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Evaluation of salinity and temperature as stress factors affecting the enumeration of fecal coliforms by the electrochemical detection method

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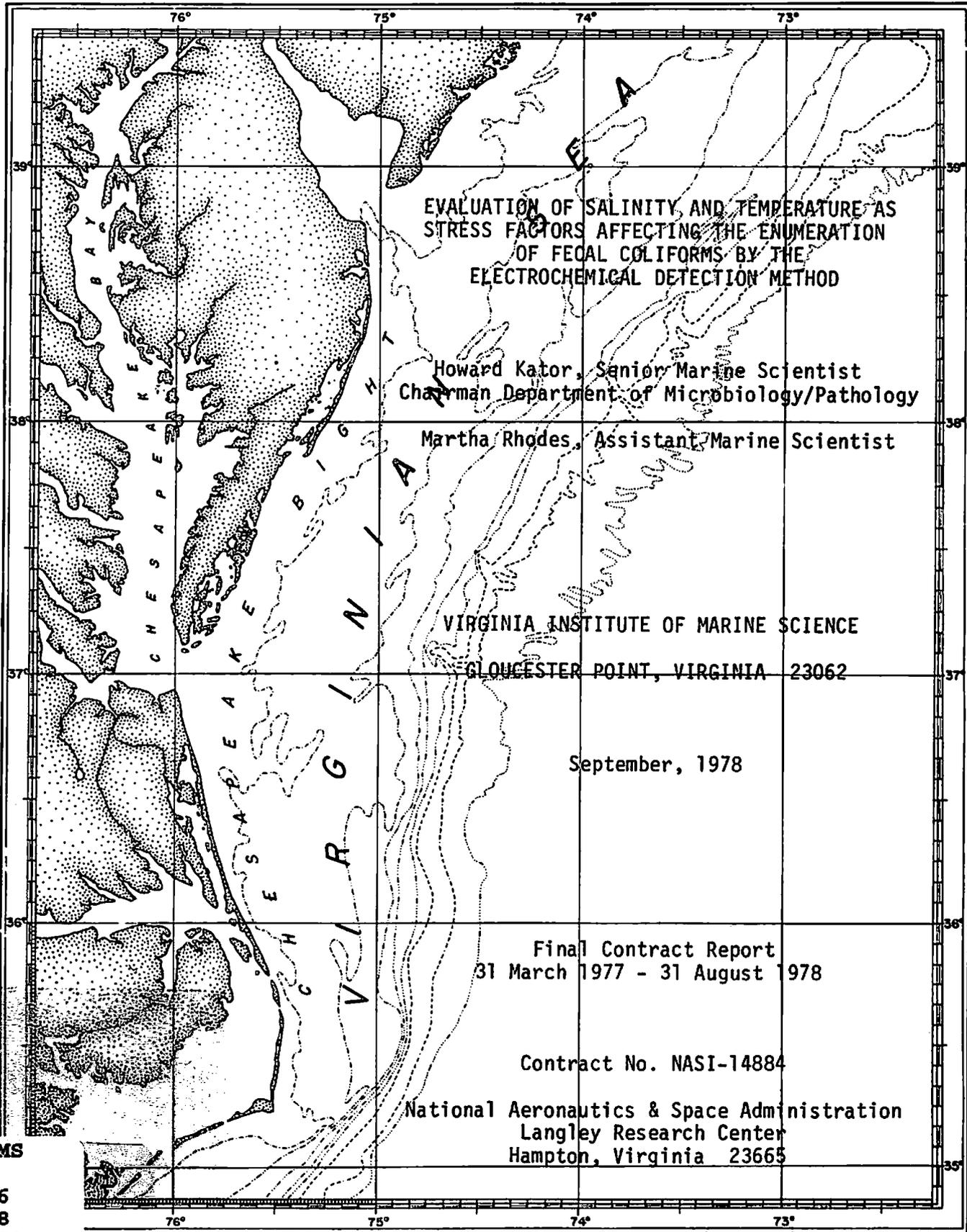*Virginia Institute of Marine Science*

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EVALUATION OF SALINITY AND TEMPERATURE AS STRESS FACTORS AFFECTING THE ENUMERATION OF FECAL COLIFORMS BY THE ELECTROCHEMICAL DETECTION METHOD

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ABSTRACT

The ability of an electrochemical detection method to predict viable numbers of fecal coliforms was evaluated under laboratory conditions with respect to seawater adjusted to various salinities and temperatures. The viability of an _Escherchia coli_ isolate as measured by the spread plate technique utilizing non-selective media was unaffected after 12 wk exposure at 2°C and 25 ^{o}/oo salinity. At higher temperatures (15-30°C) both the total decrease in cell numbers as well as the rates of die-off were greater than at 2°C. There was little apparent difference in viability across the temperature range 15-30°C. Viability was observed to be inversely related to salinity over the range 10-30 ^{o}/oo. Stress was measured using the electrochemical detection method (ECDM) and defined as the difference between the predicted endpoint response time (ER) calculated from a standard curve and the observed ER time. Seawater of higher salinities generally produced greater stress. With respect to temperature, stress was greater at 20°C than at 30°C, while at 2°C stress occurred after a prolonged period of starvation. Delayed ER times were attributed to (1) a reduction in viable cells upon inoculation of starved bacteria into media at 44.5°C and/or (2) an extended lag phase prior to logarithmic growth. Medium A-1 was superior to EC for enumeration of fecal coliforms in estuarine water samples by the ECDM method. ER times occurred sooner and the results were more predictable with the former medium.

In addition to salinity and temperature, other factors including isolate source, age, growth phase, culture media, and diluent are discussed as to their effects on viability/stress studies.

