phosphorus and was used for all further work. Because of the high phosphate contamination of solidification agents this isolate has not been streaked on solid phosphite media, but staining procedures have demonstrated it to be a pure culture of a *Bacillus sp.* Complete characterization of this isolate is now being performed and will be reported in our next report. Several different sets of isolation experiments have been performed, and the phosphite utilizing isolates all show similar morphology, staining characteristics, and spore location. It appears that this is a single organism that is isolated repeatedly, but this will be investigated more completely. Controls for this experiment consisted of identical sets as described with the exception that phosphate was included in one set to prove that the basal medium would support growth, and one set had no added phosphorous source to prove that the phosphorous-deficient medium would not support growth. In all cases the phosphate controls produced abundant growth, and the phosphorous-deficient controls produced no growth. Several different isolation procedures have been employed and all procedures have yielded the same results, i.e. isolation of an organism that appears to be utilizing hypophosphite anaerobically. This isolate is a spore-former and grows aerobically as well as anaerobically. Due to the limited available nutrients in the basal medium, the nutritional requirements of this isolate are not complex. This has special significance to potential contamination of Jupiter because of the possibly limited nutrients on the planet.

Basal media enriched with 175 ppm phosphorus as sodium hypophosphite was inoculated with the organism shown to grow in hypophosphite. The samples were grown under anaerobic conditions. Samples were taken at various time intervals after inoculation, killed with formalin solution, and filtered to remove the organisms. The growth media was then analyzed for hypophosphite, phosphate, and phosphite content.

Sensitive quantitative methods for the determination of hypophosphite, phosphate, and phosphite individually in the presence of each other are not available; therefore, chromatographic separation must be carried out prior to quantitative analysis. The gradient elution, anionic exchange method of Pollard, *et al* (*J. Chrom.*, 9, 1962, 227-230) was used for separation. Dowex 1-X8 Anion Exchange Resin (100-200 mesh) was slurried with distilled water and poured into a glass column (1 cm diameter, 50 cm height). The packed column was washed with 200 ml 4N hydrochloric acid, then with distilled water until no chloride ion could be detected. Finally the column was washed with 200 ml of 0.05M KCl buffered to pH 6.8 (the initial eluent to be used).

Samples of the filtered growth media were pipetted onto the column and washed in with a small volume of 0.05M buffered KCl. Gradient elution was utilized with KCl concentrations beginning at 0.05M and rising to a (theoretical) limit of 0.2M, using the technique of Grande and Beukenkamp (*Analytical Chem.*, Vol. 28, No. 9, Sept., 1956). Using a flow rate of 1.0 ml/min., 10 ml fractions were collected.

The phosphorus content of each fraction (measured as phosphate) was determined by oxidation of the sample with potassium persulfate and sulfuric acid, followed by colorimetric determination using the vanadomolybdophosphoric acid method (*Standard Methods for the Examination of Water and Wastewater*, 13th Ed.). Figure 1 shows the separation of a standard mixture containing a total of 500 ppm phosphorus made by mixing equal volumes of 500 ppm P solutions of sodium hypophosphite, sodium dihydrogen phosphate, and sodium phosphite.

Once the separation was completed, and the phosphorus content of each fraction determined, it was possible to calculate the total amount of hypophosphite (or phosphate, or phosphite) in the sample.

Early attempts to isolate anaerobic hypophosphite-utilizing organisms demonstrated that growth will not occur at high phosphite concentrations. For this reason an investigation was performed to determine the optimum phosphite concentration, and this concentration will be used in future investigations. In this procedure separate solutions of 1000 μg/ml of disodium phosphate and 1000 μg/ml of sodium hypophosphite were serially diluted using a two-fold dilution scheme to 0.06 μg/ml and incorporated into the basal medium described above. As in all work related to phosphorous metabolism, all glassware is washed in hot HCl and rinsed with

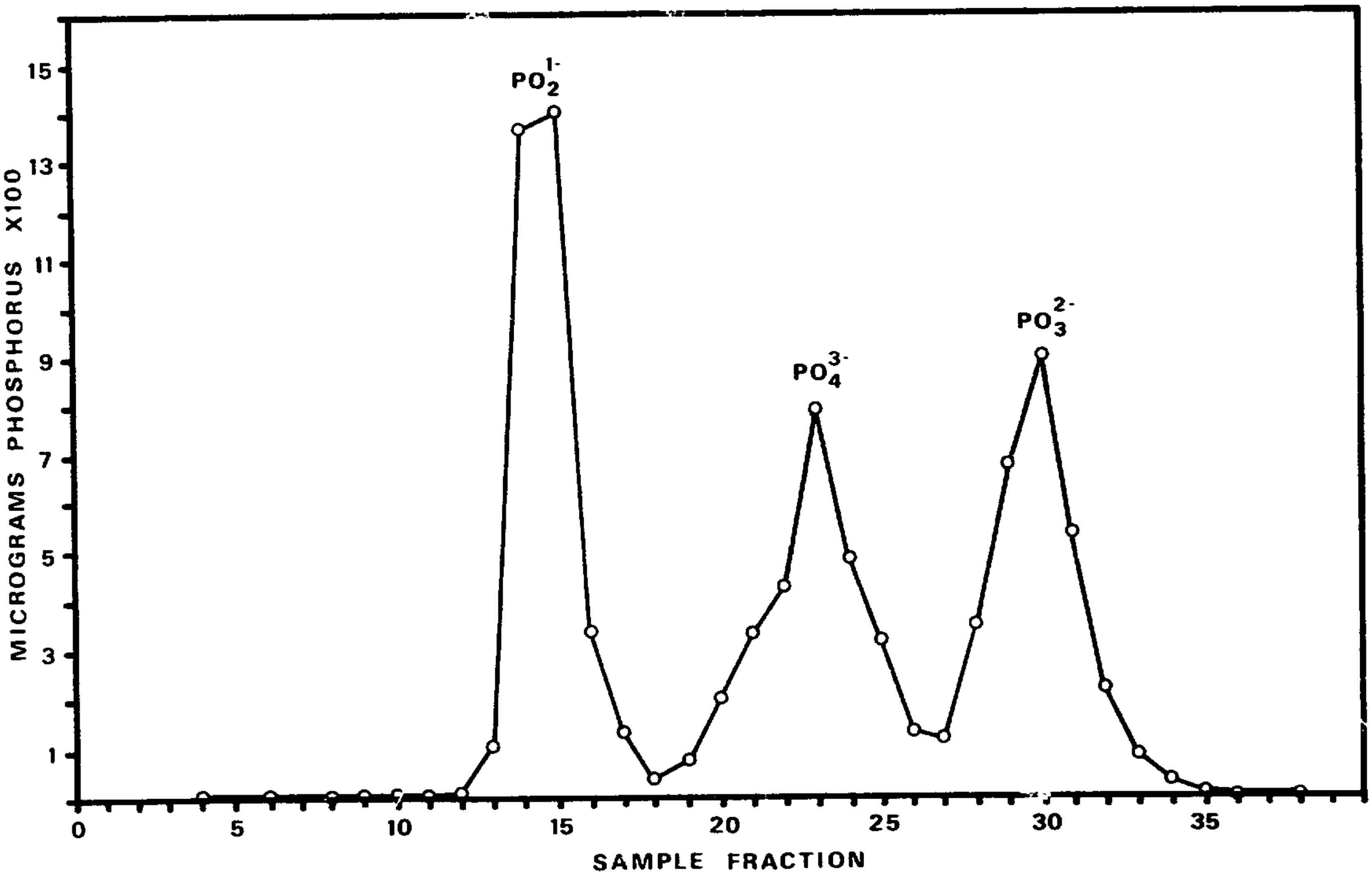

FIGURE 1: SEPARATION OF KNOWN HYPOPHOSPHITE (PO_2^{1-}), PHOSPHATE (PO_4^{3-}), AND PHOSPHITE (PO_3^{2-}) SAMPLES THROUGH A CHROMATOGRAPHIC COLUMN FOR QUANTITATIVE AND QUALITATIVE PROCEDURES.

double-distilled water, all manipulations are performed under laminar flow, and all solutions are assayed for phosphorous content.

