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RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

Submitted by

Terry L. Foster, Ph. D.
Microbiology Laboratories
Department of Biology

Hardin-Simmons University
Abilene, Texas

RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

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by

Terry L. Foster, Ph.D.
Microbiology Laboratories
Department of Biology
Hardin-Simmons University
Abilene, Texas 79601

RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

Including

A Study of Psychrophilic Organisms Isolated from the Manufacture and Assembly Areas of Spacecraft to be Used in the Viking Mission

Semiannual Progress Report No. 5 of
Planetary Quarantine Activities
July 1, 1974 - December 31, 1974

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Department of Biology
Hardin-Simmons University
Abilene, Texas 79601

Report Prepared and Submitted by:

Terry L. Foster, Ph.D.
Principal Investigator

Luther Winans, Jr.

and

Raymond Carroll Casey
Research Associates

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FOREWORD

This fifth semiannual progress report summarizes work performed for the National Aeronautics and Space Administration by the Department of Biology at Hardin-Simmons University supported by NASA Grant NGR 44-095-001, and covers the period July 1, 1974 - December 31, 1974. As indicated on the title page, the title of this project has been changed to "The Response of Selected Microorganisms to Experimental Planetary Environments." This change is considered appropriate because the project had been asked to investigate organisms other than psychrophiles, and future investigations will have application to missions other than the Viking. The general thrust of the project will continue to be to isolate organisms of planetary quarantine significance and to determine their response in experimental planetary environments.

This report includes investigations of hardy organisms and Teflon ribbons from Cape Kennedy. One investigation involves the storage of heat-treated Teflon ribbons under conditions that the Viking spacecraft will be exposed after dry-heat sterilization (nitrogen-vacuum) and under conditions predicted for the surface of Mars. These experiments were designed to help answer the question of probability of contamination and growth of hardy organisms in the biopackage of the Viking spacecraft. Results indicate that storage under nitrogen-vacuum has little or no effect on the recovery of hardies, but storage of the ribbons under experimental Martian conditions at 15°C appears to reduce the probability of recovery of hardy organisms. Details of identification of these isolates and moderate characterization of

other hardy organisms are presented. Similar environmental investigations were performed using spore suspensions of hardy organisms, and the results indicate that some of these can survive the complete heat cycle, storage in nitrogen, effect of space vacuum, and storage in a dry Martian environment. Also, these will then grow in liquid media in the Martian atmosphere.

Another experiment reported here is a detailed investigation into the response of five hardy organisms to various conditions (moisture and nutrients) in the simulated Martian environment. Results indicate that all organisms tested can survive the dry state, and most of them can grow when moisture is added. All of these experiments indicate that hardy organisms will likely grow in the Martian environment if moisture is available, and these organisms definitely present a threat to contamination of the biopackage if they are transported to the surface of Mars.

The NASA Technical Officer for this grant is Lawrence B. Hall, NASA Planetary Programs, Code SL, Washington, D.C.

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RESPONSE OF HARDY ORGANISMS TO EXPERIMENTAL SPACECRAFT AND MARTIAN ENVIRONMENTS

The JPL Systems Test and Operations Lab at Cape Canaveral (formerly the Planetary Quarantine Unit, Phoenix CDC at Cape Kennedy) has been involved for quite some time with the study of fallout samples onto Teflon ribbons from various spacecraft areas. These ribbons are dry-heat-treated at 111.7°C following the proposed temperature profile that will be used on the Viking spacecraft. They are then covered with enriched TSA broth in an attempt to recover heat-resistant microorganisms, and the results indicate that such organisms are consistantly recovered. (For procedures and results, see Report No. 42, Apr.-June, 1973, Environmental Microbiology Section, Phoenix CDC, Phoenix Arizona, and others of their reports). These results led to a more extensive study of the problem and various other experiments were planned. At a meeting in February, 1974, at Ames Research Center such plans were discussed further, and it was agreed that certain aspects of the problem be investigated at Hardin-Simmons. One planned experiment included subjecting the heat-treated ribbons to conditions that the spacecraft would encounter followed by addition of the recovery medium to the ribbons. Another experiment included the addition of recovery medium to the ribbons followed immediately by their exposure to an artificial Martian environment and incubation at 15°C. This is done in an attempt to approximate the conditions of the Viking Lander Biological Instrument (VLBI) on the surface of Mars.

A. Recovery of Hardy Organisms from Teflon Ribbons after Storage Under Dry Nitrogen Followed by Vacuum

Working with John R. Puleo of the JPL lab at Cape Canaveral, it was decided to use one-half of the ribbons in five individual runs at H-SU for the nitrogen gas-vacuum study while processing the other half by the usual procedures at Cape Canaveral. Each run of the Teflon ribbon experiments includes 32 test ribbons which are exposed to air fallout, 8 of which are selected at random for N_o determinations. The remaining 24 ribbons and 9 sterile control ribbons are subjected to the 111.7°C heat cycle. Immediately upon their removal from the oven, the ribbons were placed into sterile jars with filtered, vented lids which allowed the aseptic exchange of gas inside the jar. These were then placed into sterile, metal pressure-vacuum containers (obtained from Tesco Equipment Company, Abilene, Texas) which are large enough to hold 18 jars. The containers were then sealed, flushed several times with dry nitrogen, and finally stored under five inches water pressure nitrogen (this is the condition under which Viking will be stored after sterilization). When all five runs had been performed and stored under nitrogen (2 1/2 weeks) they were transported by car to Hardin-Simmons for completion of the experiment. This mode of transportation was chosen to assure that no damage occurred to the ribbons and to avoid possible contamination due to air shipment.

From each run, three of the sterile controls were covered with the enriched TSA broth used at Cape Canaveral (0.1% soluble starch and 0.2% yeast extract) and placed in the incubator for 30 days at 32°C. All transfers were performed under laminar flow using sterile

gloves, gowns, caps, and masks. Other technique controls included sterilization of strips of dialysis tubing of proportionate dimensions to the teflon ribbons, placing in sterile jars, and adding recovery medium to these. One such technique control was used per Teflon ribbon control. None of these controls showed contamination.

At the end of 3 weeks at room temperature in the nitrogen atmosphere, the ribbons were slowly evacuated and subjected to a vacuum of 10^{-6} Torr. They were maintained under this vacuum for 3 weeks at room temperature. At the end of this treatment, they were brought to normal atmospheric pressure with sterile nitrogen, opened under laminar flow, and removed from the vacuum containers. The lids were removed and recovery medium added under laminar flow. They were then placed in the incubator for 30 days at 32°C.

At the end of the incubation time the jars were examined for turbidity. Samples were plated from jars showing growth to determine purity of the culture. Isolated colonies were then identified according to the method of the JPL lab, with each isolate also being sent to the JPL-PQ lab for identification. A flow diagram of these procedures is shown in Figure 1 (p. 5). Results of recovery of hardy organisms after storage in dry nitrogen followed by vacuum are shown in Table 1 (p. 6).

B. Recovery of Hardy Organisms From Teflon Ribbons after Storage in an Experimental Martian Environment at 15°C

Hardy organisms being transferred to the Martian atmosphere in association with the Viking biopackage will have nutrients and moisture available, some in an artificial atmosphere, others in the Martian

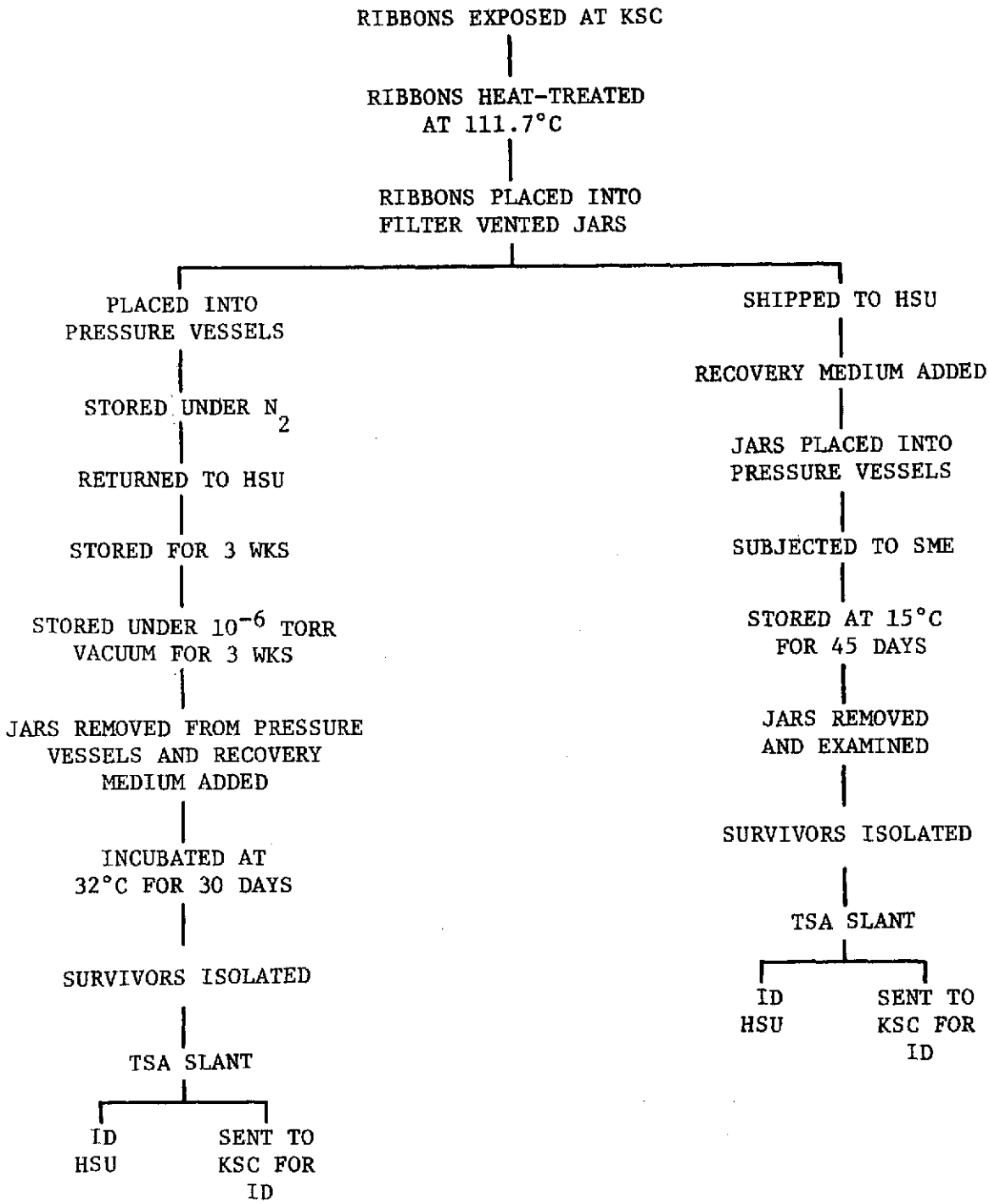
atmosphere, both maintained at approximately 15°C or less. To determine the response of hardy organisms from Teflon ribbons under these conditions, H-SU again worked in cooperation with the JPL lab at KSC.

In this experiment 5 KSC Teflon ribbon runs were divided into two groups, one being processed according to standard procedures at KSC, the other being shipped to H-SU. Control ribbons were covered immediately with TSA broth, and none of these were contaminated during shipping.

Of the five runs shipped to H-SU, one (T-10) had recovery medium added and received no treatment. This was done to determine the effect of shipping on recovery of hardies. The other four runs had recovery added, then were slowly evacuated with an increasing vacuum for 24 hours. They were then flushed several times with a gas mixture of 99.9% CO_2 plus 0.1% O_2 and were finally sealed under this mixture at a pressure of 7 mb (5.2 mm Hg). They were then stored at 15°C for 30, 35, or 40 days. This treatment was performed to approximate conditions the VLBI will experience on the surface of Mars. After incubation the containers were pressurized to normal atmospheric pressure with filtered nitrogen, opened, and the jars removed for macroscopic and microscopic examination. Samples from jars showing no turbidity were plated, and no growth was observed after 7-10 days incubation at 32°C. The protocol for this experiment is shown in Figure 1, and the results are presented in Table 1.

Summary of Teflon Ribbon Experiments

It can be seen in Table 1 that subjecting the ribbons to nitrogen-vacuum to approximate spacecraft conditions appears to have little



*SME = Simulated Martian Environment

Fig. 1. Schematic outline of procedures used in survival studies of hardy organisms from KSC Teflon ribbon experiments

Table 1. Survival of hardy organisms from KSC Teflon ribbons after storage under different conditions

Storage Conditions	Experiment Number	Positive/Total	Controls Positive/Total	Days Required to See Turbidity (number of ribbons)
N_2 (3 weeks) followed by vacuum (3 weeks)	T1	0/12	0/3	
	T2	6/12	0/3	5(4), 17(1), 23(1)
	T3	4/12	0/3	11(4)
	T4	4/12	0/3	5(2), 11(2)
	T5	6/12	0/3	5(3), 11(3)
	Total	20/60	0/15	5(9), 11(9), 17(1), 23(1)
Simulated Martian environment at 15°C	T6	0/12	0/4	30
	T7	0/12	0/4	30
	T8	2/12	0/4	40
	T9	0/12	0/4	35
	Total	2/48	0/16	
No treatment	T10	3/12	0/4	7(3)

effect on the recovery of hardy organisms. Twenty of the sixty ribbons demonstrated the presence of survivors. These isolates were identified according to KSC procedures and were found to be the same types of organisms routinely isolated as hardies.