ACKNOWLEDGMENTS

INTRODUCTION

An electrochemical detection method (ECDM) for the enumeration of various enteric bacteria (37) was recently described, the method is based on the existence of a linear relationship between inoculum size and the time of detection of an increase in potential difference in standard media. Pure culture studies were performed using combination electrodes and subsequently platinum electrodes of different surface areas (35) to detect potential changes in carbohydrate containing media. When this test system was evaluated as a tool for detecting and enumerating coliforms present in estuarine and freshwater samples differences in detection times between stock cultures and environmental coliforms were observed. Wilkins and Boykin (36) suggested that a portion of the coliform population present in the aquatic environment may be stressed with delayed detection times due to exposure of sensitive cells to selective media and elevated incubation temperatures. Data resulting from studies using coliforms exposed to freezing (29, 34), chlorination (17) and freshwater environments (4, 13, 16) suggested that injured bacterial cells may remain undetected by routine enumeration procedures.

The following study was undertaken to evaluate the responses of pure cultures of Escherichia coli with different cultural histories to seawater of various salinities and temperatures. Responses were measured using standard enumeration techniques. Furthermore, we attempted to determine if the ECDM could be used to detect sublethal stress in cultures exposed to various conditions of temperature and salinity.

MATERIALS AND METHODS

Organisms. Fecal coliforms employed in this study were isolated by enrichment in lactose broth at 35°C for 24 h followed by inoculation into EC broth at 44.5°C for 24 h. Isolates were obtained from tubes which produced gas at the elevated temperature. All isolates were identified using API-20E (Analytab Products, Inc., Plainview, N.Y.), a miniaturized multiple test system for identification of enteric bacteria. API profile recognition numbers for Escherichia coli isolates were 5 044 552, human fecal isolate; 5 044 562, human urinary tract infection isolate; and 1 144 562, estuarine water isolate obtained from a sample collected from the York River, Gloucester Point, Virginia. Stock cultures were maintained on trypticase soy agar (TSA) at 4°C. During the course of experimentation isolates were subcultured on TSA three times or less.

Cell Preparation. Inocula for seawater survival experiments were obtained from exponential phase cultures unless otherwise specified. Isolates were grown at 35°C in either trypticase soy broth (TSB) or M-9 minimal medium which consisted of 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl, 0.13g $MgSo_4 \cdot 7H_2O$ and 5 ml glycerol in 1 liter distilled water. The latter two components were sterilized separately and added to the remaining components of minimal medium after autoclaving. The final pH of the minimal medium was 7.0. Bacteria were harvested by centrifugation (1,000 x g) at room temperature for 10 min and washed three times in phosphate buffered saline (PBS) at pH 7.2. Cells were resuspended to an optical density of 0.2 - 0.3 (540 nm) in seawater of selected salinities and temperatures.

Survival Experiments. A ten-fold dilution of the final cell suspension was made into 90 ml seawater previously equilibrated to the test temperature in 125 ml sterile screw-capped flasks. Seawater at specified salinities was pre-

pared by diluting aged filtered ocean water (35 ^{o}/oo) with reverse osmosis/ glass distilled water and sterilized by autoclaving. Samples of inoculated seawater were removed at various time intervals for enumeration by spread plating on TSA and determination of endpoint response (ER) in EC and TSB at 44.5°C. Colony counts were made after ca. 24 h incubation at 35°C. Total exposure time to seawater prior to 0 h sampling ranged from 2-5 min while exposure time to media at 44.5°C prior to dilution for plate counts was less than 1 min. All dilutions were performed using PBS at room temperature unless otherwise specified.

Endpoint Response (ER) Determination. The experimental design for detecting ER time consisted of a test tube (25 mm X 100 mm) containing two platinum electrodes and 18 ml medium to which 2 ml E. coli seeded seawater was added. Tubes were fitted with a No. 4 rubber stopper containing electrodes of grade A platinum alloy wire, 24 gauge (0.508 mm), Engelhard Industry, Carteret, N.J.). Electrodes were inserted into slits made in the stopper and the stopper bound with wire and/or epoxy to prevent electrode slippage. Electrodes were designed such that the ratio of their lengths below the surface of the medium was 1:4. Sterilization was by flaming over an alcohol lamp. The longer elect-rode was connected to the negative terminal and the shorter one to the positive terminal of a strip chart recorder (Model 194, Honeywell Industrial Division, Fort Washington, Pa. or Model SR-204, Heath Company, Benton Harbor, Mich.). Recorders were set at a chart speed of 10 min/in and operated at 0.5 volts (Honeywell) or 1.0 volts (Heath) full scale. ER times were measured as the time elasped between challenge and the initial increase in potential difference.

Environmental Samples. Estuarine water samples were collected from the York River at Gloucester Point or from its nearby tributaries which included Yorktown Creek, Sarah Creek as well as the Northwest and Northeast Branches of Sarah

Creek. Sampling sites at all localities were subject to varying degrees of pollution from domestic sewage and boats. Water samples were collected in sterile 500 ml erlenmeyer flasks and processed within 60 min. Simultaneous temperature and salinity measurements were made. Parallel fecal coliform enumerations were performed using a five-tube most probable number (MPN) technique (1) with lactose enrichment at 35°C for 48 h with subsequent inoculation of positive tubes into EC at 44.5°C for 24 h or direct inoculation into Medium A-1 (2) at 44.5°C for 24 h. Corresponding ER determinations were made by addition of a 100 ml sample into each of two 250 ml erlenmeyer flasks containing 100 ml single strength EC or Medium A-1 prewarmed to 44.5°C. Flasks were fitted with rubber stoppers containing the appropriate sterile platinum electrode configuration as specified above.