The basal media containing decimally diluted phosphate or hypophosphite were inoculated with 0.1 ml of a water suspension of washed cells originally grown from 7-10 days in phosphite medium. These were grown anaerobically at 32°C for 7 days and results determined spectrophotometrically. The results of this investigation demonstrated that growth in the phosphate medium was best at the most concentrated solution (1000 μg/ml). Growth in the hypophosphite medium was best at 62 μg/ml (Figure 2). These results indicate that higher levels of hypophosphite may be toxic to the isolate. This phenomenon is currently being investigated to verify these results.

In order to evaluate the sensitivity and resolution of the experimental procedures, we inoculated our isolate into a known concentration of hypophosphite in basal medium. This was killed and filtered at various time intervals beginning at T_0, and the filtrate was separated with column chromatography. Each fraction was then analyzed for phosphorous concentration. Figure 3 shows the results of the T_0 determination, and the concentration demonstrated agrees with the initial concentration of hypophosphite. This method comes out of the column in fractions 12-19 which coincides with the hypophosphite peak in Figure 1. Figure 3 also shows a very low level of phosphate contamination which supports the sensitivity of the procedures being used. The results presented in Figure 4 (86 hours incubation) shows an obvious decrease in hypophosphite concentration indicating that the organism is utilizing this phosphorous source anaerobically.