In contrast to this, the ribbons subjected to the experimental Martian environment at 15°C showed a marked decrease in the recovery of survivors with only 2 of the 48 ribbons being positive. The hardy organisms are typically considered to be fairly slow-growers, and at this low temperature, they would be expected to be even slower. These ribbons were incubated for 30, 35, or 40 days, and the only survivors appeared in the set incubated at 40 days. Because of time constraints on completion of this investigation, prolonged incubation was not possible. Most of the survivors in the first part of this experiment showed turbidity in 5-11 days, and it seems that 30-35 days should be sufficient to demonstrate growth even at the lower temperature. Upon completion of this part of the investigation, these ribbons were incubated at ambient conditions for an additional 30 days to determine if survivors might be present that did not show turbidity originally. Prolonged incubation of these ribbons did not result in the recovery of additional survivors. It appears then that subjecting heat-treated Teflon ribbons to recovery medium and to the experimental Martian atmosphere at 15°C does decrease the recovery of hardy organisms, but does not eliminate them completely. The fact that two of the ribbons showed survivors indicates that the Martian atmosphere alone cannot be depended upon to prevent growth of survivors in the VLBI.

The final set of ribbons (T-10) in Table 1 was performed to deter-

mine the effect of shipping the ribbons from KSC to H-SU. As can be seen, it appears that there is no appreciable loss of recovery due to shipping. It is important to note that none of the controls showed contamination which indicates that the shipping containers designed for transport of ribbons during this investigation are satisfactory. It is possible that such containers can also be used to ship Teflon ribbons from JPL, Martin-Marietta, etc., to KSC.

Table 2 is a comparison of treatments of Teflon ribbons at H-SU to the standard procedures at KSC. From this comparison it can again be seen that nitrogen-vacuum has little effect on recovery of survivors with KSC recovering 32 of 60 ribbons, and H-SU recovering 20 of 60. However, the simulated Martian atmosphere appears to have a definite effect with KSC recovering 21 of 48 ribbons, and H-SU recovering only 2 of 48. As can be seen, the only survivors recovered at H-SU were from the set which showed the highest recovery rate at KSC (T-8). The low N_o's in these experiments can be accounted for by the fact that the sampling area inside the VAB was changed just prior to obtaining these samples.

This was a limited experiment to answer some specific questions in a short period of time. Because of the small sample sizes, no statistical evaluation will be made from these experiments. In summary, it appears that the conditions which the spacecraft will encounter will have little effect on the recovery of hardy organisms, whereas the proposed conditions on the surface of Mars may lower the probability of recovery of hardies but will not eliminate it completely.

Table 2. Comparison of results of VAB Teflon ribbon experiments performed according to standard procedures at KSC and experimental spacecraft and Martian environments at H-SU (111.7°C and 1.2 mg/L water)

H-SU Experimental Groups	Run No.	N_o-Spores (KSC)	KSC Results*			H-SU Results		
			Positive/Total	MPN For Ribbon	Survivor Fraction (N_H/N_o)	Positive/Total	MPN For Ribbon	Survivor Fraction (N_H/N_o)
Nitrogen-Vacuum	T-1	2.3×10^2	2/12	0.182	7.9×10^{-4}	0/12	0	0
	T-2	6.7×10^2	4/12	0.406	6.1×10^{-4}	6/12	0.693	1.0×10^{-3}
	T-3	8.0×10^2	9/12	1.387	1.7×10^{-3}	4/12	0.406	5.1×10^{-4}
	T-4	1.7×10^3	9/12	1.387	8.2×10^{-4}	4/12	0.406	2.4×10^{-4}
	T-5	1.1×10^3	8/12	1.099	9.7×10^{-4}	6/12	0.693	6.3×10^{-4}
Simulated Martian Environment (15°C)	T-6	3.6×10^2	2/12	0.182	5.1×10^{-4}	0/12	0	0
	T-7	6.5×10^2	5/12	0.539	8.3×10^{-4}	0/12	0	0
	T-8	6.1×10^2	10/12	1.792	3.0×10^{-3}	2/12	0.182	3.0×10^{-4}
	T-9	1.0×10^3	4/12	0.406	4.0×10^{-4}	0/12	0	0
No Treatment	T-10	8.8×10^2	4/12	0.406	4.6×10^{-4}	3/12	0.287	3.2×10^{-4}

*Using standard KSC procedures

C. Characterization of Hardy Organisms

Organisms isolated from the Teflon ribbon experiments described in sections A and B were identified and found to be of the same types as those routinely isolated at KSC. The results of identification are presented in Table 3, and complete biochemical characterization of these is shown in Table 4. As can be seen, two of the ribbons with survivors showed mixed populations (T2-22 and T7-18). This phenomenon has occurred more frequently since the temperature was lowered from 113°C to 111.7°C. Although this is a small sample, the results are compared to previous experiments at KSC, and it can be seen in Table 5 that the percent distribution is comparable. This, and the total absence of contamination of controls, emphasizes that these isolates are almost certainly typical hardy organisms.

During the course of study of hardy organisms, the efficiency of the enriched anaerobic agar was discussed. Some of the hardies were subjected to media in Brewer anaerobe jars to determine their ability to grow anaerobically. The results are shown in Table 6, and it appears that the enriched anaerobic agar may be toxic because it routinely cultured fewer organisms than the anaerobe jar, and in no case did an organism exhibit a positive result on anaerobic agar and a negative result in the jar.

Because of the low temperature to be encountered on the Martian surface and in the biopackage, several isolates were examined for their ability to grow at low temperature. The results of growth at 3°C, 15°C, and 24°C are presented in Table 7. This demonstrates that at least four of the 24 isolates examined grow at 3°C, 10 at 15°C, and 20 at 24°C.

Table 3. Identification of hardy organisms recovered from KSC Teflon ribbons after storage of ribbons under different conditions

Isolate No.	Identification
T2-4	Atypical *Bacillus* (unreactive)
T2-10	Atypical *Bacillus* (reactive)
T2-22A	Atypical *Bacillus* (reactive)
T2-22B	Atypical *Bacillus* (unreactive)
T2-22D	*Bacillus pumilus*
T2-27	*B. brevis*
T2-30	*B. lentus*
T3-17	Atypical *Bacillus* (unreactive)
T3-23	Atypical *Bacillus* (unreactive)
T3-31	*B. lentus*
T4-9	Atypical *Bacillus* (unreactive)
T4-13	*B. pumilus*
T4-22	*B. lentus*
T4-28	Atypical *Bacillus* (unreactive)
T5-2	*B. lentus*
T5-15	Atypical *Bacillus* (unreactive)
T5-20	*B. subtilis*
T5-24	*B. subtilis*
T5-26	Atypical *Bacillus* (unreactive)
T5-28	*B. lentus*
T7-18A	Atypical *Bacillus* (reactive)
T7-18B	Atypical *Bacillus* (unreactive)
T7-20	*B. sphaericus*
T8-18	Atypical *Bacillus* (reactive)
T8-28	Atypical *Bacillus* (unreactive)
T10-6	*B. lentus*
T10-10	*B. lentus*
T10-30	*B. lentus*

Table 4. Biochemical test reactions of heat-stressed isolates after storage of Teflon ribbons in nitrogen-vacuum or simulated Martian environment at H-SU

Organism	No. of Isolates	Starch	Casein	Mannitol	V.P.	Citrate	Nitrate	Anaerobic Growth	Tyrosine	Phenyl-alanine
B. brevis	1	-*	-	+	-	-	+	-	-	-
B. lentus	5	-	-	+	-	-	-	-	-	-
	1	+	-	-	-	-	-	-	-	-
	1	+	-	+	-	-	-	-	-	-
	1	+	-	+	-	-	+	-	-	-
B. pumilus	2	-	+	+	+	+	-	+	-	-
B. sphaericus	1	-	-	-	-	+	-	-	-	-
B. subtilis	2	+	+	+	-	+	+	-	-	-
Atypical Bac.	10	-	-	-	-	-	-	-	-	-
	1	+	+	-	-	-	-	-	-	-
	1	+	-	-	-	+	-	-	-	-
	1	-	-	-	-	-	+	-	-	-
	1	+	+	+	-	+	-	-	-	-

*Duplicate samples

Table 5. Comparison of distribution of different species of hardy organisms isolated from KSC and H-SU

Organism	KSC Percent	H-SU Percent
B. lentus	43.0	29.0
B. brevis	7.5	3.6
B. circulans	2.5	0
B. coagulans	2.5	0
B. macerens	2.5	0
B. subtilis	2.5	7
B. pumilis	0	7
B. sphaericus	0	3.6
Atypical (Reactive)	30.0	14.3
Atypical (Unreactive)	10.0	36.0

Table 6. Results of anaerobic growth of hardy organisms in the Brewer GasPak jar and in enriched anaerobic agar (KSC)

Isolated At KSC	GasPak	Anaerobic Agar	Isolated At H-SU	GasPak	Anaerobic Agar
M1-12	+	-	T2-4	-	-
M1-29	-	-	T2-10	-	-
M2-18	+	+	T2-22A	-	-
M4-6	-	-	T2-22B	-	-
M5-19	-	-	T2-22D	+	+
M6-11	-	-	T2-27	-	-
M6-12	+	+	T2-30	-	-
M6-28	-	-	T3-17	-	-
M8-25	+	+	T3-23	-	-
M8-28	+	-	T3-31	+	-
M10-2	+	-	T4-9	-	-
M10-20	+	-	T4-13	+	-
M10-30	-	-	T4-22	+	-
M13-20	+	-	T4-28	-	-
M16-16	+	+	T5-2	-	-
M16-23	-	-	T5-15	-	-
M18-10	+	-	T5-20	+	-
M18-29w	-	-	T5-24	+	-
M18-29c	-	-	T5-26	-	-
M20-27	-	-	T5-28	-	-
V1-6	-	-	T7-18A	+	-
V1-9	-	-	T7-18B	-	-
V2-20	-	-	T7-20	+	+
V3-13	-	-	T8-18	-	-
V3-28	-	-	T8-28	-	-
V5-8	-	-	T10-6	-	-
			T10-10	-	-
			T10-30	-	-
Total (26)	10	4	(28)	8	2

Table 7. Growth of KSC hardy organisms at temperatures below 32°C after 14 days

Culture	3°C %T	15°C %T	24°C %T
M1-12	100	100	82
M1-29	100	100	78
M2-18	100	28	29
M4-6	81	38	27
M5-19	100	48	27
M6-11	100	98	75
M6-12	90	58	28
M6-25	90	52	34
M6-36	100	100	88
M8-14	99	84	96
M8-25	100	26	37
M8-28	98	97	68
M9-12	96	96	87
M10-2	98	86	53
M10-20	100	100	94
M10-30	97	97	76
M13-20	90	84	59
M16-16	100	99	77
M16-23	99	99	95
M18-10	96	96	47
M18-29c	98	97	97
M20-27	99	99	78
V1-9	98	97	86
V2-20	100	48	40

Three of them failed to grow. Organism M4-6 appears to grow quite well at lower temperatures, and this is especially interesting because this isolate has been demonstrated to be extremely dry heat resistant at 113°C (Cinn. - FDA). Two of the isloates M8-14 and M8-25 appear to have an optimum growth temperature at approximately 15°C.

D. Response of Hardy Organism Spore Crops to Experimental Spacecraft and Martian Environments

To further demonstrate the response of hardy organisms to the prescribed experimental conditions, an experiment was designed to subject spore suspensions of previous isolates to the same conditions as those used for the Teflon ribbons. Spore suspensions of five hardy organisms isolated at KSC were prepared, titered, and approximately 10^5 spores were deposited into sterile glass vials. The alcohol was evaporated and the vials were sent to KSC for subjection to the dry-heat cycle at 111.7°C. This investigation included seven different experimental groups, each in triplicate, for each of the five organisms. One experimental group was used as a control to determine the effects of shipping and handling of samples between H-SU and KSC. The vials were returned to H-SU after heat-treatment and subjected to various experimental groups as shown in Figure 2. The seven groups are as follows (Groups 2-7 were processed through the heat cycle):

1. N_o - Untreated
2. N_o - After heat-treatment
3. Stored in dry simulated Martian environment (SME) at 15°C
4. Stored in TSB under SME - Counted periodically
5. Stored under nitrogen-vacuum
6. Stored under nitrogen-vacuum, then dry SME at 15°C for 30 days
7. Stored under nitrogen-vacuum, then in TSB in SME - Counted periodically