Standard Curves. Endpoint response times were determined in EC, TSB and Medium A-1 at 44.5°C for various inocula. Ten-fold dilutions of an E. coli suspension (urinary tract isolate) prepared as for survival experiments were made in 20°C seawater (25 °/oo) and inoculated into test media. Exponential cultures were pregrown in either TSB or M-9 media. The relationship between inoculum size and ER time was determined by linear least-squares regression analysis and confidence belts for the linear regression were established at the 95 percent confidence level (28).

Data Analysis. During the course of experiments designed to assess the effect of seawater exposure on ER time the following data were collected: coliforms/ml seawater, coliforms/ml medium following a ten-fold dilution from seawater and ER time in the respective media. Standard curves of ER vs. coliforms/ml medium were analyzed by linear regression techniques. The ER, predicted from the linear regression line and based on the number of cells/ml medium calculated to have resulted from a ten-fold dilution from seawater, was subtracted from

the observed ER. This relationship of observed ER minus predicted ER as a function of time in seawater was graphically presented with 95% confidence limits for y. Stress was defined as the time delay between observed ER and predicted ER and was considered significant when this value exceeded the 95% confidence limits.

RESULTS

Linear regression analysis of standard curve results indicated that both the previous cultural history of the inoculum and the medium used to measure endpoint response affected ER detection times. Using TSB as the test medium to measure ER times, cells pregrown in either TSB or M-9 gave standard curves with similar slopes, correlation coefficients, and standard errors of the estimate (Figs. 1 and 2). In contrast, using EC as the test medium, TSB pregrown cells gave a standard curve with a smaller correlation coefficient and a higher standard error of the estimate (Fig. 3). When transferred into EC both M-9 and TSB pregrown cells produced standard curves with steeper slopes and greater ER time intervals compared with cells transferred into TSB (Figs. 3 and 4).

A series of experiments were conducted to evaluate E. coli viability in seawater and post-starvation behavior in various test media as a function of salinity and temperature. E. coli (urinary tract) growing exponentially in M-9 was inoculated into seawater of various salinities. The greatest rate of decline in viable counts occurred during the first 2 da; rates of which increased directly with salinity (Fig. 5). Although two experiments each were conducted at 20°C and 30°C, no clear effect of temperature with respect to viability was evident. Average E. coli decreases after 2 da were 0.1, 0.6, 1.1, and 1.9 log units at 20°C and 0.3, 0.7, 1.2, and 1.7 log units at 30°C in 10, 15, 25, and 30 °/oo respectively.

When the data were analyzed with respect to ER determinations, stress as previously defined was evident as a function of salinity, temperature, and test media (Figs. 6 and 7). In starvation studies conducted at 20°C stress was more pronounced in EC than TSB and increased with increasing salinity.
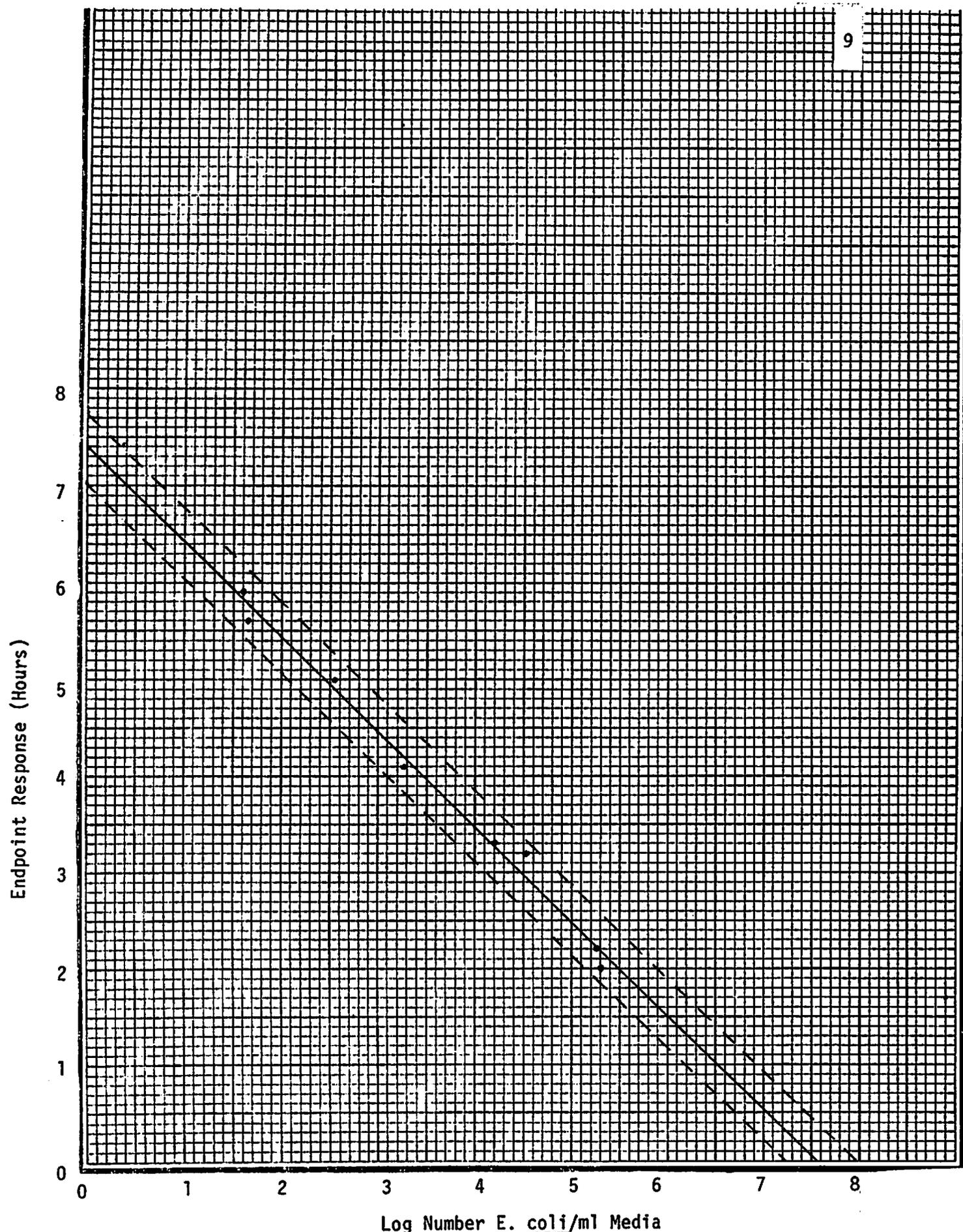
Figure 1. Relationship between inoculum size and the time of endpoint response in TSB at 44.5°C when inocula (urinary tract isolate) were pregrown in TSB at 35°C. A linear least-squares regression calculation gave a correlation coefficient of -0.99 (N = 8) with an intercept of 7.39 and a slope of -0.98. The standard error of estimate at the 95 percent confidence level was ± 0.37.