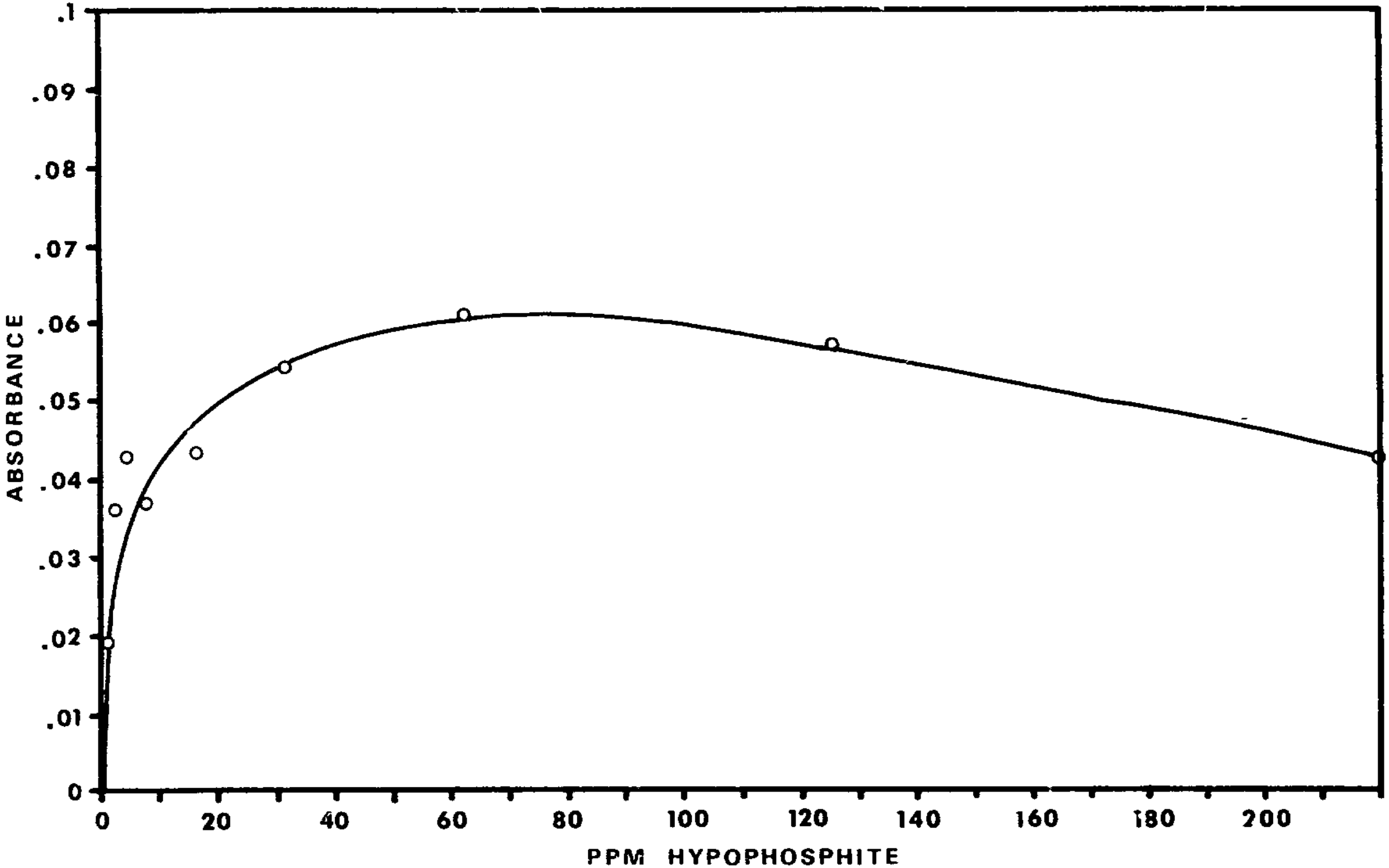

FIGURE 2: THE EFFECT OF HYPOPHOSPHITE CONCENTRATION ON THE GROWTH OF AN ANAEROBIC ISOLATE FROM CAPE CANAVERAL SOIL

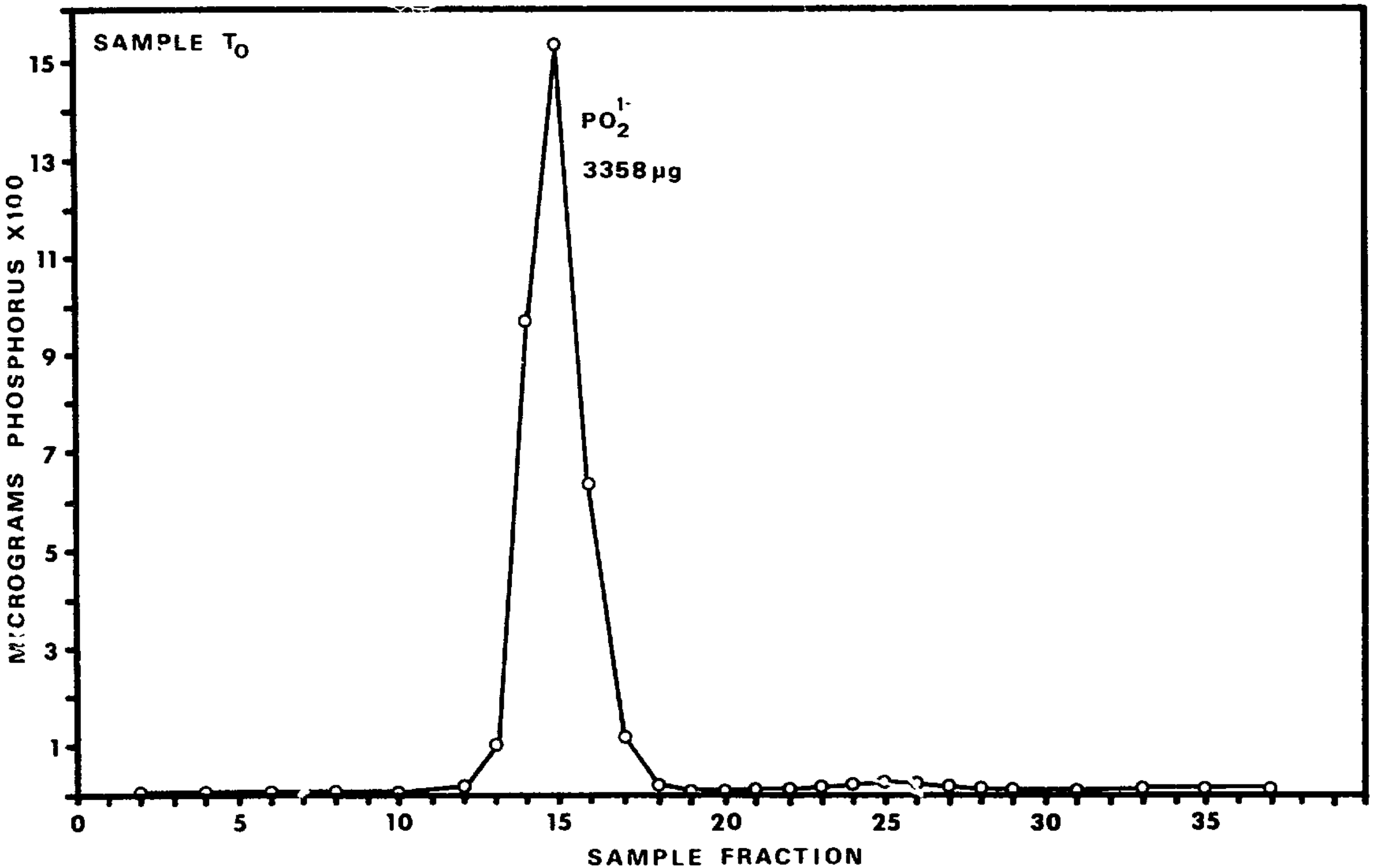

FIGURE 3: SEPARATION OF SAMPLE T_0 THROUGH CHROMATOGRAPHIC COLUMN AND ANALYSIS OF FRACTIONS FOR TOTAL PHOSPHOROUS CONTENT

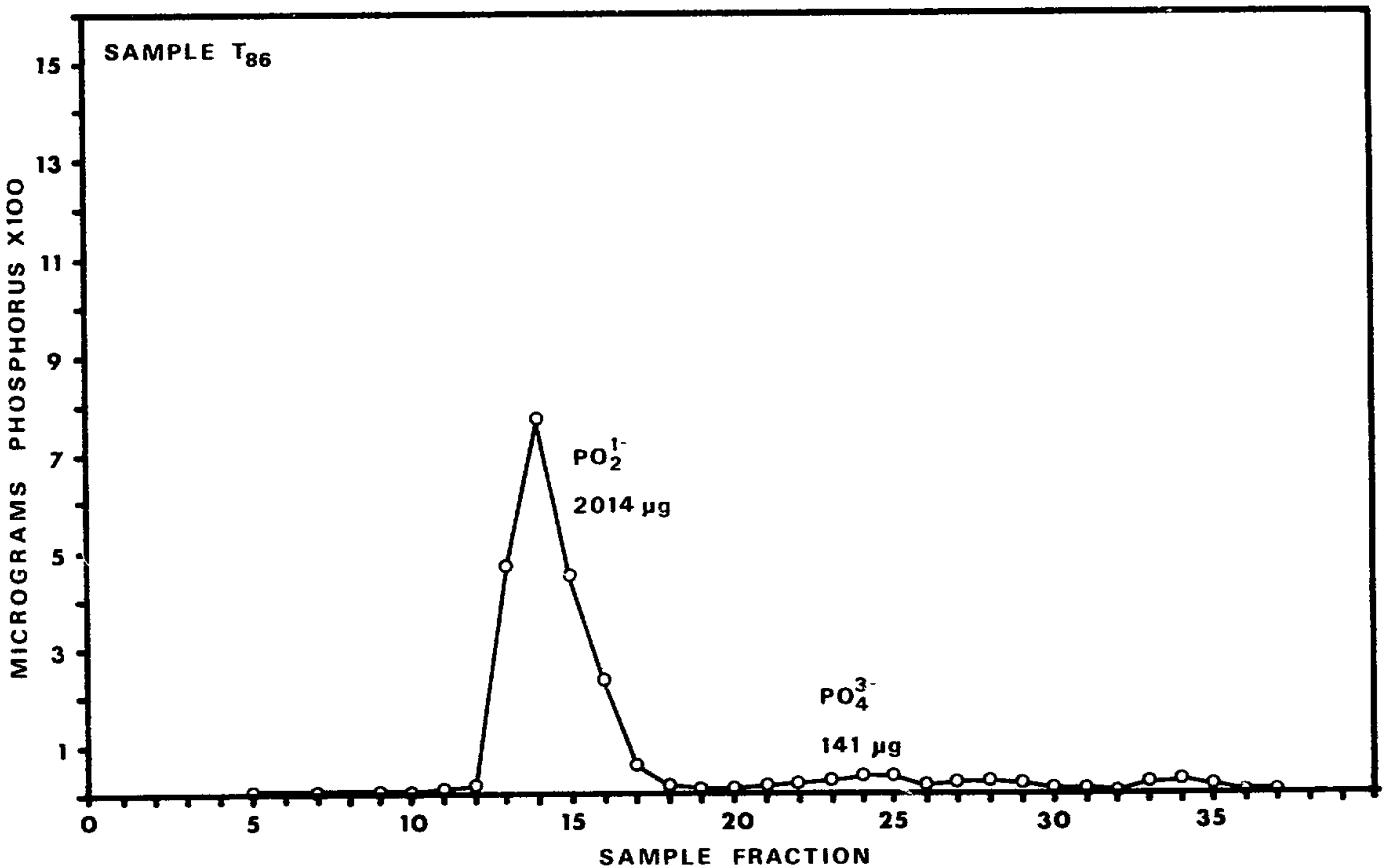

FIGURE 4: SEPARATION OF SAMPLE T_{86} (86 HOURS INCUBATION) THROUGH CHROMATOGRAPHIC COLUMN AND ANALYSIS OF FRACTIONS FOR TOTAL PHOSPHOROUS CONTENT

This is demonstrated more graphically in Figure 5A where the hypophosphite concentration is plotted over the 86-hour period. As can be seen, the concentration decreases in a consistent manner with time, again indicating that the hypophosphite is being utilized anaerobically. When this is compared with Figure 5B which shows the turbidity of the culture and the phosphate concentration, it can be seen that hypophosphite concentration decreases as turbidity increases. After a period of growth the phosphate concentration shows a slight, but sharp increase followed very quickly by a decrease. It is theorized that as the organism begins to utilize hypophosphite and grow that cells soon begin to die, thus releasing phosphate into the medium. As this phosphate becomes available at least a part of the population utilizes this as a preferential phosphorous source, and its concentration decreases. It is noted that the phosphate concentration is on an expanded scale, and its concentration does not account for the amount of hypophosphite utilized. This is due to our experimental procedure because our analysis is performed only on the filtrate, and much of the utilized hypophosphite will be bound as organic phosphate in the cells which have been filtered out of the system.