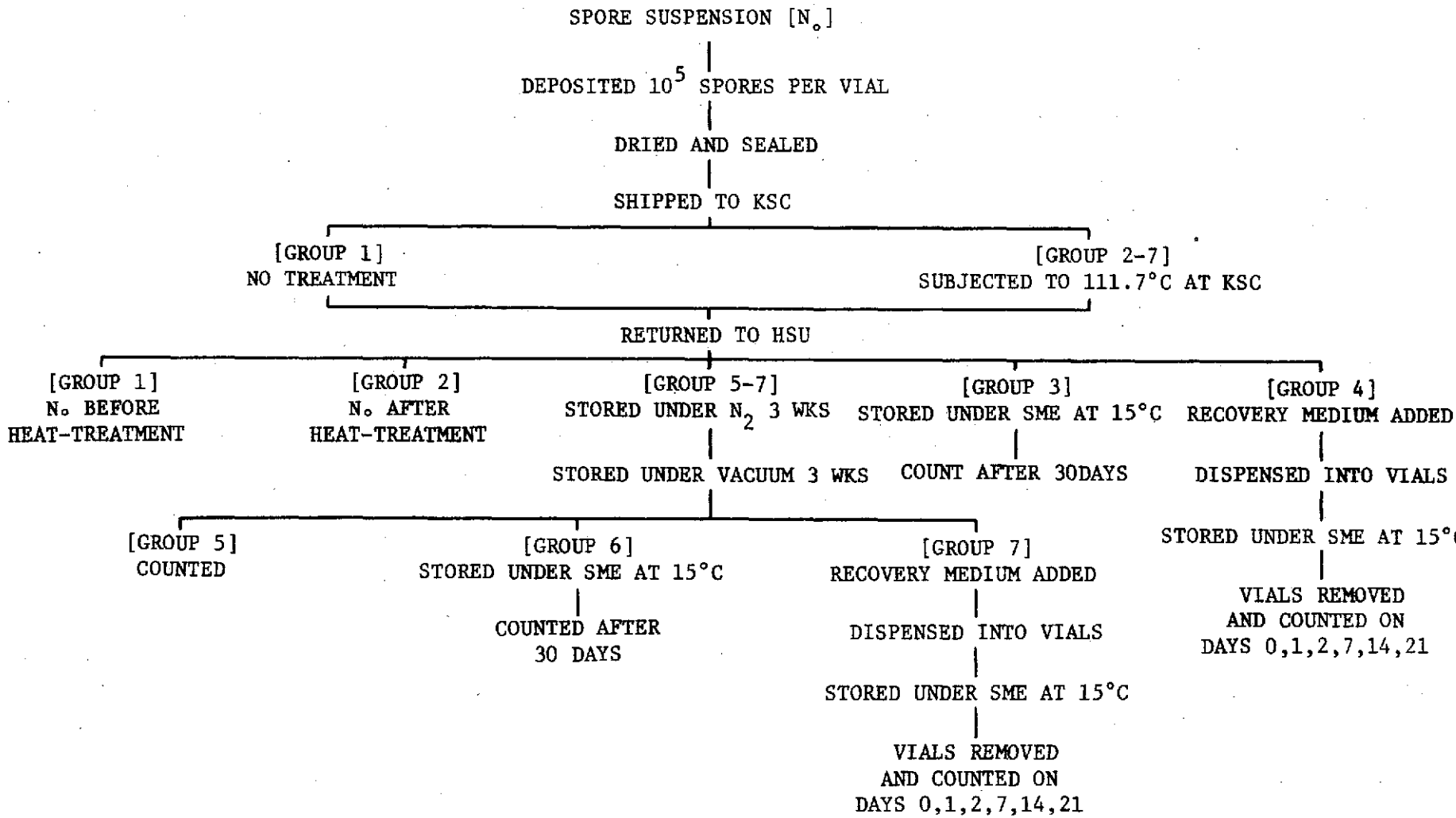


Fig. 2. Schematic outline of experimental conditions used in survival and growth studies of selected hardy organisms from KSC

Not only is this experiment designed to demonstrate the response of hardy organism spore crops to experimental environments, it also shows that heat-resistant spore crops can be successfully prepared from hardy organisms which have been subcultured numerous times. Hardy organisms M6-12, M6-25, M18-10, M20-27, and V2-20 had been obtained from KSC for earlier experiments, and they had been subcultured many times before spore suspensions used in this experiment were prepared.

The results of this experiment are presented in Table 8. The comparison of the original deposit and the N_o after shipping (Group 1) shows that shipping the vials and holding for 2-3 weeks has no appreciable effect on the recovery of spores. A comparison of untreated and heat-treated N_o's (Group 1 and 2) shows that 3 of the 5 samples experienced only a one log drop after heating at 111.7°C for 30 hours. This demonstrates that these are truly hardy organisms. The other two experienced decreases below our countable level, but surviving colonies were apparent on the least dilute plates. Storage of the samples in the dry state in the experimental Martian environment (Group 3) showed little change from the heated N_o except for one of the sublethally injured isolates which showed some recovery (M6-25). Storage of the heat-treated spore crops in TSB in the Martian environment at 15°C (Group 4) allows for rapid growth of the hardy organisms as demonstrated by increases of several logs after only seven days. In other words, it appears that in this experiment with spore suspensions, the experimental Martian environment has no lethal or inhibitory effect on the hardy organisms. This is in contrast to what was demonstrated in the experiment with the Teflon ribbons where only a few hardies grew out in the experi-

Table 8. Survival of heat-treated hardy organisms from KSC following different methods of storage (survivors/vial)

Organism	Amount Deposited	Group 1 N_o Not Heated	Group 2 Heat-treated N_o	Group 3 Stored in Dry SME
M6-12	5.0×10^5*	1.9×10^5	2.1×10^4	8.3×10^3
M6-25	3.5×10^5	2.9×10^5	$< 1.0 \times 10^1$	3.3×10^3
M18-10	3.0×10^5	5.2×10^4	4.2×10^3	8.7×10^3
M20-27	1.2×10^5	7.9×10^4	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
V2-20	1.1×10^5	1.4×10^4	1.4×10^3	4.2×10^3

	Group 4 - Stored in TSB under SME for 21 days					
Organism	Day 0	Day 1	Day 2	Day 7	Day 14	Day 21
M6-12	7.9×10^3	1.0×10^4	4.7×10^4	9.4×10^6	2.6×10^8	9.1×10^5
M6-25	$< 3.0 \times 10^2$	8.7×10^3	8.0×10^4	1.5×10^7	3.7×10^6	2.1×10^5
M18-10	5.7×10^3	7.8×10^3	1.5×10^5	6.4×10^7	2.6×10^7	6.2×10^5
M20-27	$< 3.0 \times 10^2$	4.4×10^3	5.3×10^4	1.2×10^8	1.0×10^8	6.2×10^8
V2-20	1.4×10^3	3.3×10^3	2.4×10^3	5.3×10^7	3.0×10^7	2.0×10^6

(continued)

*Each count is the average of triplicate vials (6 plates)

Table 8. (cont.)

Organism	Group 5 N_2-Vac	Group 6 N_2-Vac-SME
M6-12	$7.0x10^1$	$3.9x10^2$
M6-25	0	$2.3x10^3$
M18-10	$1.9x10^3$	$1.6x10^3$
M20-27	0	$1.8x10^3$
V2-20	$5.1x10^2$	$4.1x10^3$

Group 7 - N_2-Vac-TSB-SME

Organism	Day 0	Day 1	Day 2	Day 7	Day 21
M6-12	$7.0x10^1$	0	$8.0x10^2$	$3.0x10^2$	$1.7x10^3$
M6-25	0	0	$2.3x10^2$	$2.0x10^2$	$1.0x10^4$
M18-10	$5.7x10^2$	$3.5x10^2$	$2.5x10^2$	$5.0x10^2$	$5.0x10^5$
M20-27	0	0	0	0	0
V2-20	$1.6x10^2$	$9.5x10^2$	$9.6x10^2$	$6.5x10^2$	0

mental Martian environment. This could be an effect of the size of the hardy population because the N_o of hardies on the ribbons would be extremely low while the N_o of the pure spore suspensions was 10^5 spores per vial, thus allowing for an increased probability of isolating a subpopulation which could survive and grow in the simulated Martian environment.

The final three groups of this experiment were subjected to nitrogen (3 weeks) then vacuum (3 weeks). Group 5 was then counted, and the results show that the two organisms which were almost destroyed by the heat cycle (M6-25 and M20-27) did not appear to survive the nitrogen-vacuum treatment. Organism M6-12 also showed a marked decline. However, Group 6 was treated in the same way as Group 5, except it was then stored dry in the experimental Martian environment at 15°C. This treatment shows that the organisms generally recover to approximately the same level as organisms in Group 3. Group 6 is indicative of what might happen to hardies from the oven to the dry surface of Mars. Group 7 is indicative of what might happen to the hardies from the oven to the culture medium in the biopackage. It can be readily seen that the organisms in this group exhibit variable behavior, but the final results indicate that at least three of the organisms will grow in this environment. The results of the hardy organism spore suspensions in the experimental spacecraft and Martian environments indicate that the hardies can likely survive the storage under nitrogen, the hard vacuum, the Martian environment, and that some can likely grow in nutrient and moisture conditions (such as the VLBI) on the surface of Mars. This experiment strongly emphasizes what was stated earlier, that hardies will likely

survive nitrogen-vacuum (transport to Mars), and that the Martian environment cannot be relied upon to retard growth of these hardy organisms.

E. Response of Hardy Organism Spore Crops to the Simulated Martian Environment

In this experiment, spore suspensions of the five hardy organisms used in experiment D were used. These were inoculated into sterile VAB soil under various experimental conditions, subjected to the artificial Martian environment, and samples counted periodically for 45 days to determine changes in population. The Martian environment in this experiment consisted of 99.9% CO_2 + 0.1% O_2, 7 mb pressure, -65°C for 16 hours and 20°C for 8 hours, and variable moi ture and nutrients. The procedures for establishing this atmosphere have been described in our earlier reports.

The substrate used in this experiment was VAB soil which was deposited into stainless steel cups, sterilized, and dried at 170°C for 4 hours. The cups were then placed into vacuum dessicators containing activated alumina. At the desired time, a 0.1 ml. anhydrous alcohol suspension of spore crops was deposited into the cups, the cups then being placed back into the dessicator and into a 60°C oven to evacuate the alcohol. The five organisms used in the experiment are the same as those used earlier (M6-12, M6-25, M18-10, M20-27, and V2-20), and the vials were set up in different groups as shown in Table 9. Duplicate samples of each organism from each of the groups were counted periodically in duplicate so that each point is an average of four counts. The groups with excess moisture (C, D, E, F)had 0.1 ml. of water or 0.1 ml. of TS broth added per gram of soil.

Table 9. Experimental conditions used in growth studies of selected KSC hardy organisms under a simulated Martian environment

GROUP	TREATMENTS
A	Dry soil only - Incubated 42 days.
B	Dry soil with nutrients - Incubated 42 days.
C	Soil with excess moisture - Incubated 42 days.
D	Soil with excess moisture and nutrients - Incubated 42 days.
E	Dry soil only - 28 days - Add excess moisture - 17 days.
F	Dry soil only - 28 days - Add excess moisture & nutrient - Incubated 17 days.
CONTROLS	
C1-A	Dry soil - Ambient Atmosphere - Incubated 42 days.
C1-B	Dry soil - Simulated Atmosphere - Incubated 42 days.
C2-A	Soil with excess moisture & nutrients - Ambient Atmosphere Incubated 42 days.
C2-B	Soil with excess moisture & nutrients - Simulated Atmosphere Incubated 42 days.

The results of this experiment are shown in Figures 3-20. There are three figures for each organism, the first showing dry conditions, the second showing moist conditions, and the third showing dry for 28 days followed by addition of moisture on day 28. To assure proper treatment, all vials were opened and resealed on day 28 even if they had no moisture added.

Several important conclusions can be reached by viewing these growth curves. First, in no case was there a change in population in the dry samples. As expected, the organisms cannot grow without moisture, but neither do the organisms decrease in number. In other words, in the dry state the Martian environment is not toxic to any of the five organisms over a 42 day period. This indicates that the organisms may survive on Mars until a favorable microenvironment may be reached.

Another important point to mention in the graphs is the fact that two of the five organisms (M6-12 and M20-25) appear to decrease their populations quite rapidly upon the addition of moisture. This lethal effect seems to be a factor of the freeze-thaw cycle, and not the Martian atmosphere because Figures 18 and 19, which show controls incubated at 24°C, demonstrated obvious growth of these two isolates in the Martian atmosphere. The other three test organisms demonstrate obvious population increases in the simulated Martian environment in the presence of moisture. The control experiments demonstrate that the three organisms examined can grow in the simulated atmosphere at 24°C, but they do so for a shorter period of time than in the ambient atmosphere which maintains its high population.

In summary it appears that individual hardy organisms will respond differently to the experimental Martian environment with excess moisture, some growing, others being killed. In the dry atmosphere, it appears that the organisms will likely survive, and may indeed be preserved for prolonged periods of time. This may indicate that bacterial spore contaminants may survive on the surface of Mars until they can be distributed to a favorable microenvironment. This information may also be useful in calculation of P_G on Mars after Viking lands. If more moisture is found then is presently predicted, the probability that growth might occur would be increased.

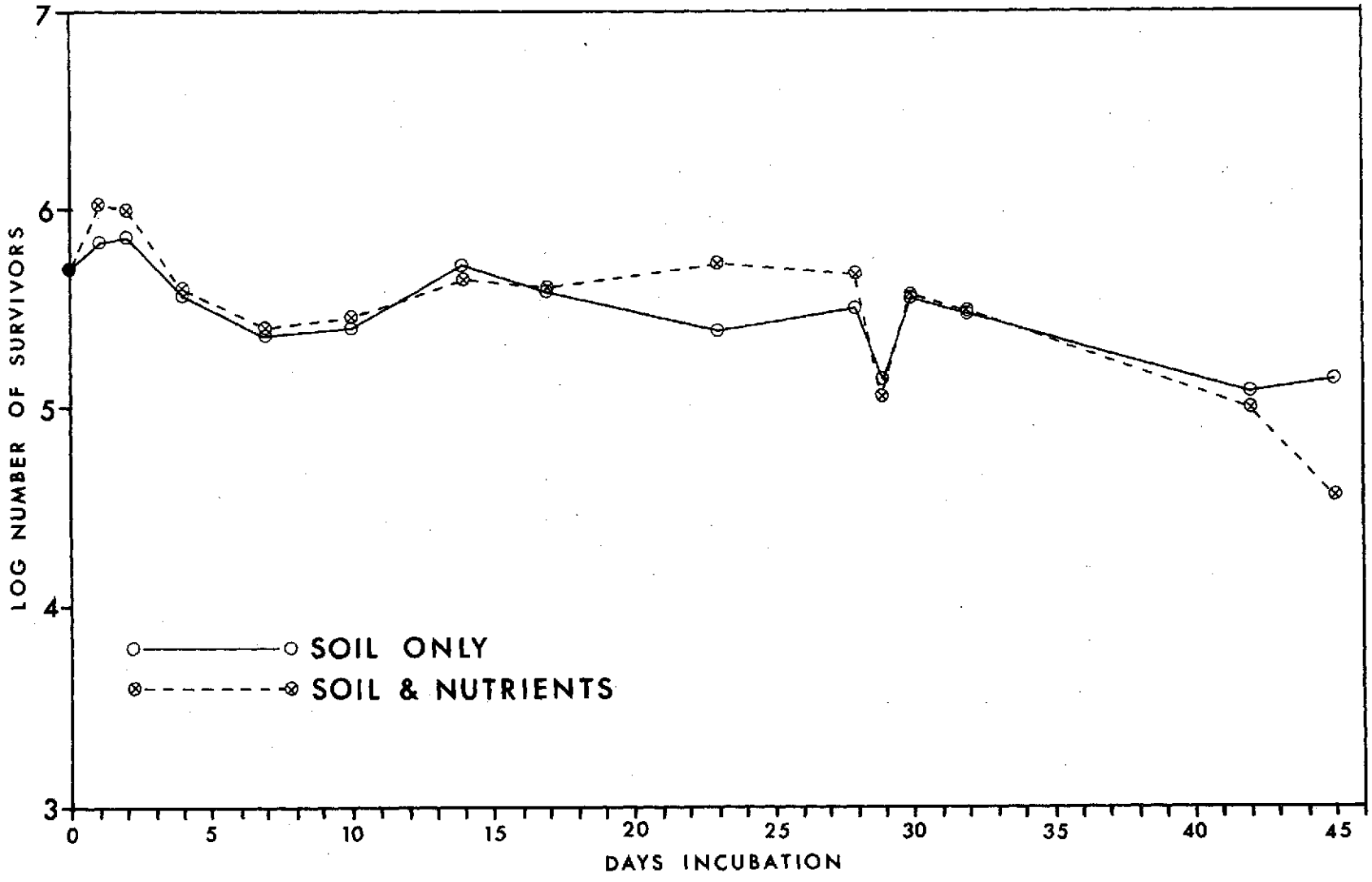


Fig. 3. Response of organism M6-12 in VAB soil to a dry simulated Martian environment