Figure 2. Relationship between inoculum size and the time of endpoint response in TSB at 44.5°C when inocula (urinary tract isolate) were pregrown in M-9 medium at 35°C. A linear least-squares regression calculation gave a correlation coefficient of -0.98 (N = 22) with an intercept of 8.07 and a slope of -1.05. The standard error of estimate at the 95 percent confidence level was ± 0.54.
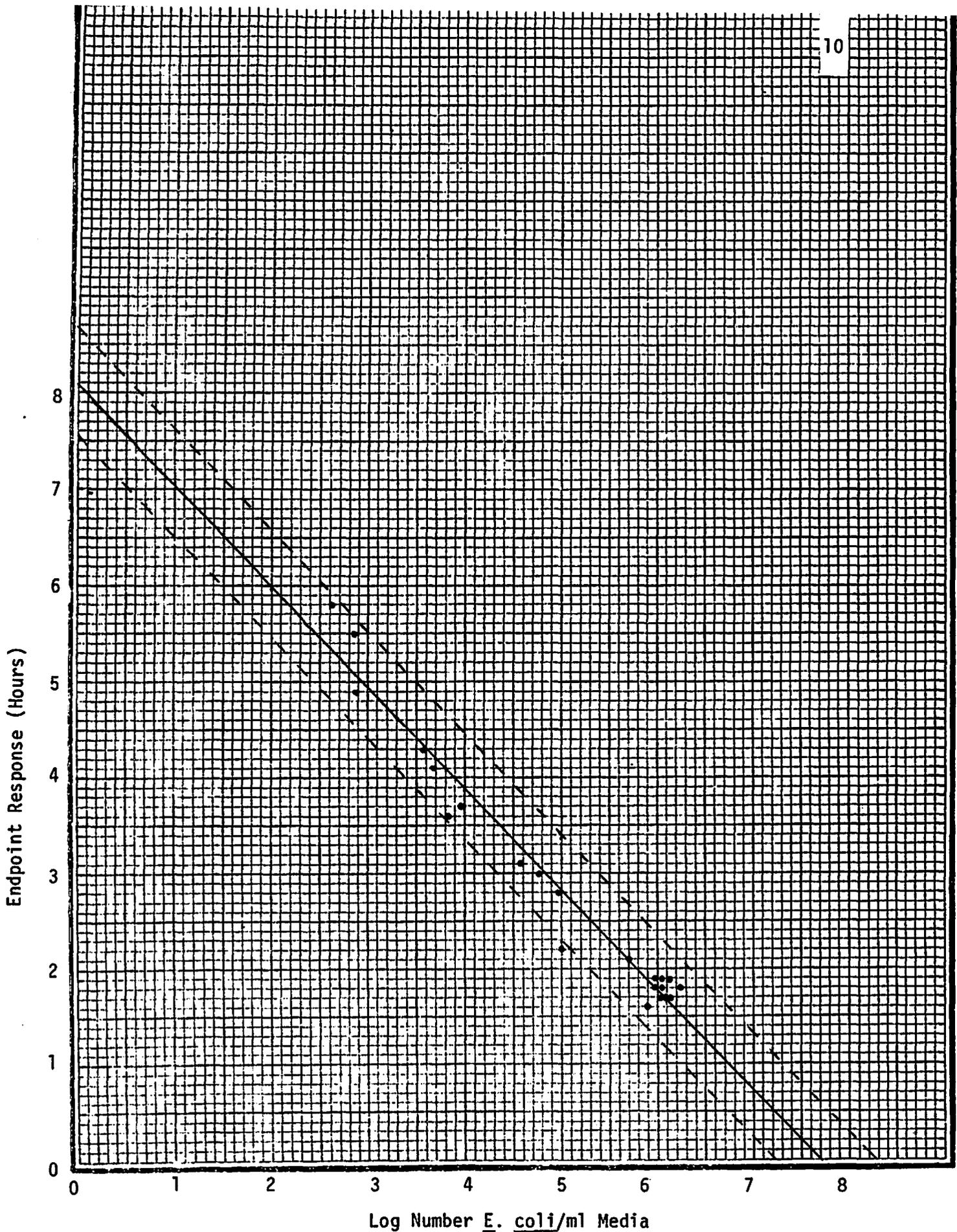