Figure 6 shows results when the organisms are grown in a medium containing phosphate as the phosphorous source. As can be seen, the phosphate concentration decreases during the first few hours. Although a curve is not presented, the phosphate decrease coincides with increasing turbidity. After about 48 hours the phosphate concentration increases again for a few hours and then becomes stationary. The phosphate increase in Figure 6 is similar to that which occurred in Figure 5A

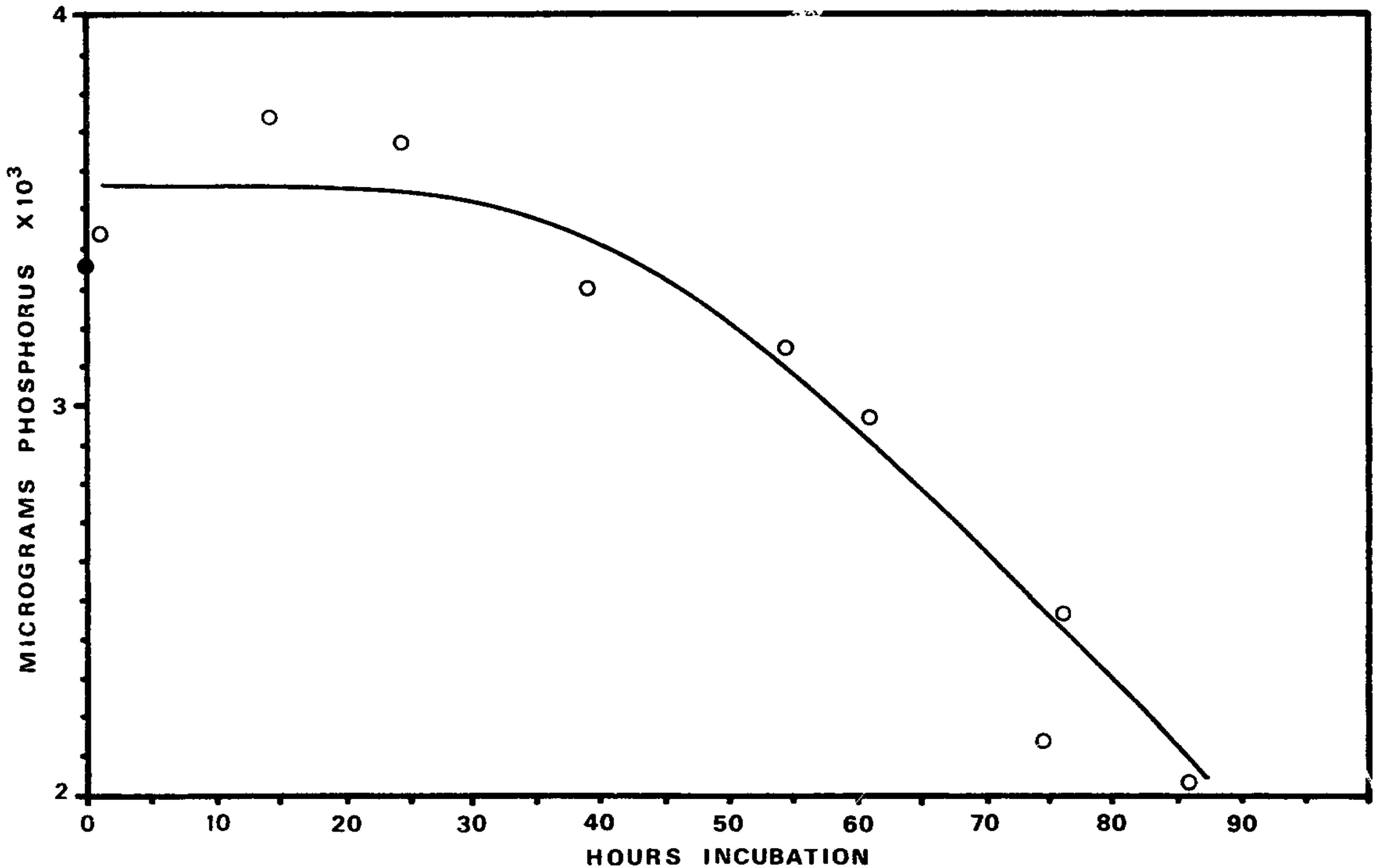

FIGURE 5A: ANAEROBIC-HYPOPHOSPHITE UTILIZATION BY A SOIL ISOLATE FROM CAPE CANAVERAL OVER AN 86 HOUR INCUBATION PERIOD

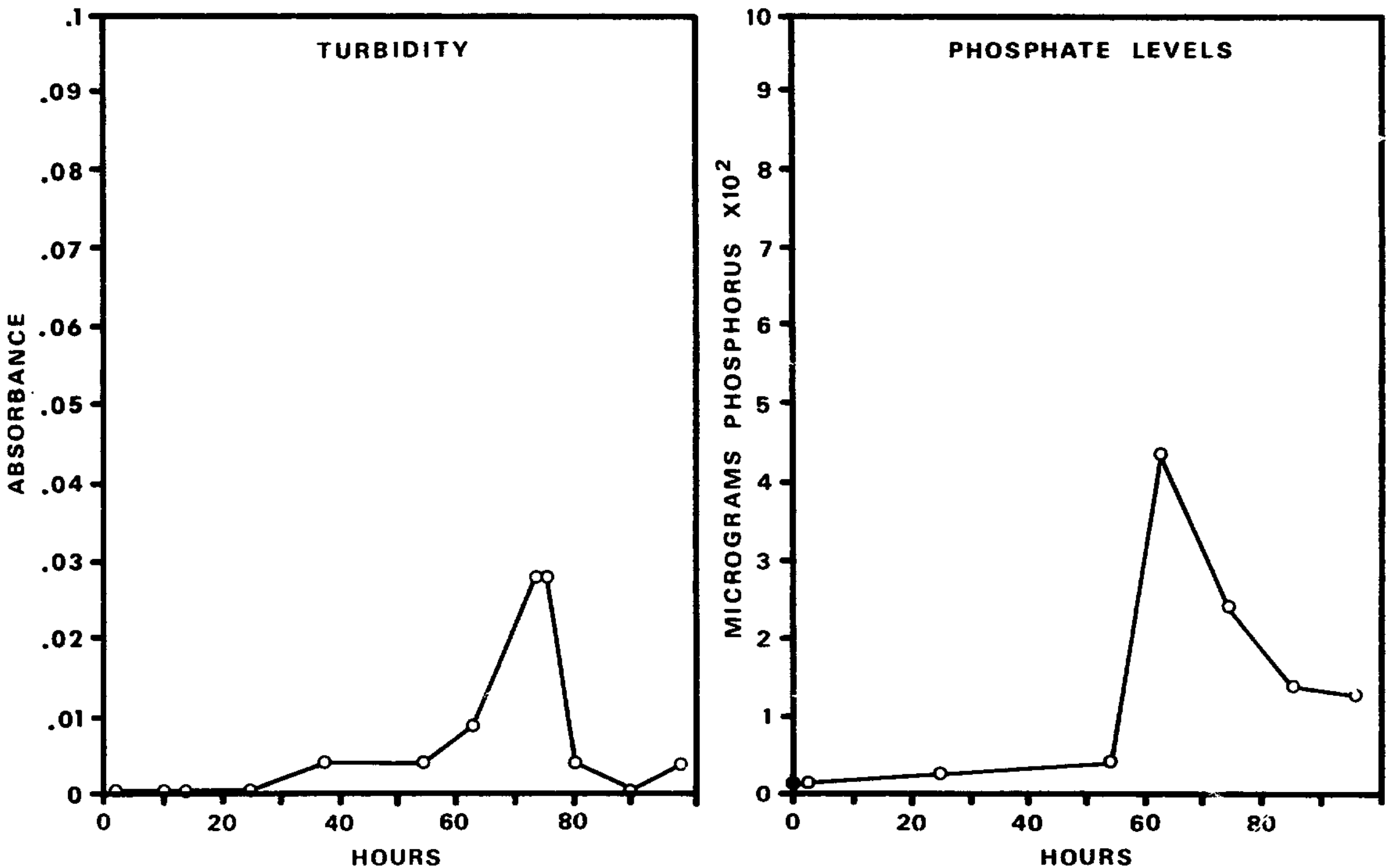

FIGURE 5B: TURBIDITY AND PHOSPHATE ACCUMULATION OF AN ANAEROBIC ISOLATE GROWN ON HYPOPHOSPHITE MEDIUM OVER AN 86 HOUR INCUBATION PERIOD