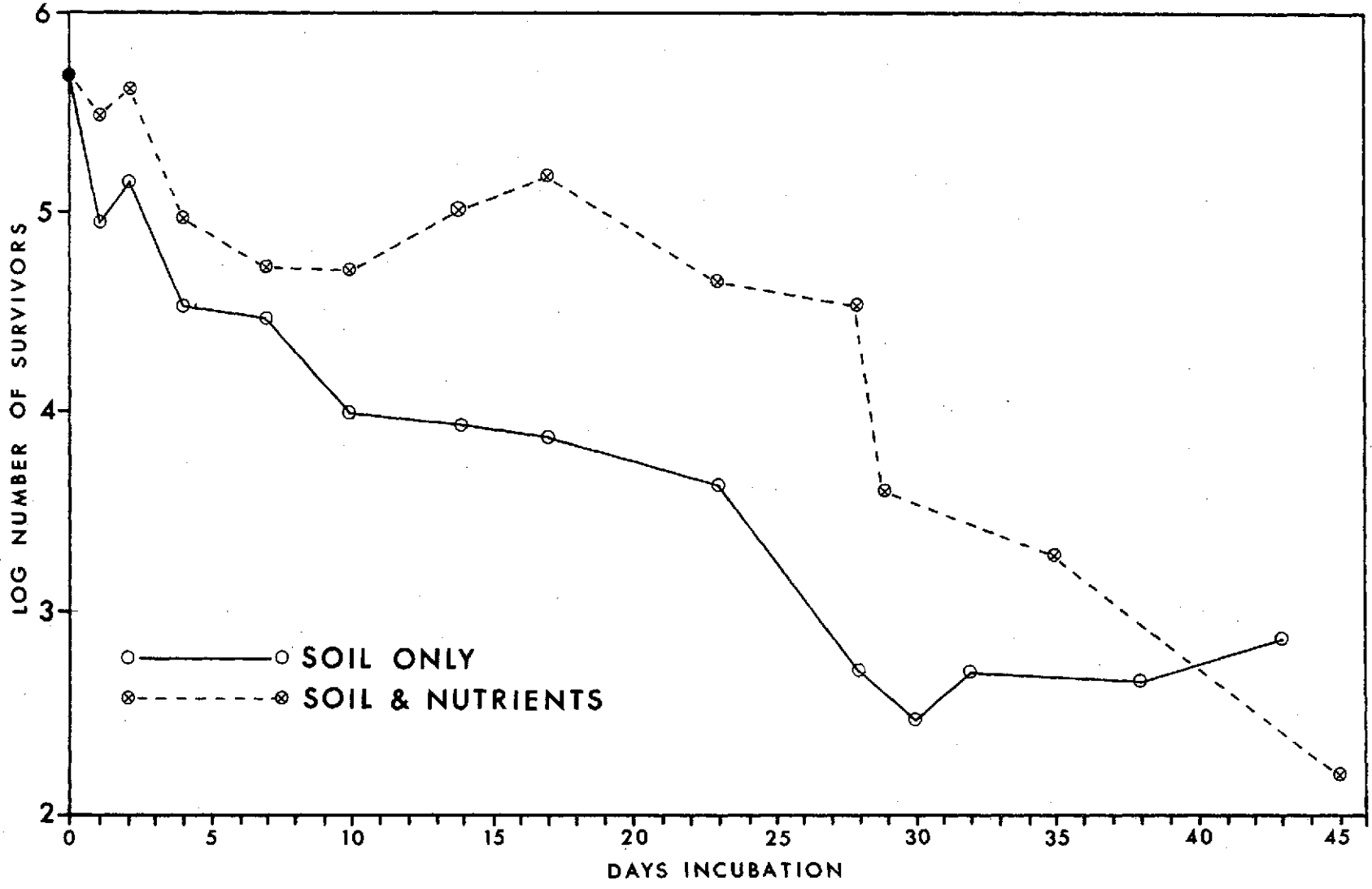


Fig. 4. Response of organism M6-12 in VAB soil to a simulated Martian environment containing excess moisture

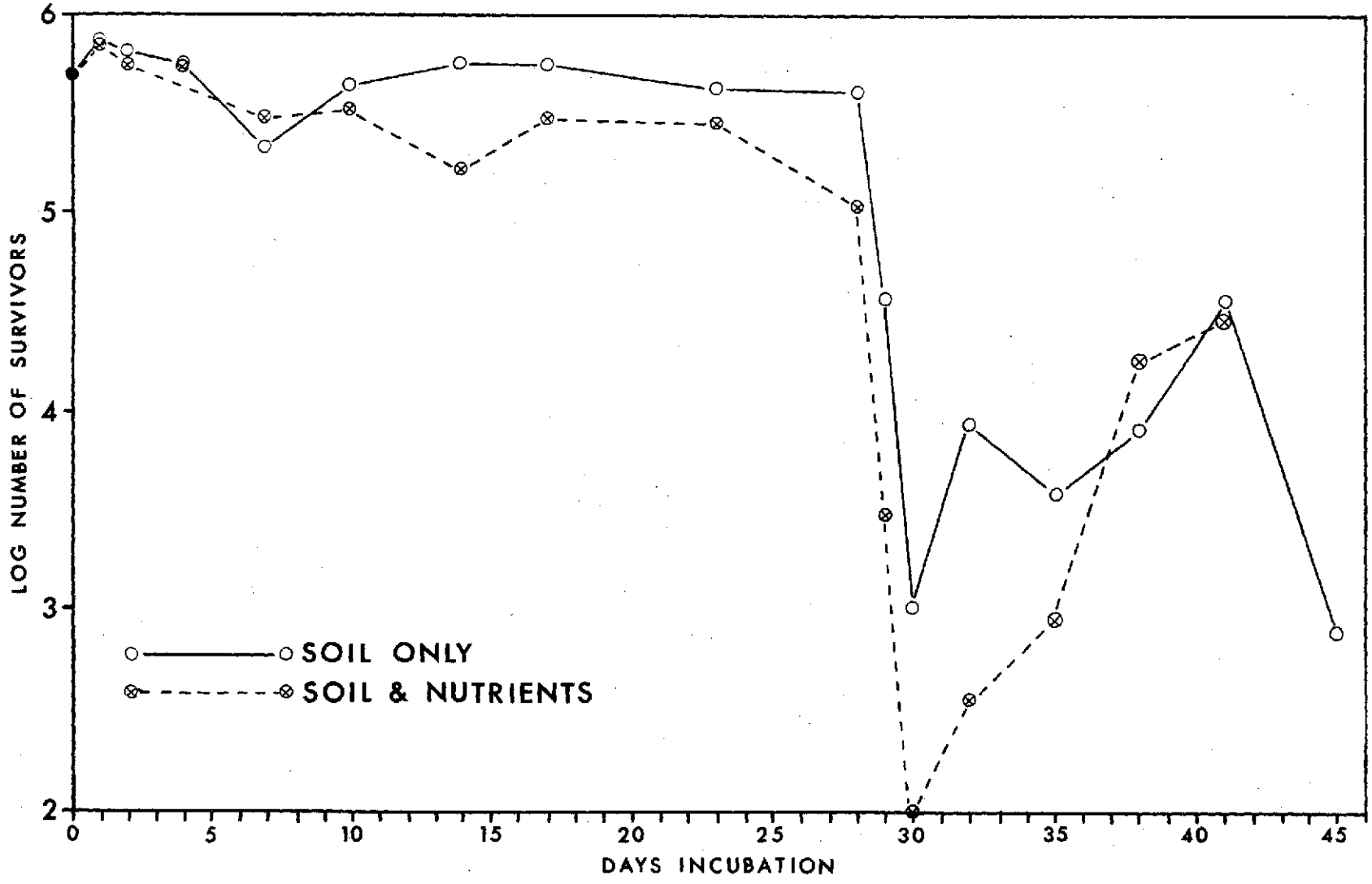


Fig. 5. Response of organism M6-12 in VAB soil to a simulated Martian environment. Environment was dry the first 28 days and contained excess moisture the last 17 days

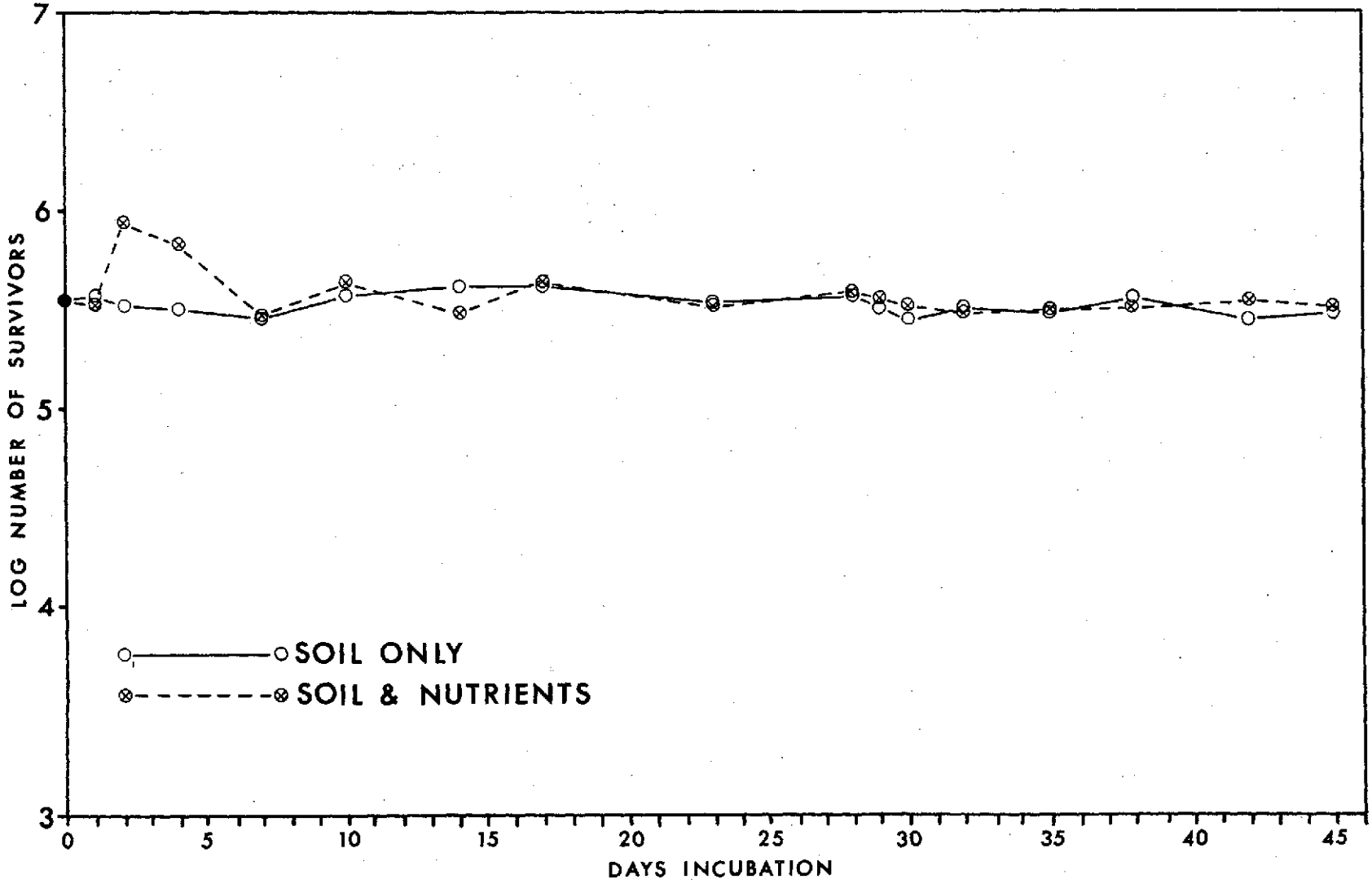


Fig. 6. Response of organism M6-25 in VAB soil to a dry simulated Martian environment