Figure 3. Relationship between inoculum size and the time of endpoint response in EC at 44.5°C when inocula (urinary tract isolate) were pregrown in TSB at 35°C. A linear least-squares regression calculation gave a correlation coefficient of -0.77 (N = 12) with an intercept of 9.97 and a slope of -1.16. The standard error of estimate at the 95 percent confidence level was + 2.67.
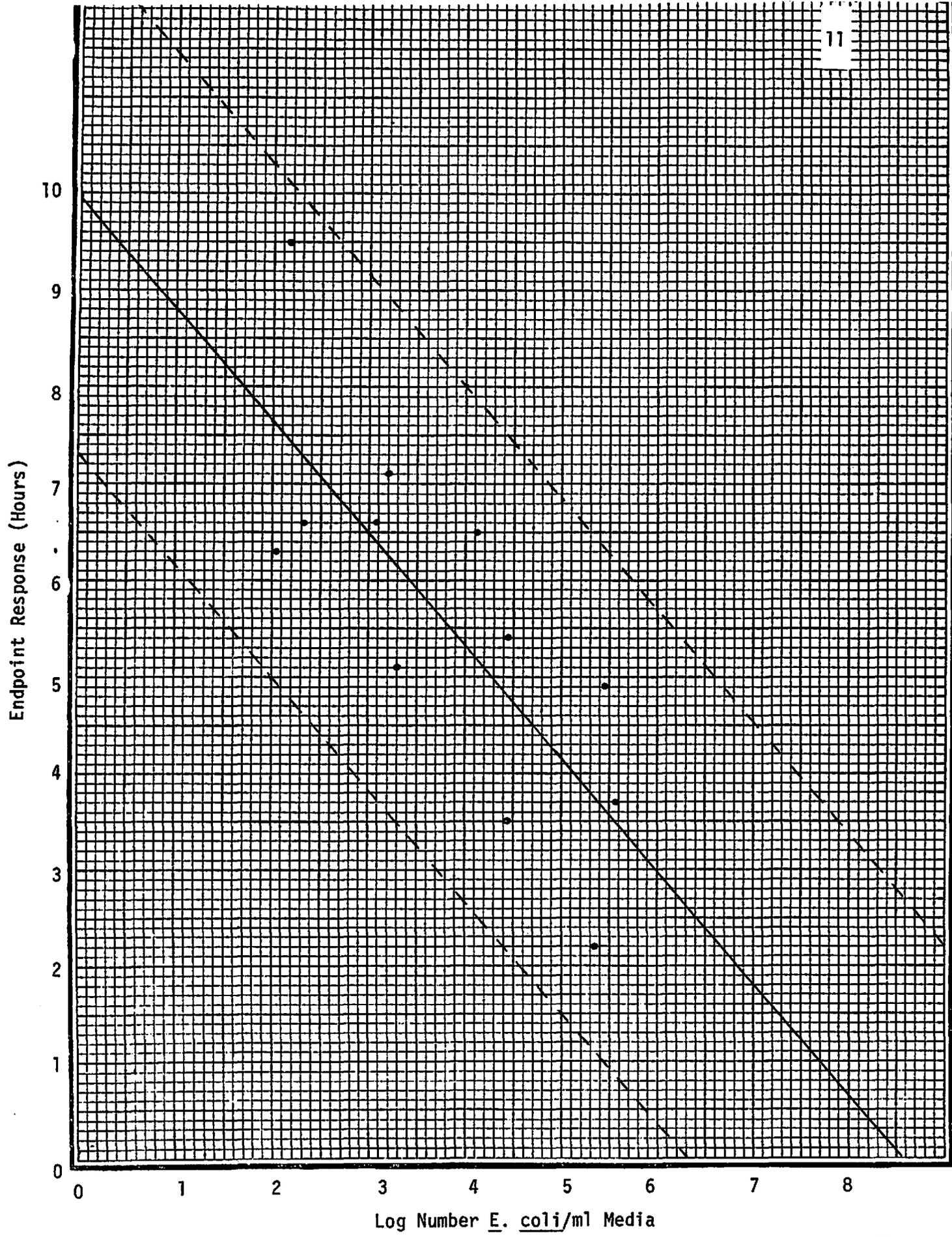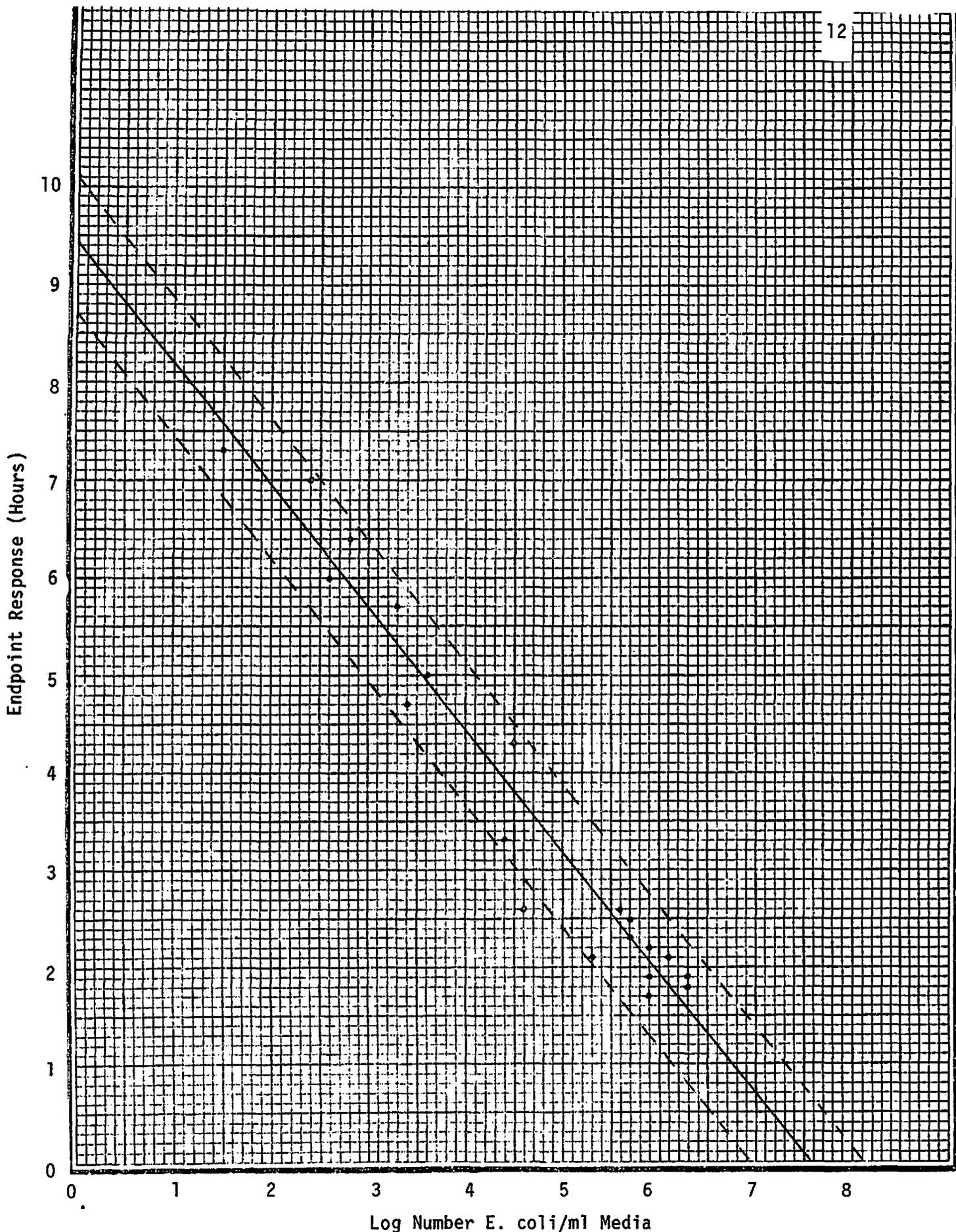