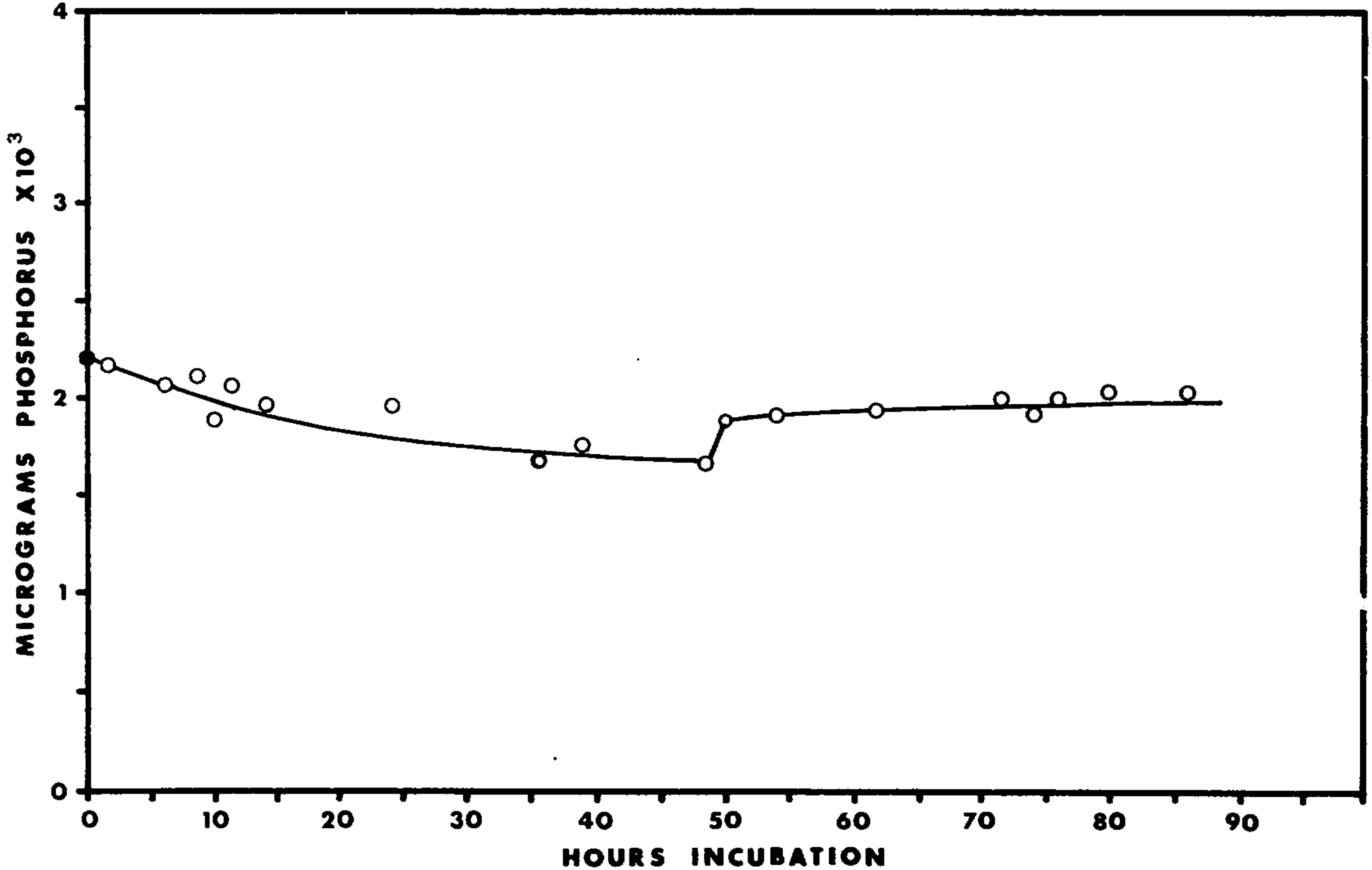

FIGURE 6: ANAEROBIC PHOSPHATE UTILIZATION BY A SOIL ISOLATE FROM CAPE CANAVERAL WHICH ALSO APPEARS TO BE CAPABLE OF UTILIZING HYPOPHOSPHITE AS A PHOSPHOROUS SOURCE

(cells grown in hypophosphite), except the increase in 5A does not occur until 60 hours. This indicates that cells are dying and releasing free phosphate into the medium, and it is postulated that phosphate is probably more preferred than hypophosphite as the phosphorous source because of the faster rate of growth and phosphorous depletion when phosphate is present. This hypothesis is currently being investigated, and these results will be presented in the next report.

Because of the possibility that the hypophosphite may have been oxidized to phosphate during the incubation period a set of uninoculated controls were run in parallel with the inoculated samples. The uninoculated control samples were identical to the inoculated samples except they were not inoculated with the isolate. These controls were divided into two groups with the first group being analyzed with T_0 (0 hours) and the second group being analyzed at the end of the incubation period (86 hours). There was no change in the hypophosphite level during the incubation period and therefore demonstrated that there was no oxidation of hypophosphite to phosphate. The drop in the hypophosphite levels in the inoculated samples was due to growth.

CHARACTERIZATION OF OMNITHERMS

In our previous report (no. 6), a group of isolates capable of growth at 3°, 32°, and 55°C was described. At that time, these were called omniphiles, but the term omnitherm seems to be more appropriate. A review of the literature failed to locate a term to describe organisms growing over such a broad temperature range, and further investigations will likely determine what to call this group.

Since the last report a great deal of time has been spent in reexamining these cultures for purity and ability to repeat this characteristic growth. Of the original 34 isolates only a few appeared to possess mixed colonies, with these being isolated and examined separately. Upon further investigation, it was found that in almost all cases, these colonies demonstrated identical characteristics, and it is now felt that the apparently mixed colonies are merely colonial variants of the same organism. Some of the original 34 isolates have been lost on subculture, and at present there are 28 isolates which are being investigated.

Although cardinal temperatures have not been determined for all of these isolates, it has been determined that some of these will grow at 0°C in 14-21 days. At the present time, none have been found to grow at 65°C, and the optimum temperature for most of these is 32-34°C. The isolates under investigation have been grown at 3°C, transferred to fresh medium, incubated at 55°C, and shown growth (and vice versa). In other words, they have undergone a change in incubation temperature of 52°C, and continued to grow. They do not have to be slowly acclimated to the opposite extreme temperature in order to grow.