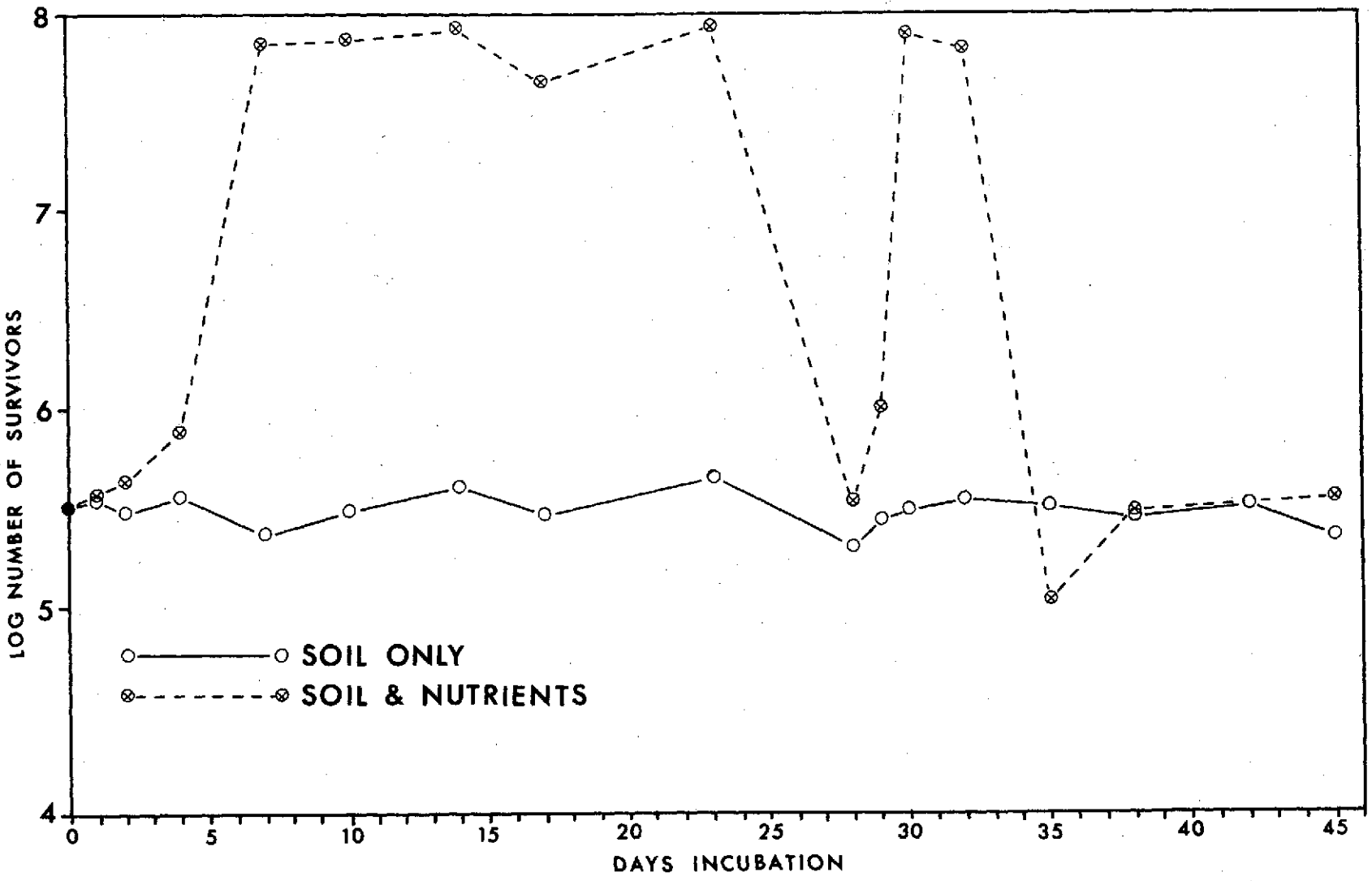


Fig. 7. Response of organism M6-25 in VAB soil to a simulated Martian environment containing excess moisture

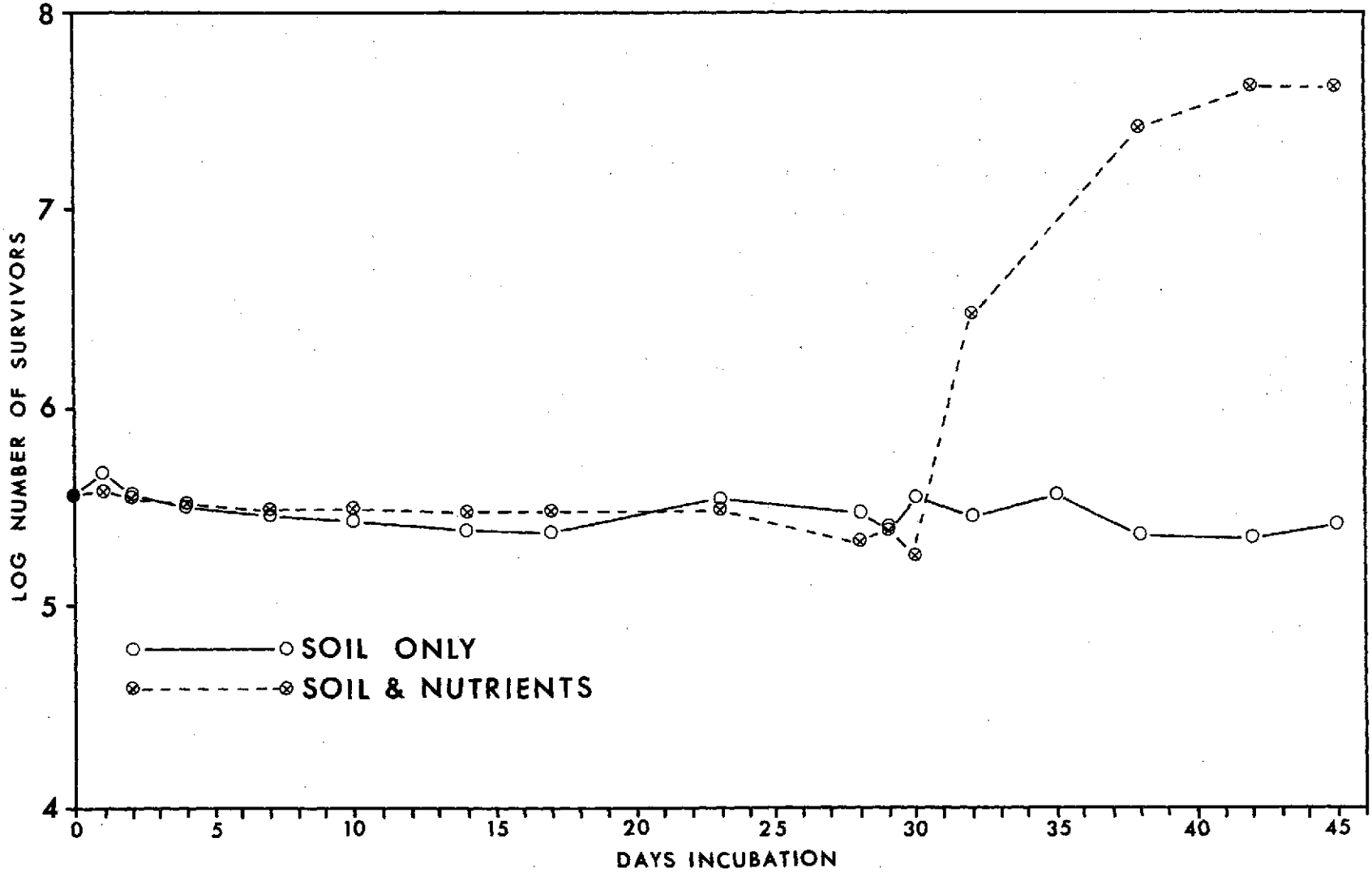


Fig. 8. Response of organism M6-25 in VAB soil to a simulated Martian environment. Environment was dry the first 28 days and contained excess moisture the last 17 days

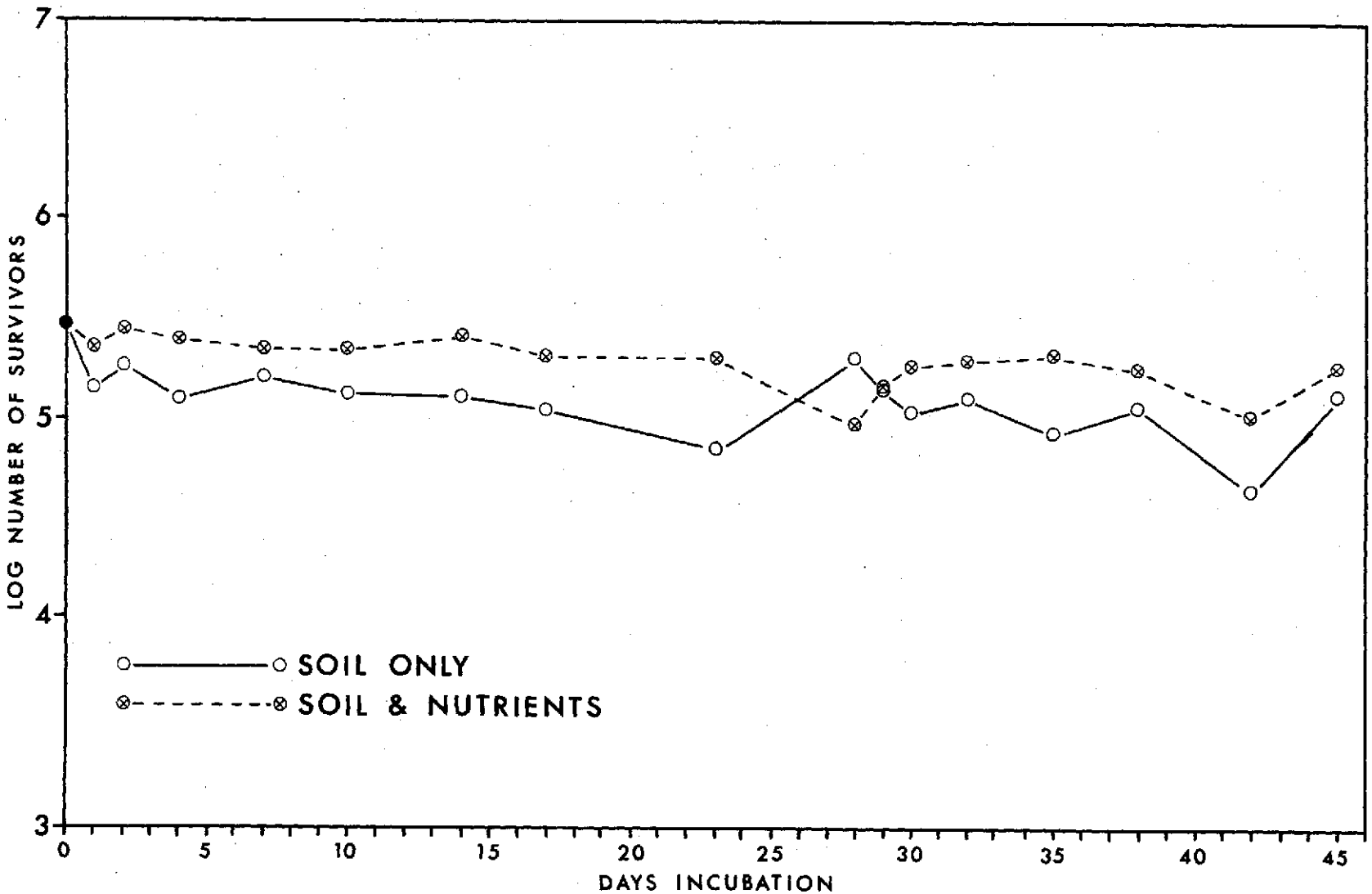


Fig. 9. Response of organism M18-10 in VAB soil to a dry simulated Martian environment

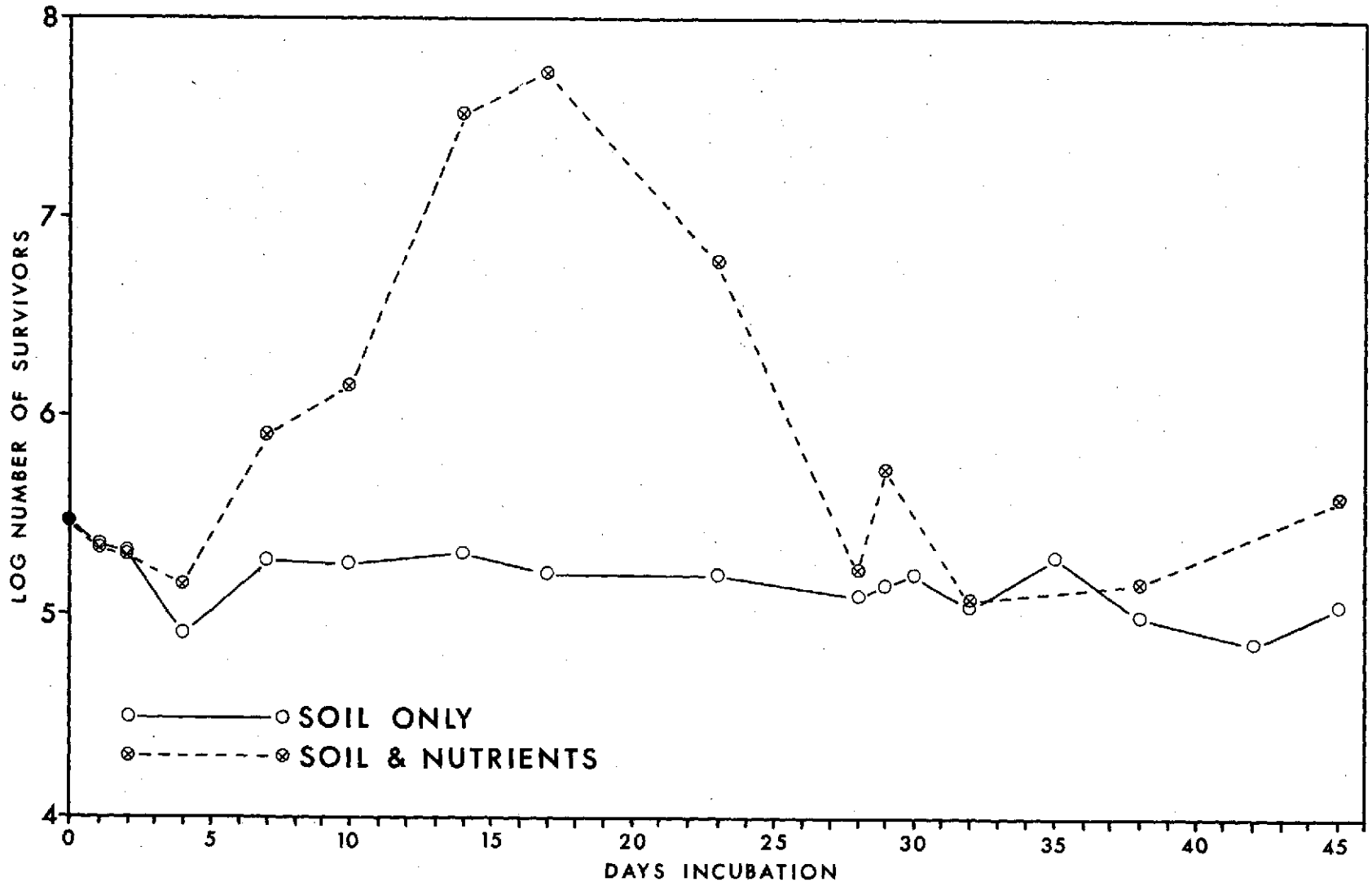


Fig. 10. Response of organism M18-10 in VAB soil to a simulated Martian environment containing excess moisture