Figure 4. Relationship between inoculum size and the time of endpoint response in EC at
44.5°C when inocula (urinary tract isolate) were pregrown in M-9 medium at
35°C. A linear least-squares regression calculation gave a correlation coeffi-
cent of -0.97 (N = 20) with an intercept of 9.41 and a slope of -1.25. The
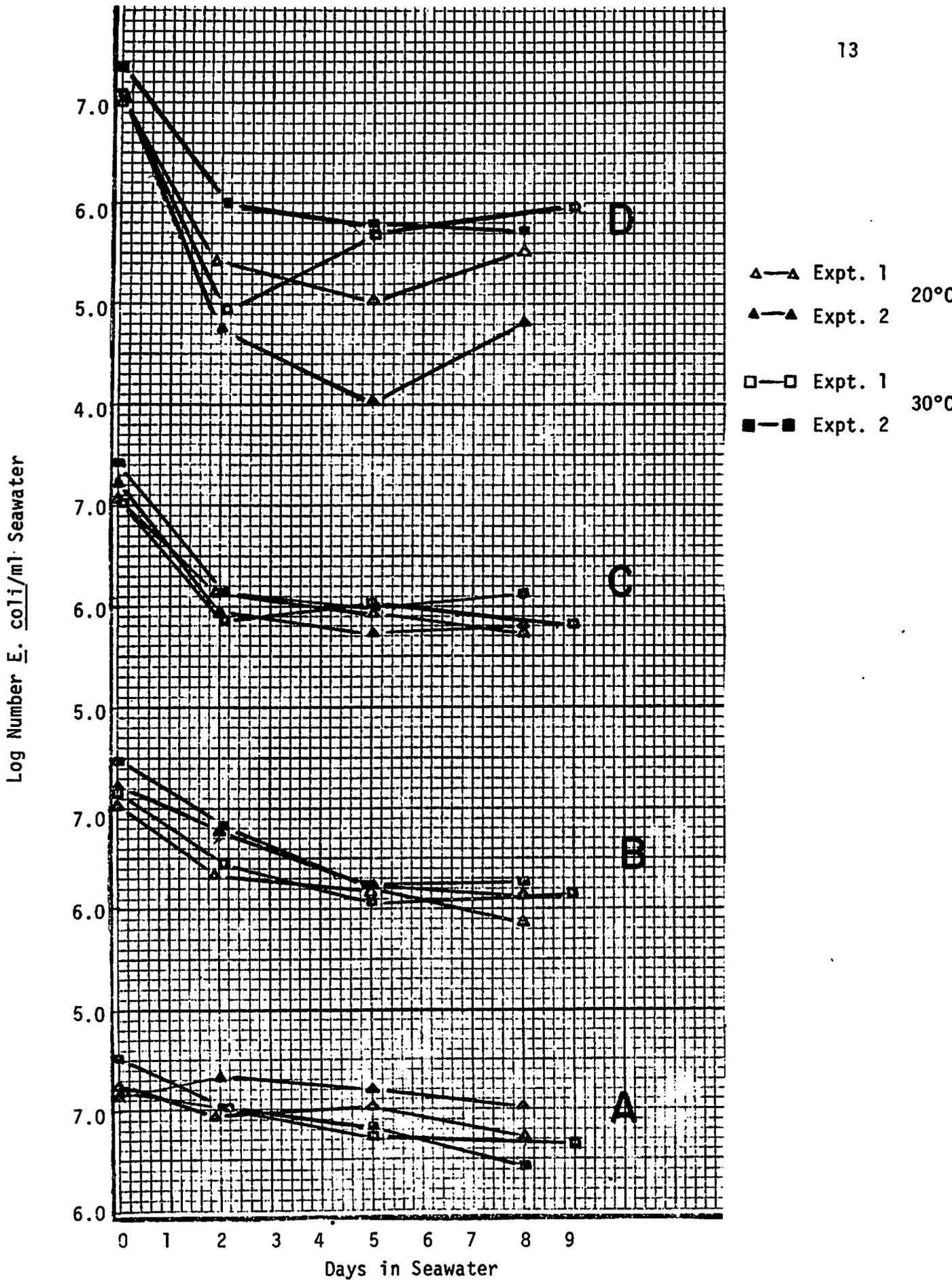standard error of estimate at the 95 percent confidence level was ± 0.73.