In order to characterize the omnitherms, they were gram-stained and measured. In doing so it was found that many of them have spores, and many in which spores have not been demonstrated appear to be *Bacillus* species. The results of the cellular morphology are presented in Table 1. Because the majority of these appear to belong to the genus *Bacillus*, they were subjected to heat-shock on two separate occasions, and all of them survived heat-shock. For this reason standard *Bacillus* identification schemes have been used to determine their biochemical activity. Standard inocula of each isolate was prepared, and these were used to inoculate biochemicals for incubation at 32°C. These results are shown in Table 2. All tests were performed in duplicate and showed consistency of results. Even though tentative identification could be made on the basis of these results, identification will not be attempted at this time because of the unusual temperature ranges of growth of this group of organisms.

In performing biochemical reactions on these organisms it was observed that several of them gave positive results on the test for anaerobic growth. Because this characteristic is important in potential contamination of planets, these organisms are currently being investigated for their ability to grow anaerobically at the three different temperatures. Preliminary results indicate that many of these are facultative anaerobes, some of which grow anaerobically at all three temperatures.

Now that the ability of these organisms to grow over a broad temperature range has been satisfactorily verified and their morphology and preliminary biochemical activity has been investigated, detailed investigations into their general physiology are now in progress. These investigations include generation times at all temperature ranges,

TABLE 1: Gram Stain and Morphology of Omnitherms

Strain	Gram Reaction	Cell Shape	Dimensions	Endospore
A-28	-	rod	0.7 µm × 1.8-2.8 µm	central, 1.4 µm long
C-16	+	rod	0.7-1.3 µm × 1.7-2.4 µm	yes*
G-20	+	rod	0.7 µm × 1.4-2.8 µm	
G-28-2	+	rod	0.4 µm × 1.4 µm	
G-33	+	rod	0.7 µm × 1.8-3.5 µm	yes
G-33A	+	rod	0.7 µm × 1.4-2.1 µm	
G-33B	+	rod	1.1 µm × 2.8 µm	
G-38A	+	rod	0.7 µm × 2.8 µm	central to subterminal, 1.4 µm long
G-38B	+	rod	0.7 µm × 2.1 µm	central, 1.1 µm long
G-38C	-	rod	0.4 µm × 1.4-2.8 µm	
J-21	+	rod	1.1 µm × 1.4 µm	
AA-6	-	rod	0.5 µm × 0.9 µm	yes
AA-10	+	rod	1.1 µm × 1.4-2.1 µm	yes
AA-12	+	rod	1.1 µm × 3.2 µm	
BB-1	-	rod	0.7 µm × 2.6 µm	central to terminal, slightly swollen, 1.4 µm long
GG-30	-	rod	0.7 µm × 2.8-3.3 µm	
GG-41	+	rod	1.1 µm × 3.5-4.2 µm	
GG-44	-	rod	0.4 µm × 1.4-2.1 µm	yes
HH-1	+	rod	0.7 µm × 1.7 µm	
HH-1B	-	rod	0.7 µm × 2.1 µm	yes
HH-34	-	rod	0.7 µm × 2.1-3.5 µm	central, slightly swollen, 1.4 µm long
HH-66	+	rod	0.7 µm × 1.4-2.1 µm	
JJ-28	+	rod	0.7 µm × 1.4 µm	
JJ-29	-	rod	1.4 µm × 1.8-2.4 µm	
JJ-30A	+	rod	0.7 µm × 3.2-5.6 µm	yes
JJ-30B	-	rod	0.7 µm × 3.5-5.6 µm	yes

* Yes means spores present but size and location not determined.

TABLE 2: Biochemical Reactions of Omnitherms

Isolate Number	Biochemical Reactions										
	Catalase	Starch	Casein	P.R. Mannitol	V.P.	Citrate	Nitrate	Anaerobic	Tyrosine	Phenylalanine	Sabouraud
A-28	+	-	+	+	-	+	-	-	-	-	-
C-16	+	+	+	+	+	+	-	-	-	-	+
G-20	+	-	-	-	-	+	-	-	+	-	+
G-28-2	+	+	+	-	+	+	+	+	-	-	+
G-33	+	-	+	+	-	+	-	-	-	-	+
G-33A	+	+	+	-	-	-	-	-	+	-	+
G-33B	+	+	+	-	-	+	-	-	+	-	+
G-38A	+	+	+	+	-	+	-	-	-	-	+
G-38B	+	-	+	+	-	+	-	-	-	-	+
G-38C	+	+	+	-	-	+	-	-	+	-	+
H-86-1	+	+	+	+	-	+	-	-	+	+	+
J-21	+	+	+	+	+	-	-	+	-	-	-
AA-6	+	-	-	+	+	-	+	+	-	-	+
AA-10	+	-	+	-	+	+	+	+	-	-	+
AA-12	+	-	+	+	-	-	+	-	-	-	-
BB-1	+	+	+	-	+	+	+	+	+	-	+
GG-30	+	-	+	+	-	+	-	-	-	-	+
GG-41	+	-	-	-	-	+	-	-	+	-	+
GG-44	+	-	-	-	-	+	-	-	+	-	+
HH-1	+	-	-	-	-	-	-	-	+	-	+
HH-1A	+	-	-	-	+	-	+	-	+	-	+
HH-1B	+	-	-	-	-	-	-	-	+	-	+
HH-34	+	-	+	+	+	-	-	-	-	-	+
HH-66	+	-	+	-	-	+	+	+	-	-	+
JJ-28	+	+	+	+	+	-	-	+	-	+	+
JJ-29	+	+	+	+	+	-	+	+	-	+	+
JJ-30A	+	+	-	+	-	-	-	+	-	-	-
JJ-30B	+	+	-	+	-	-	-	-	-	-	-

All reactions were inoculated in duplicate.

metabolic activity at the different temperature ranges, and product analysis, resistance to physical and chemical controls, and other characteristics.

EVALUATION OF REPLICATE-PLATING TECHNIQUES

One of the inherent disadvantages of soil population profile studies with respect to a limiting factor such as incubation temperature is the large number of colonies that must be processed. If one selects duplicate countable plates (30-300 colonies) for one soil dilution, he would have 60 to 600 colonies to transfer. If these colonies were to be replated for incubation at three temperatures, the number of transfers would jump to 180 to 1800. In one recent study of mixed Cape Canaveral soil samples, there were 8 growth conditions. If appropriate plates from each growth condition were subjected to the above treatment, it would mean a potential of 1440 to 14,000 transfers per day.

Previously, all transfers were made with an inoculating needle and the transfer of over a thousand colonies in an eight hour work day was virtually impossible for one person, which meant that some of the plates had to be stored at low temperatures until personnel was available to process them. This was not only time consuming, but a certain percentage of the colonies died while in storage. One study showed a loss of 22% of the 32° aerobic isolates. It is likely that with enough samples to give statistically valid data, an investigation would soon be hopelessly bogged down with unprocessed plates. Apparently then, a fast, precise replicate-plating technique would be highly advantageous for any study involving multiple transfers of large numbers of colonies.

This investigation deals with the statistical evaluation and comparison of several such methods, involving the use of the Lederburg velvet replicate-plating technique. Pasteur pipets with pour plate samples, and toothpicks and straight pins with pour plates and spread plates.

Soil samples were obtained from an Atlantic dredge site at Cape Canaveral where a submarine base is being constructed. These samples, which consisted predominately of sand, were diluted in 1.0% phytone peptone broth, then sonicated for 2 minutes. Pour plates were made using approximately 25 ml per plate of Trypticase Soy Agar containing an additional 3.0% NaCl. Spread plates were made by spreading 0.1 ml of a 10^2 soil dilution onto the surface of a solidified 25 ml TSA + 3% NaCl plate. All transfers were made under laminar flow and all plates were incubated at 32° for 10 days. This extended incubation time was employed because these isolates are typically slow-growers.