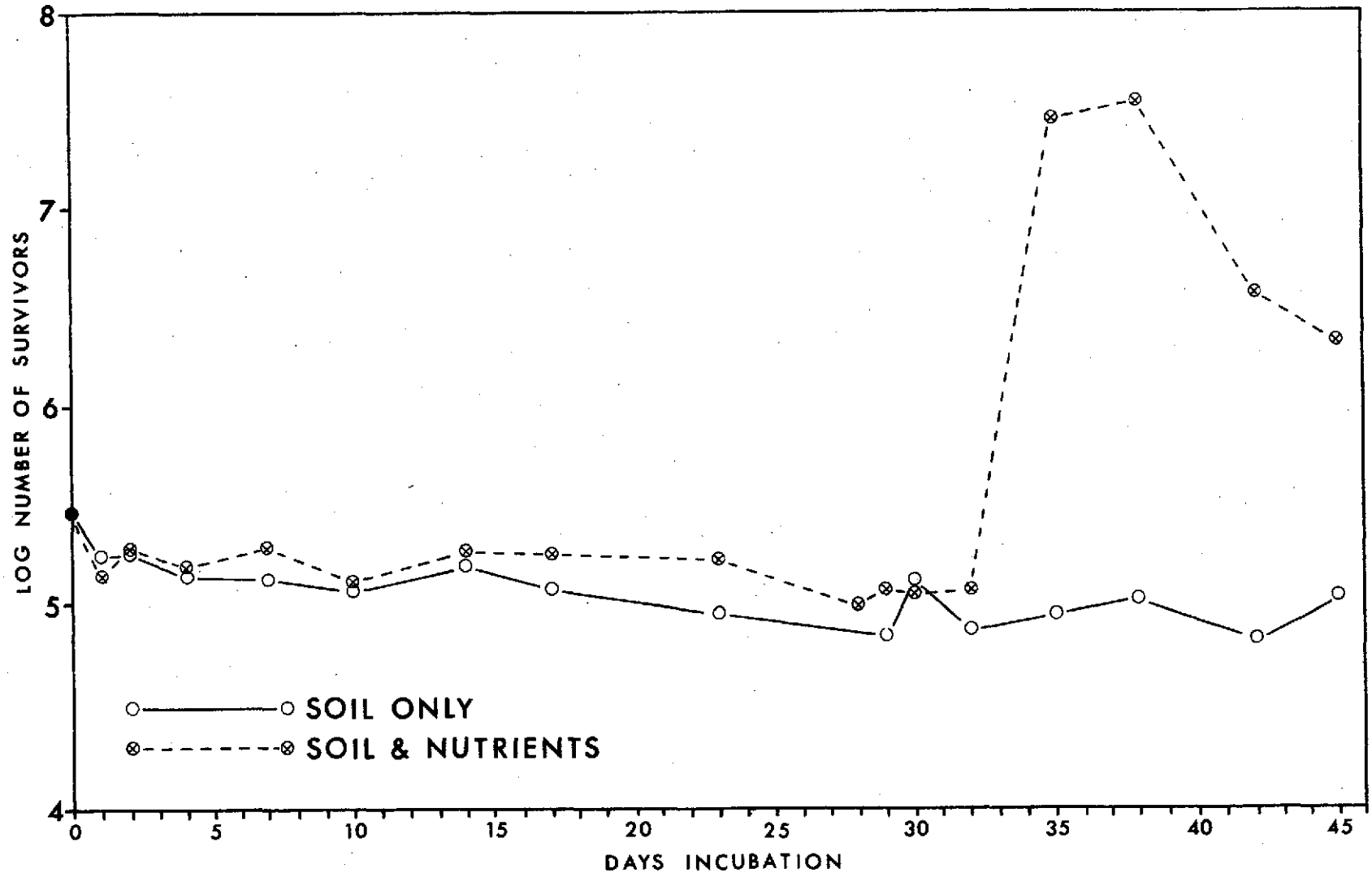


Fig. 11. Response of organism M18-10 in VAB soil to a simulated Martian environment. Environment was dry the first 28 days and contained excess moisture the last 17 days

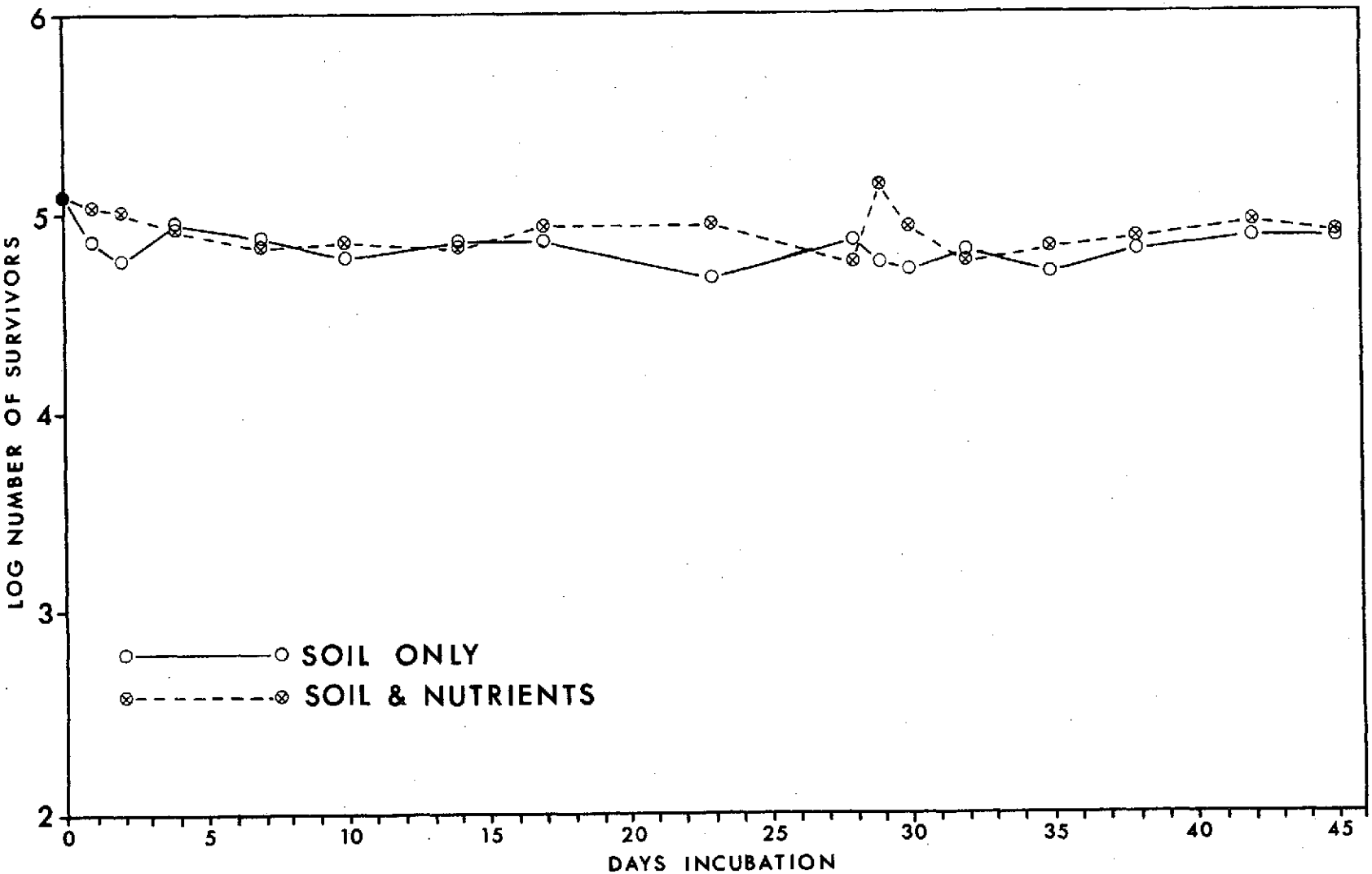


Fig. 12. Response of organism M20-27 in VAB soil to a dry simulated Martian environment

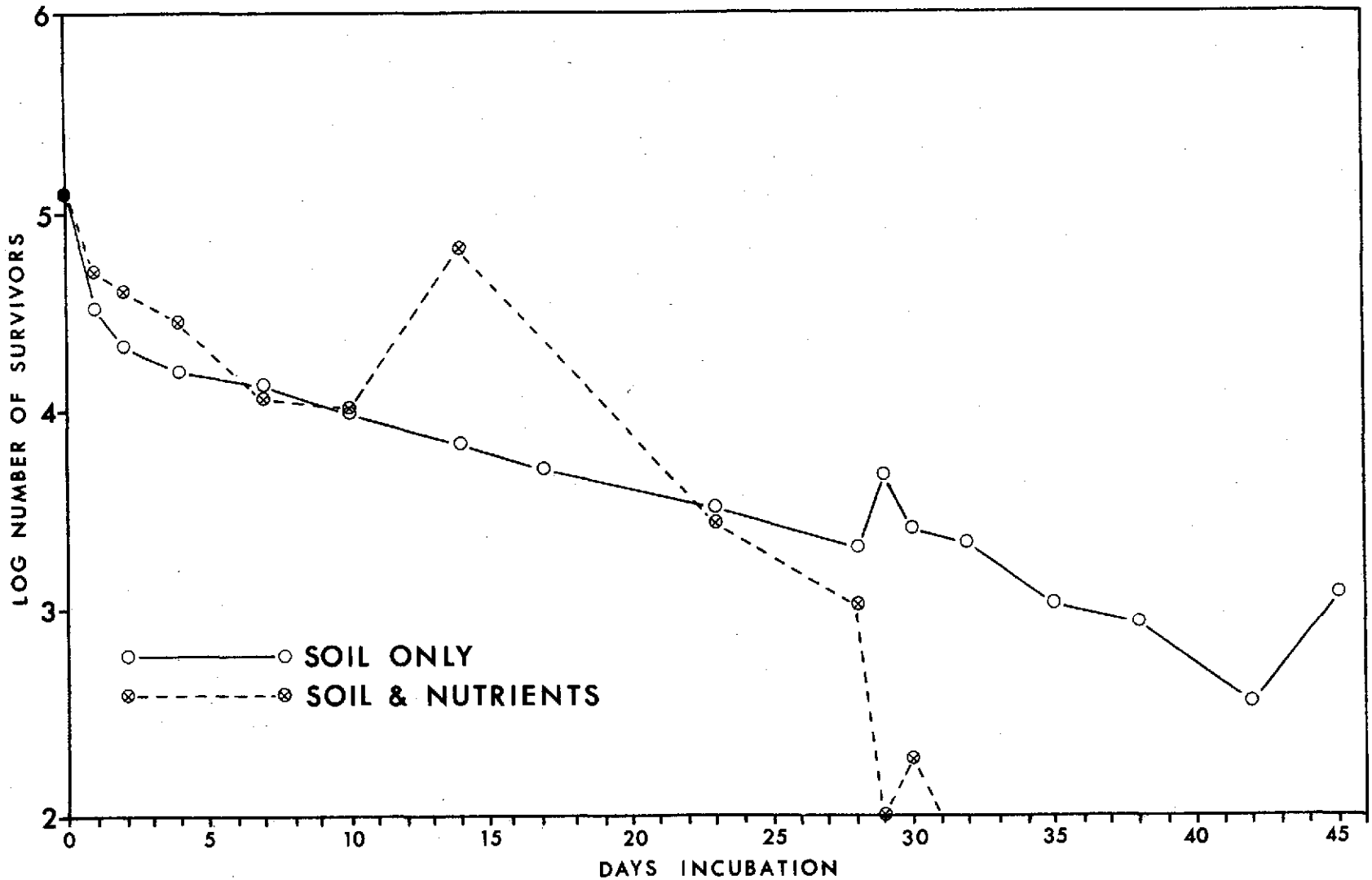


Fig. 13. Response of organism M20-27 in VAB soil to simulated Martian environment containing excess moisture