Figure 5. Comparison of viability of E. coli at 20°C and 30°C at various salinities.
Prior to starvation the isolate (urinary tract) was pregrown in M-9
medium at 35°C. Salinity: A = 10 °/oo, B = 15 °/oo, C = 25 °/oo, and
D = 30 °/oo.

Figure 6.   Effect of starvation at 20°C in seawater of various salinities on the endpoint response (ER) of E. coli inoculated into either EC or TSB at 44.5°C. Prior to starvation the isolate (urinary tract) was pregrown in M-9 medium at 35°C. Viable counts determined using PBS diluent at room temperature.

EC   TSB   15

Observed ER Minus Predicted ER (Hours)

2.0

1.5

1.0

0.5

0.0

-0.5

0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8

Days in Seawater

Salinity
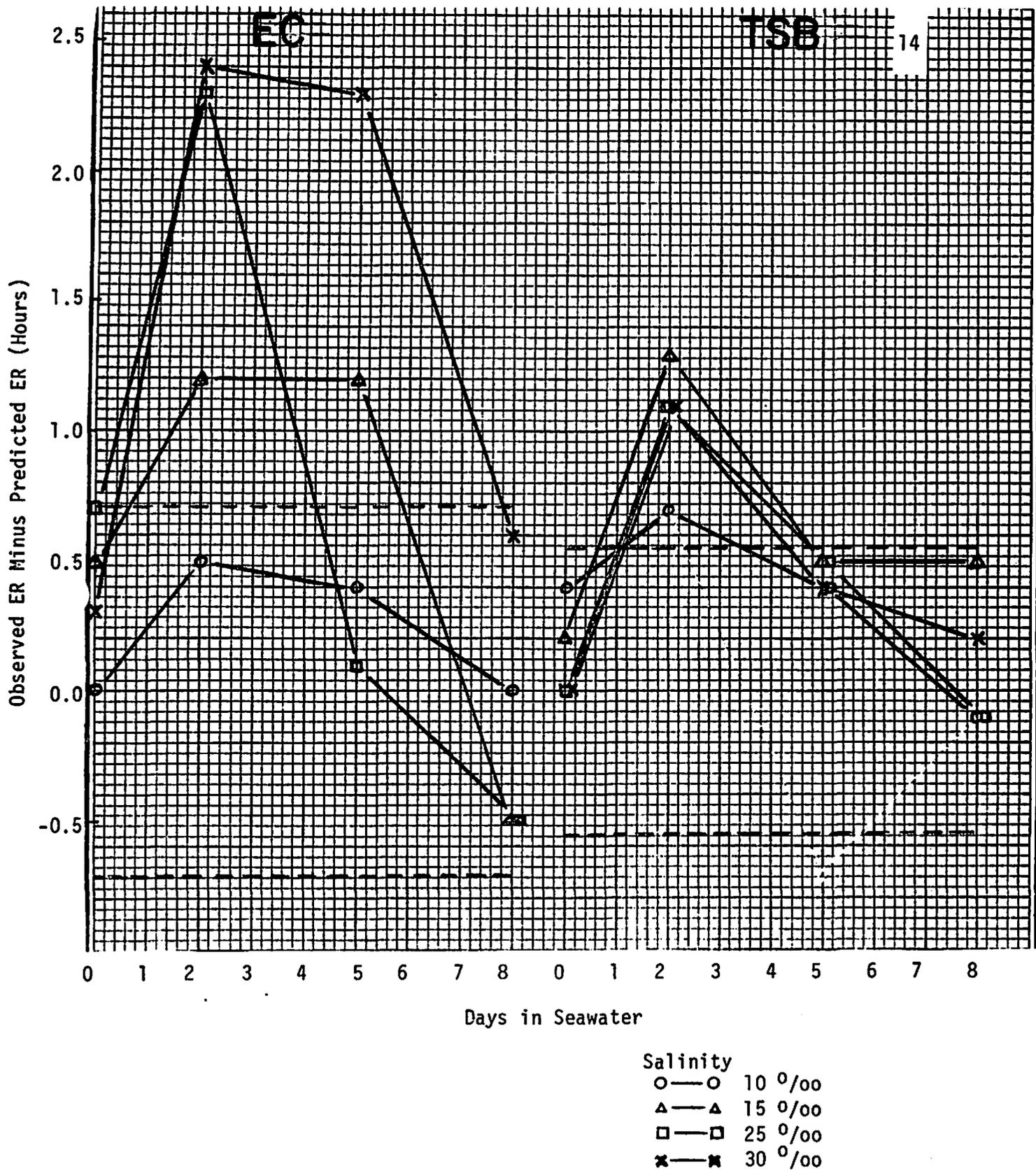
o——o 10 °/oo
△——△ 15 °/oo
□——□ 25 °/oo
x——✕ 30 °/oo