Apparatus for the velvet replicate-plate technique consisted of sterile velvet squares on flat topped plastic mandrels. The original plate was inverted onto the velvet to inoculate it and four uninoculated plates were in turn, inverted onto the same velvet square.

The pipet transfer-technique utilized the removal of colonies from pour plates with sterile Pasteur pipets, with cotton plugged rubber bulbs to draw the colonies into the pipet. Individual colonies were dispersed in 1.0 ml of 1.0% phytone peptone, 0.05 ml plates. The pipet was inserted into the agar upon inoculation of the replicate plate in order that a precise area could be observed for growth, and so that overlapping of colonies would not occur.

Colonies were located on the TSA plates by first making a reference mark at one spot on the wall of the bottom half of the Petri dish, then placing the plate on a grid. This grid consisted of four circles, each composed of 5 concentric circles. Each circle was divided into equal parts by eight radii. By aligning the reference marks of each of the replicate plates on the same respective axis, it was possible to locate the inoculation sight of each culture suspension on each plate.

Techniques utilizing toothpicks (TPT) and straight pins (PT) were identical except for the implement of transfer. Toothpicks or acid-washed brass straight pins were autoclaved, then aseptically inserted through colonies on spread plates (SP) or in pour plates (PP). The original plates containing either toothpicks or pins were inverted onto a Petri dish bottom, half filled with melted paraffin, so that the original plate rested on the bottom plate. Following solidification of the paraffin the original plate was lifted off the assembly, leaving one end of the transfer implement embedded in the paraffin and the other end inoculated with a specific colony. Four uninoculated plates were inverted and, each in turn, pressed onto the transfer implements. Identification of cultures on different replicate plates utilized the same system as with the pipet technique.

Original plates used more culture medium than normal, so that support would be provided for the transfer implements, the length of which was twice the depth of a normal Petri dish.

Ten original plates were used for each technique with replication of each plate to 4 others giving a total of 40 observations per technique. The arithmetic means for each 10 plate group and corresponding 40 plate group were taken, and the average percent transfer calculated for each technique.

Plates from the velvet replicate-plating method were uncountable; therefore, no data for them are shown. The pipet transfer method was rejected on the grounds that it was almost as slow as transferring colonies with a needle. Table 3 presents data for the remaining methods.

The results of Table 3 demonstrate that the toothpick transfer method with the spread plate technique gives a higher percentage transfer of viable cultures, with the pin method on spread plates being the second choice. Concerning reproducibility through repeated transfers the toothpick method is superior, showing consistent transfer through all four replicates. The pin transfer shows a progressive loss of reproducible results with each successive replicate, the greatest loss being after the first replicate plate is made. From these results it is apparent that the toothpick transfer is the method of choice. However, because of the irregularity of toothpicks, the need to cut them to size, and the large holes gouged into the agar, the method of choice from a practical standpoint is the pin method with the spread plate technique.

Statistical evaluation of the transfer techniques utilized a chi-square analysis of a 2×4 contingency table where original counts and replicate counts opposed the 4 transfer techniques to prove or disprove the null hypothesis, in this case, H_0: $\mu_1=\mu_2=\mu_3=\mu_4$. Then, a one tailed t-test was made to determine significant difference between original and transferred numbers of colonies. The level of significance for chi-square and t was taken as 0.05. These results are presented in Tables 4 and 5.

In this study, data were compared by a chi-square evaluation to estimate heterogenity of the procedures used. From Table 4 it can be seen that the calculated chi-square is less than the theoretical, thus indicating that no heterogenity was demonstrated with respect to the

TABLE 3: PERCENTAGE TRANSFER OF BACTERIAL COLONIES USING VARIOUS TRANSFER METHODS

TRANSFER METHOD	ORIGINAL COLONIES TRANSFERRED	FIRST REPLICATE[2]	SECOND REPLICATE	THIRD REPLICATE	FOURTH REPLICATE	AVERAGE % TRANSFER
PT/SP[1]	235	97.4	90.6	90.2	87.2	91.4[3]
PT/PP	89	86.5	79.8	76.4	76.4	79.8
TPT/SP	215	97.7	96.3	97.2	96.3	96.9
TPT/PP	461	74.4	74.6	74.6	73.0	74.2

[1]PT PIN TRANSFER
TPT TOOTHPICK TRANSFER
SP SPREAD PLATES
PP POUR PLATES
[2]PERCENTAGE TRANSFER IS AN AVERAGE OF 10 PLATES PER TRANSFER METHOD
[3]AVERAGE OF 40 PLATES PER TRANSFER METHOD

TABLE 4: CHI-SQUARE ANALYSIS TO DEMONSTRATE HOMOGENITY OF VARIOUS REPLICATE PLATING PROCEDURES

TRANSFER METHOD	PT/SP[1]	PT/PP	TPT/SP	TPT/PP	TOTALS (ORIGINAL COUNT)
ORIGINAL NUMBER	235	89	215	459	998
EXPECTED NUMBER	244.3	88.9	220.3	435.6	
NUMBER TRANSFERRED	214.8	74.4	207.2	343	839.4
EXPECTED NUMBERS	205.5	74.6	192.9	366.4	
					GRAND TOTAL (EXPECTED COLONIES)
TOTAL NUMBER OF EXPECTED COLONIES	449.8	163.4	422.2	802	1834.7

[1]PT PIN TRANSFER
TPT TOOTHPICK TRANSFER
SP SPREAD DISH
PP POUR PLATE
DEGREES OF FREEDOM = 3
CALCULATED X^2=5.48
X^2 FOR D.F=3 AT 95% (0.05) CONFIDENCE LEVEL =7.82

TABLE 5: STATISTICAL EVALUATION OF PERCENTAGE TRANSFER OF VARIOUS REPLICATE PLATING PROCEDURES

TRANSFER METHOD	PT/SP[1]	PT/PP	TPT/SP	TPT/PP
ORIGINAL COLONY NUMBER[2]	23.50	8.90	21.50	46.10
VIABLE TRANSFERS[3]	21.48	7.14	20.73	34.18
CALCULATED t-VALUE[4]	1.36	1.07	0.42	4.58
SIGNIFICANT DIFFERENCE	NO	NO	NO	YES

[1]PT PIN TRANSFER
TPT TOOTHPICK TRANSFER
SP SPREAD PLATE
PP POUR PLATE
[2]AVERAGE OF 10 PLATES
[3]t-VALUE FOR DEGREES OF FREEDOM AT 95% CONFIDENCE LEVEL = 1.83
[4]AVERAGE OF 40 PLATES (10 ORIGINALS × 4 REPLICATES)

resolution of this test. The results of the t-tests in Table 5 demonstrated a significant difference in the number of transferred colonies only with the toothpick method using pour plates. The three other methods showed no difference.

The criteria for evaluating a replicate-plating procedure must be two-fold. First, the method must be statistically sound, i.e. there should not be gross discrepancies between original counts and replicate counts. Second, a time factor must be considered. If two given procedures are statistically equivalent, then preference should be given to the faster of the two. One major objective of this study then was to find and develop a faster way of replicate plating. From a standpoint of rapidity, the Lederburg method was by far the fastest, easiest method. The problem was that although colonies transferred, they did so in a fragmented way that gave poor colony definition and obscured the count. As has been stated, the pipet method was extremely slow and presented no real advantage. Since the remaining three techniques (PT/SP, PT/PP, TPT/SP) are apparently of similar degrees of efficiency, a preferential choice could be made as to convenience and highest percent transfer.