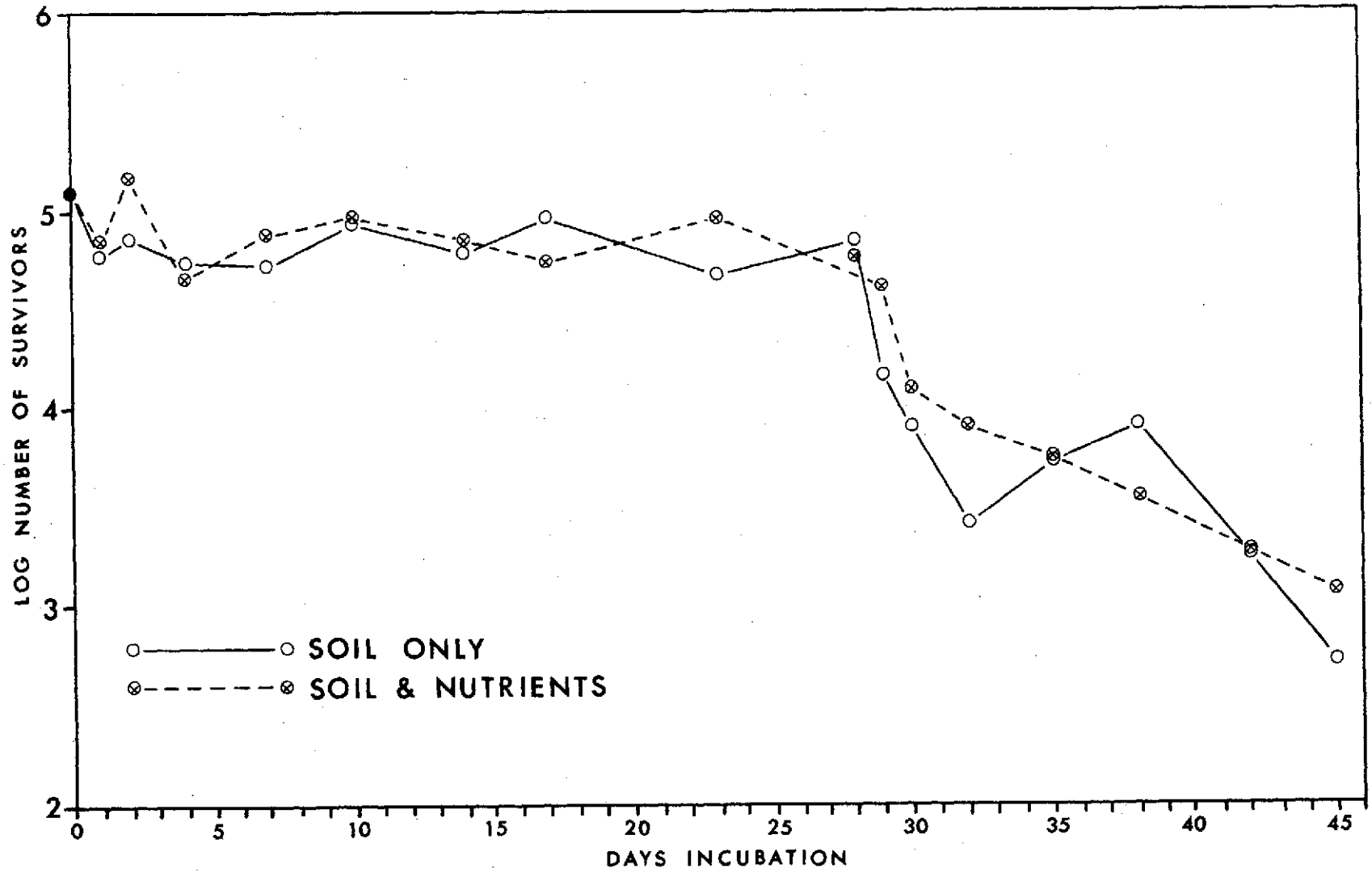


Fig. 14. Response of organism M20-27 in VAB soil to a simulated Martian environment. Environment was dry the first 28 days and contained excess moisture the last 17 days

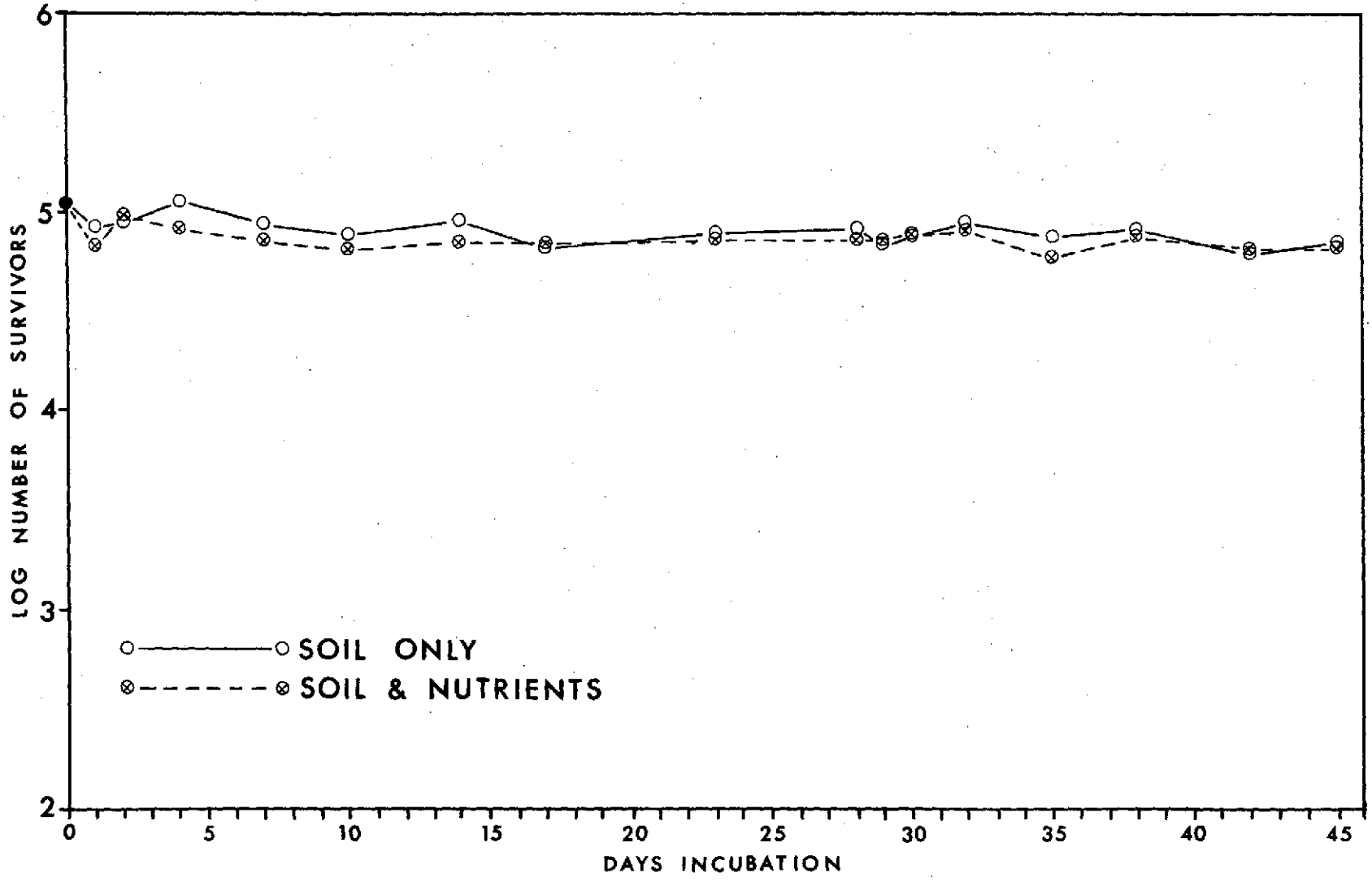


Fig. 15. Response of organism V2-20 in VAB soil to a dry simulated Martian environment

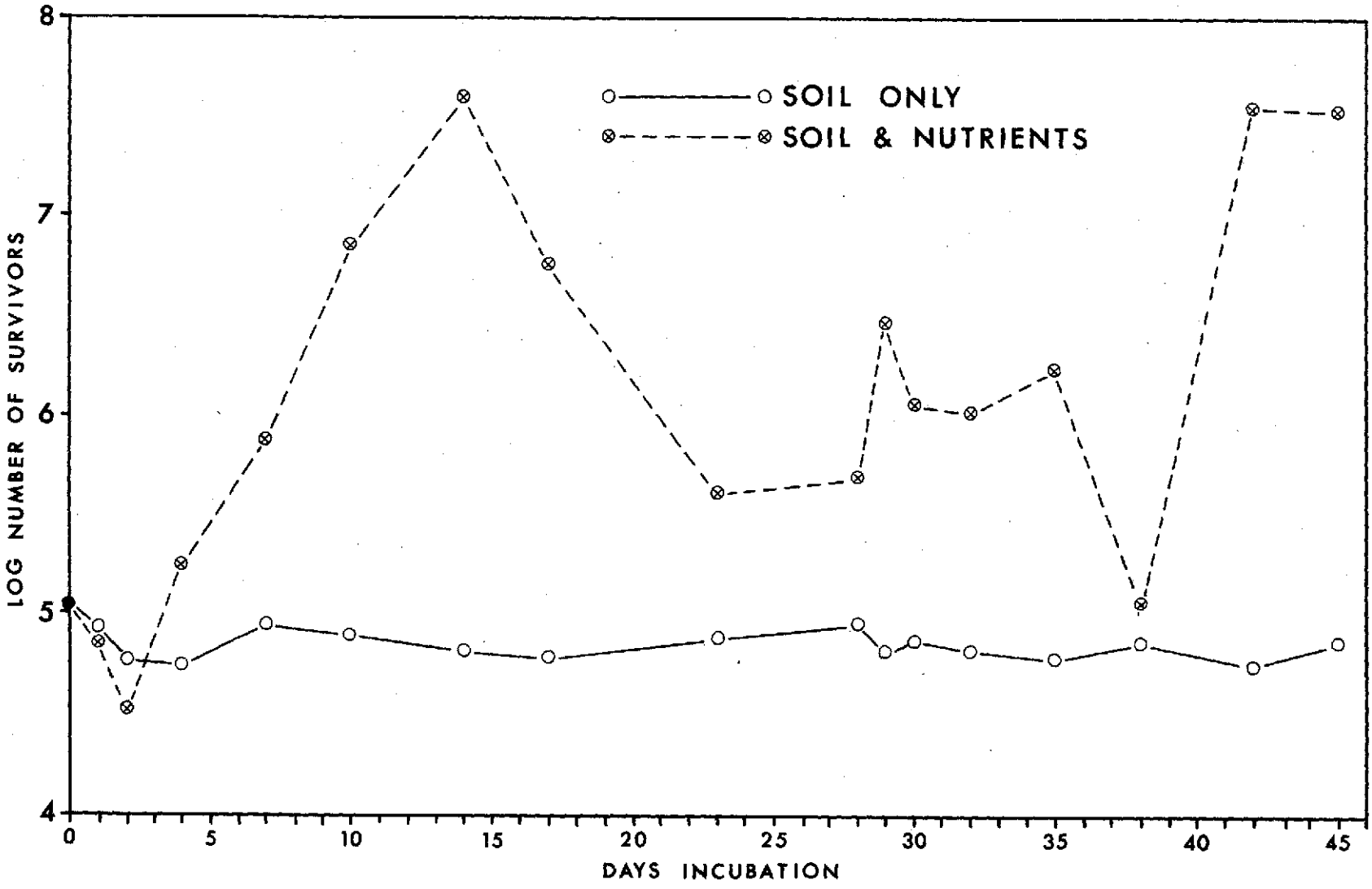


Fig. 16. Response of organism V2-20 in VAB soil to simulated Martian environment containing excess moisture

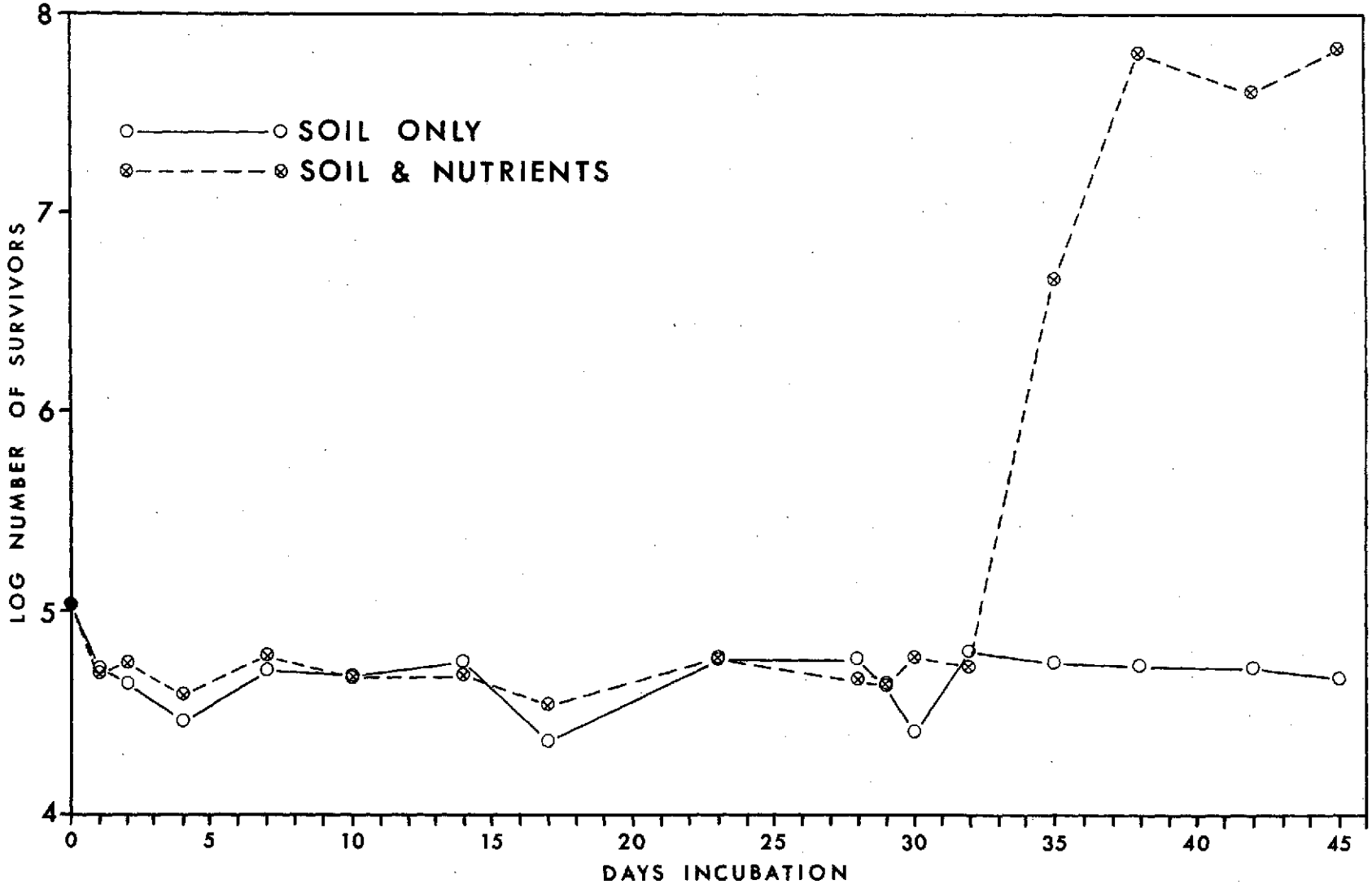


Fig. 17. Response of organism V2-20 in VAB soil to a simulated Martian environment. Environment was dry the first 28 days and contained excess moisture the last 17 days

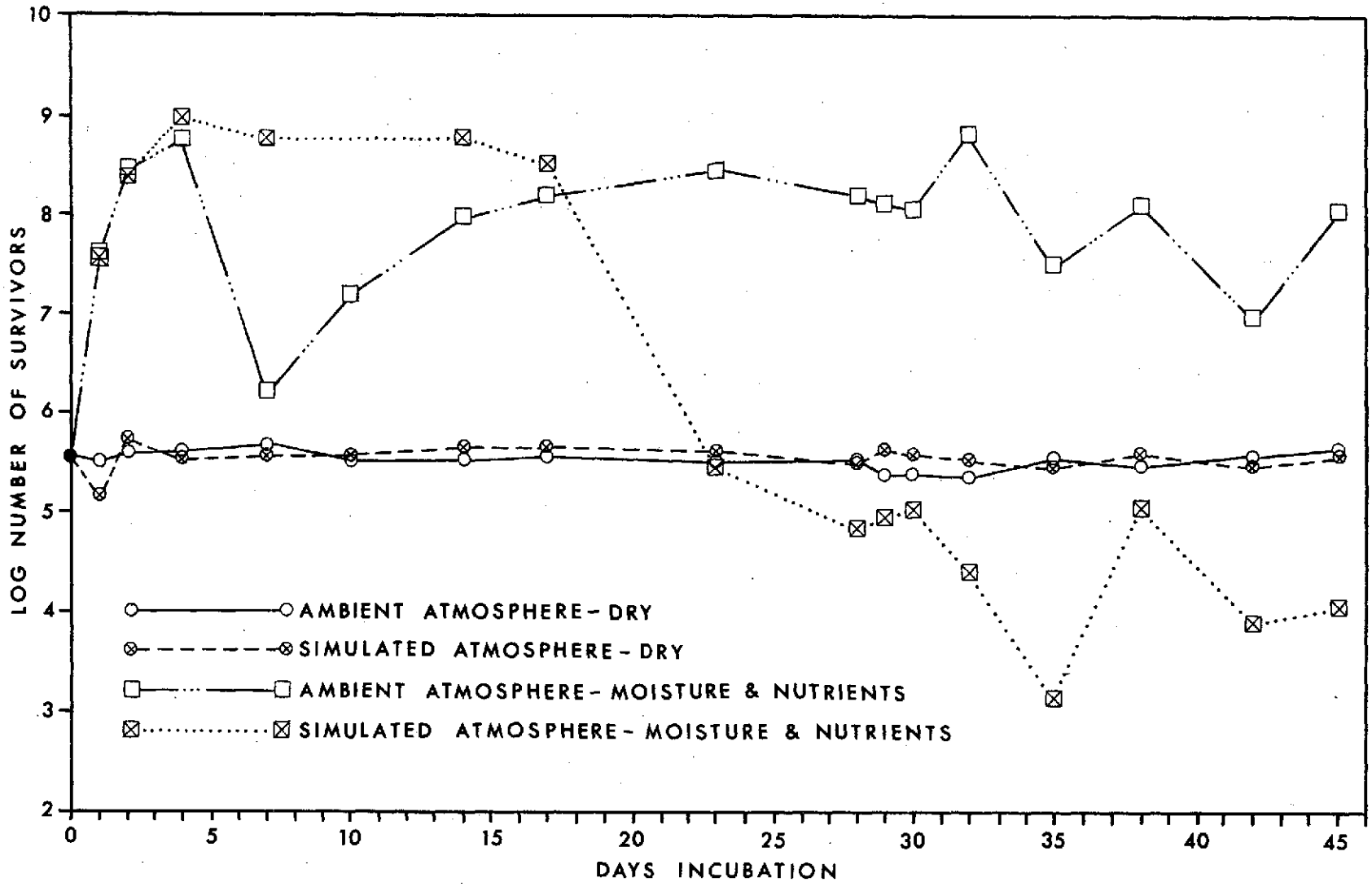


Fig. 18. Response of organism M6-25 to both ambient and simulated atmospheres at a constant 24°C

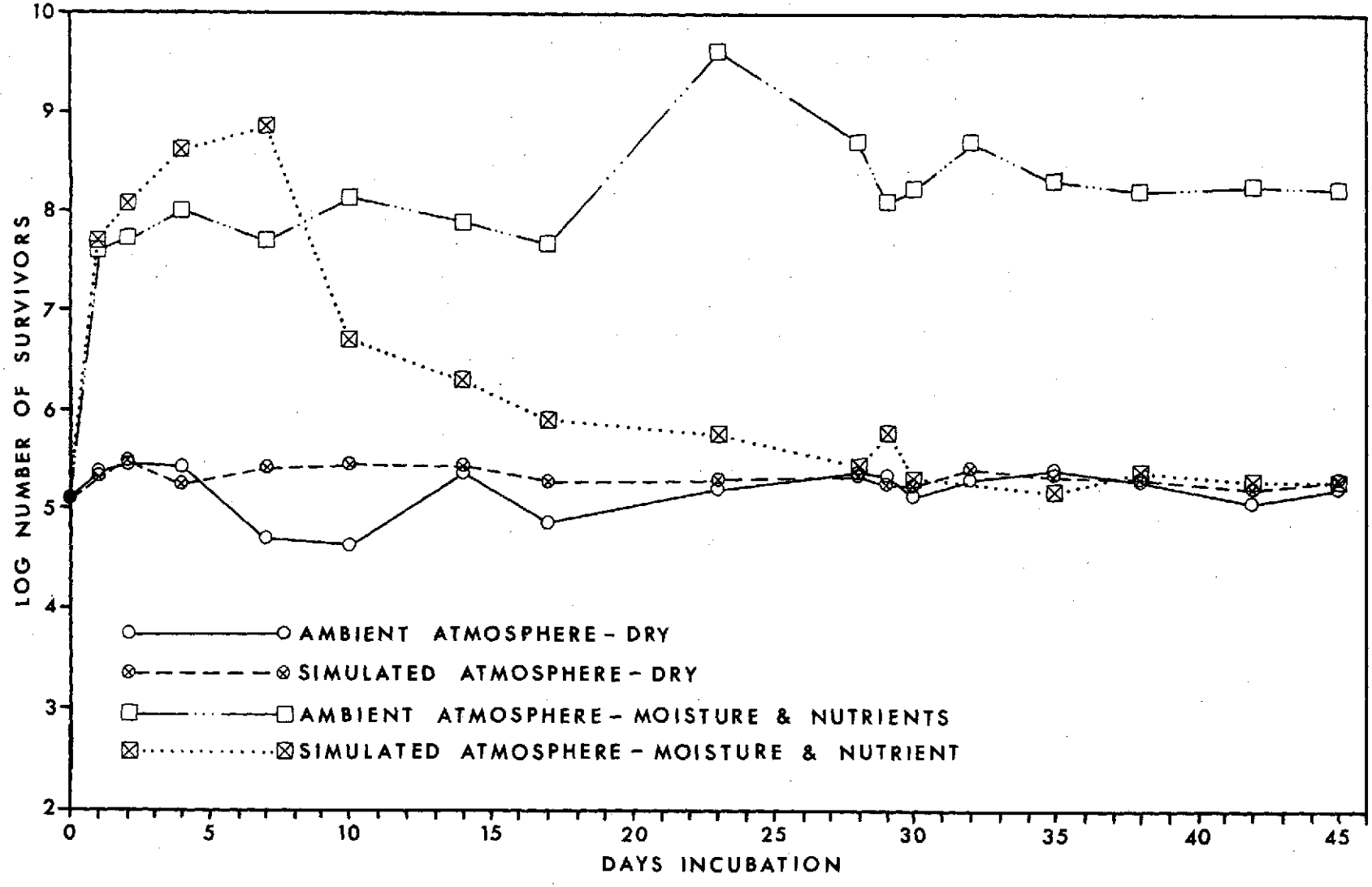


Fig. 19. Response of organism M20-27 to both ambient and simulated atmospheres at a constant 24°C

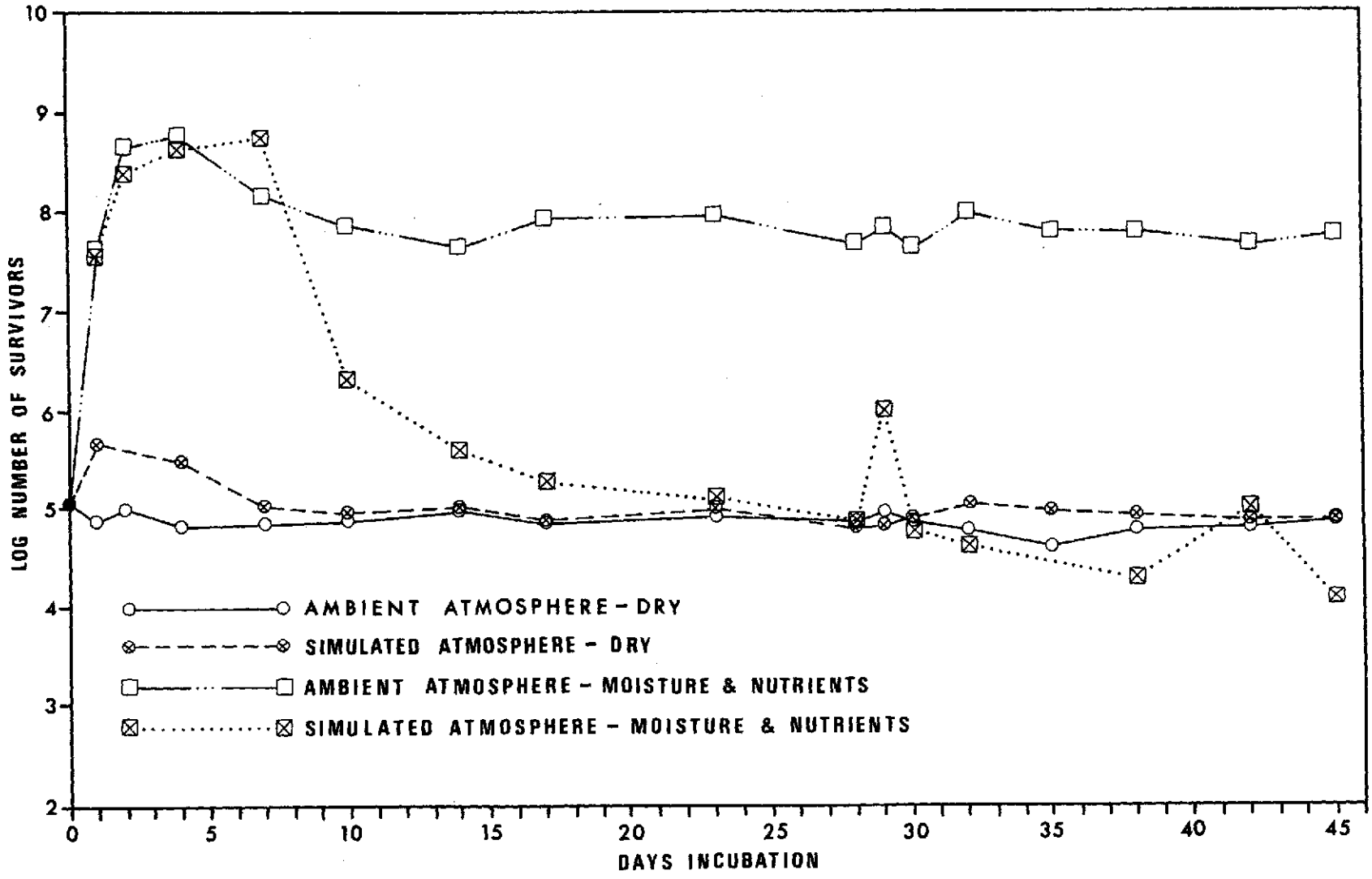


Fig. 20. Response of organism V2-20 to both ambient and simulated atmospheres at a constant 24°C

FUTURE ACTIVITIES

The investigations described in this report were performed primarily at the request of the Planetary Quarantine Office. Since these tasks are completed, our research will again emphasize the isolation and characterization of special types of microorganisms. This type of study will again include isolation and characterization of psychrophiles associated with spacecraft facilities, and a new effort will be made to isolate and characterize anaerobic isolates as well. This will be done because it is felt that the outer planets possess anaerobic atmospheres.

The modified Imshenetsky slide culture technique has been described in our earlier reports, and it appears that this may be a useful procedure for rapid detection of growth of microorganisms under different environmental conditions. We will continue to evaluate this procedure for possible application in Planetary Quarantine research. Other areas of research for the coming months will also include additional studies with the experimental Martian environments.