After evaluation of all procedures the method of choice for practical use is the pin transfer method employed with the spread plate technique. If spread plates cannot be used, pin transfer with the pour plate is also acceptable, but gives poorer transfer. The three methods giving the best percent transfer all give better results than picking individual colonies by hand with an inoculating needle. This fact plus cutting the work time from days to hours makes these procedures especially attractive.

In order to improve on all of these procedures, investigations with other transfer implements would be of value. For practical use pins are the best in this investigation, but a similar implement with greater roughness or porosity should improve the results.

ISOLATION PROCEDURES TO ENUMERATE POPULATIONS FROM OCEAN DREDGE

Previous investigations of soil samples from Cape Canaveral have demonstrated the presence of psychrophilic sporeformers. It has been postulated that these are introduced to the Cape soils from the ocean. Present construction at Cape Canaveral includes a new submarine base, and this necessitates dredging an inlet to increase its depth. This dredging provides an excellent opportunity to study the microbial populations in the sediment of the ocean floor.

Samples of the dredge material were collected in sterile jars and immediately stored in the refrigerator. The dredging provided two distinctly different types of samples which will be investigated. One is a dark sand, the other a dark, hard clay material. The clay possesses a hardness similar to that of concrete; therefore is fairly difficult to work. These samples were returned to our laboratories and stored at -65°C.

The sand samples were removed from the -65° freezer and stored at 0°C for approximately 48 hours prior to plating. Five 10 g samples were weighed into 90 ml 1.0% phytone peptone blanks and dispersed by shaking. This procedure was repeated using 90 ml 1.0% phytone peptone blanks containing 3% NaCl. All samples were then insonated for 2 minutes to further disperse samples in the deluent.

Samples were plated according to three different procedures: Group I--non-salt diluents plated onto standard TSA plates (plates a and b), and TSA plates containing an additional 3% NaCl (plates c and d); Group II--1.0% peptone plus 3% salt deluents plated onto standard TSA plates (plates a and b), and TSA plates containing an additional 3% NaCl; Group III--1.0% peptone plus 3% NaCl diluents (90 ml blank heatshocked) plated onto regular TSA plates (a and b), and TSA + 3% NaCl plates. Serial dilutions ranged from 10^1 to 10^4.

Incubation was at 32°C for 5 days, after which counts were made. Five-day incubation was required for good colony formation. Average counts for each class were considered to be independent variables and were compared as such by computer, suing a Student t-test. Comparisons of populations were: Group I (non-salt deluent) TSA vs. TSA + 3% NaCl; Group II (salt deluent) TSA vs. TSA + 3% NaCl; Group III (salt diluent--heat shock) TSA vs. TSA + 3% NaCl; Group I (non-salt), TSA (a and b) vs. Group II (salt), TSA (a and b); Group I (non-salt), TSA + 3% NaCl (c and d) vs. Group II (salt), TSA + 3% NaCl (c and d). Nine degrees of freedom were used in t-value evaluation. Calculated values were considered significant at the 95% level if greater than 1.833.

Average plate counts for each group are given in Table 6. This shows that the mesophilic, aerobic counts of this sample are lower than surface soil samples obtained from Cape Canaveral. The heat-shock count indicates quite a low population of spores in the sample.

The objective of this part of our investigation is to determine the best enumeration procedure to give optimum counts from these marine samples. For this reason, results of the t-test comparisons of the different procedures are presented in Table 7, and it can be seen that incorporation of salt into the diluent and/or recovery medium has highly significant effects on the recovery of organisms from the ocean-dredge sand. By comparing Tables 6 and 7 one can draw the following conclusions:

TABLE 6: AVERAGE COUNTS (PER GRAM OF SAMPLE) OF OCEAN-DREDGE SAND FROM CAPE CANAVERAL USING VARIOUS ENUMERATION PROCEDURES

EXPERIMENTAL GROUP	RECOVERY MEDIUM	AVERAGE COUNT[2]
I	TSA[1]	4.4×10^{4}
(NON SALT DILUENT)	TSA + 3% NACL	6.1×10^{4}
II	TSA	1.6×10^{4}
(SALT DILUENT)	TSA + 3% NACL	3.0×10^{4}
III	TSA	4.6×10^{2}
(SALT HEAT-SHOCKED)	TSA + 3% NACL	3.4×10^{2}

[1]TRYPTICASE SOY AGAR
[2]AVERAGE OF TEN PLATES

TABLE 7: COMPARISONS OF VARIOUS ENUMERATION PROCEDURES EMPLOYED ON OCEAN-DREDGE SAND FROM CAPE CANAVERAL

COMPARISON	t-VALUES 9 DEGREES OF FREEDOM THEORETICAL 0.05	0.01	CALCULATED	SIGNIFICANT DIFFERENCE 0.05	0.01
GROUP I (NON-SALT) TSA vs. TSA + 3% SALT	1.833	2.821	2.382	YES	NO
GROUP II (SALT) TSA vs. TSA + 3% SALT	1.833	2.821	8.707	YES	YES
GROUP III (SALT HEAT-SHOCKED) vs. TSA + 3% SALT	1.833	2.821	5.051	YES	YES
GROUP I (NON-SALT) TSA vs. GROUP II (SALT) TSA	1.833	2.821	6.440	YES	YES
GROUP I (NON-SALT) TSA + 3% SALT vs. GROUP II (SALT) TSA + 3% SALT	1.833	2.821	3.8618	YES	YES

1. Recovery of total populations is significantly higher when salt is incorporated into the recovery medium, both with and without salt in the diluen.

2. Recovery of spores is better without salt in the recovery medium when salt is present in the diluent. (The set without salt in the diluent was lost due to laboratory accident, and this comparison is being repeated.)

3. Recovery of total population is significantly higher when salt is omitted from the diluent, both with and without salt in the recovery medium.

4. Method of choice for total population studies of ocean-dredge samples will be standard 1.0% peptone diluent (no salt) plated onto TSA for 3% salt.

Now that the method of choice has been determined, population studies of the dredge sand and clay are in progress. This will include incubation of samples at 3°, 32°, and 55°C aerobically and anaerobically, heat-shocked and non heat-shocked. Replicate transfers will then be made to determine the distribution of anaerobes, psychrophiles, thermophiles, and omnitherms.

PLANS FOR FUTURE WORK

Investigations completed to date on the anaerobic utilization of phosphite as a phosphorous source have been performed with hypophosphite. This will be repeated with phosphite, then attempts will be made to isolate organisms which utilize phosphine anaerobically. To further demonstrate that these organisms are indeed utilizing hypophosphite, ^{32}P labeled hypophosphite is currently being incorporated into the culture medium and attempts are being made to prove that this is being incorporated into the cells. The isolate showing this capability is now being characterized, and these results plus the results with labeled hypophosphite will be presented in our next report. Additional investigations are being performed to determine the preferred phosphorous source when this isolate is grown in a mixture of hypophosphite, phosphate, and phosphite, and to determine if high concentrations of hypophosphite or phosphite are toxic.

Characterization of the omnitherms is still in progress and these results will also be included in the next report. Such investigations will include growth curves, generation times, resistance to control agents, and other physiological characterizations. Another population study is under way, again using Cape Canaveral soil, to isolate more omnitherms. Continued investigations will be performed in an attempt to determine the distribution of these organisms from various spacecraft areas.

Now that procedures have been determined for maximum microbial isolation from ocean-dredge samples, microbial population studies will be performed on the sand and clay samples in an attempt to determine if this is a source of organisms currently under investigation.