Figure 7. Effect of starvation at 30°C in seawater of various salinities on the
endpoint response (ER) of E. coli inoculated into either EC or TSB at
44.5°C. Prior to starvation the isolate (urinary tract) was pregrown
in M-9 medium at 35°C. Viable counts determined using PBS diluent at
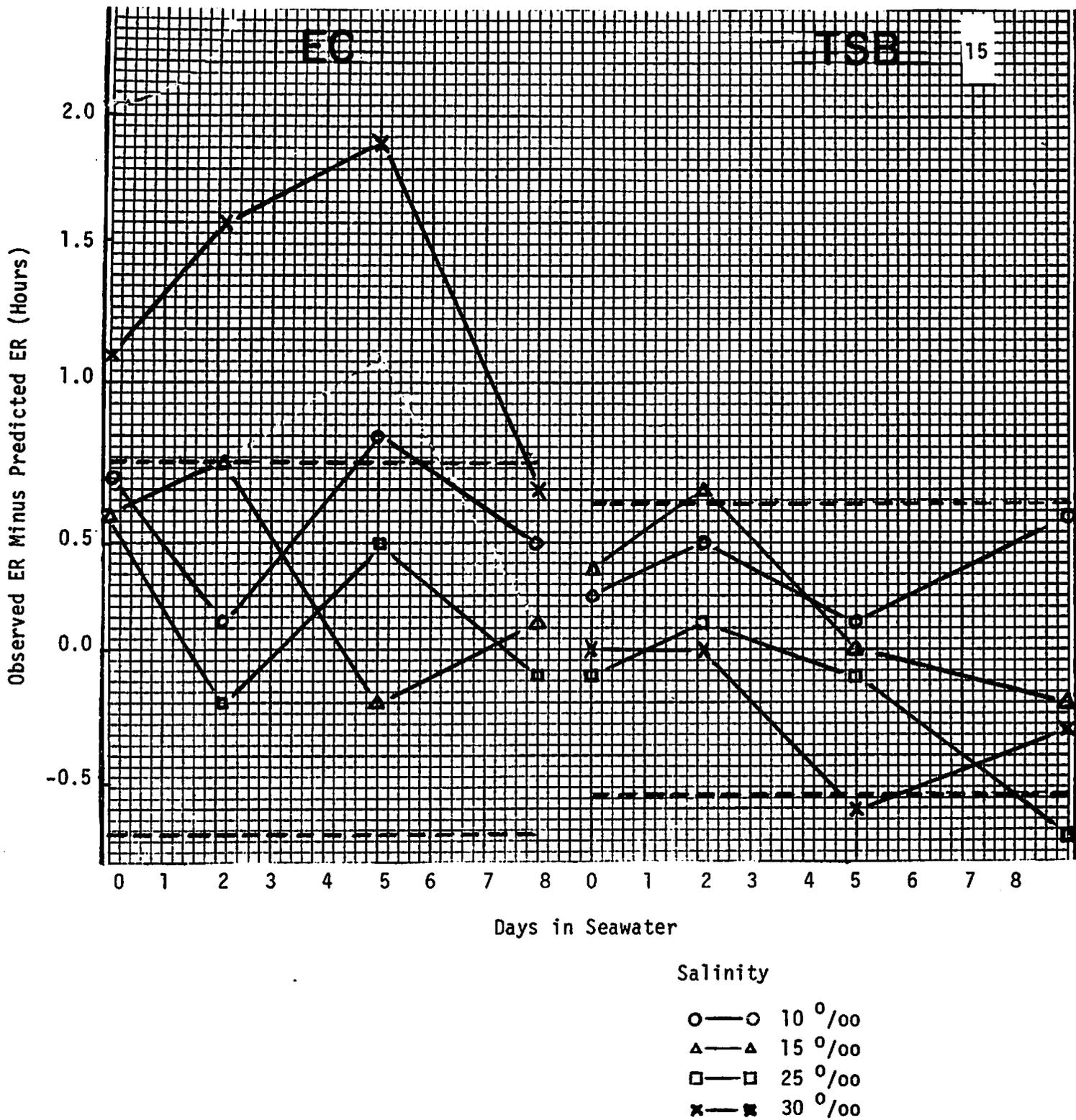room temperature.

Generally, maximum stress developed after 2 da of starvation and thereafter decreased. Cells starved at 30°C showed little evidence of stress except at 30 °/oo salinity when transferred to EC.

A similar study was conducted using an E. coli strain isolated from estuarine water, pregrown in M-9, and starved in 10 °/oo and 25 °/oo seawater at 20°C. This isolate was more refractory to starvation and salinity effects than the preceding isolate which was obtained from a human host. After 15 da in seawater there was no decrease in viable cell count in 10 °/oo seawater whereas a 0.9 log unit reduction had occurred in 25 °/oo seawater. Stress resulting from starvation at either salinity in terms of prolonged ER time was not observed (Fig. 8).

A study was conducted to determine if cell death in the test media contributed to the increases in ER seen under different conditions. The observed log number of E. coli in the test medium minus the predicted E. coli number calculated from the viable count in seawater was plotted against time of starvation. With respect to the urinary tract isolate, the greatest differences between observed and anticipated bacterial levels occurred in EC as opposed to TSB (Figs. 9A and 9B) and were generally accompanied by prolonged ER values (Figs. 6 and 7). In contrast, the estuarine water isolate, which showed no evidence of starvation stress also showed little if any reduction in cell number upon exposure to EC or TSB (Fig. 10). Although the data suggested that delayed ER times were related to cell death upon exposure primarily to the selective medium EC at the elevated temperature, all the results could not be explained solely on this basis. For instance, cell death in EC after exposure to 30 °/oo seawater at 20°C and 30°C was approximately the same after 5 and 8 da starvation whereas significantly prolonged ER times were observed only at the earlier time interval. Therefore, growth
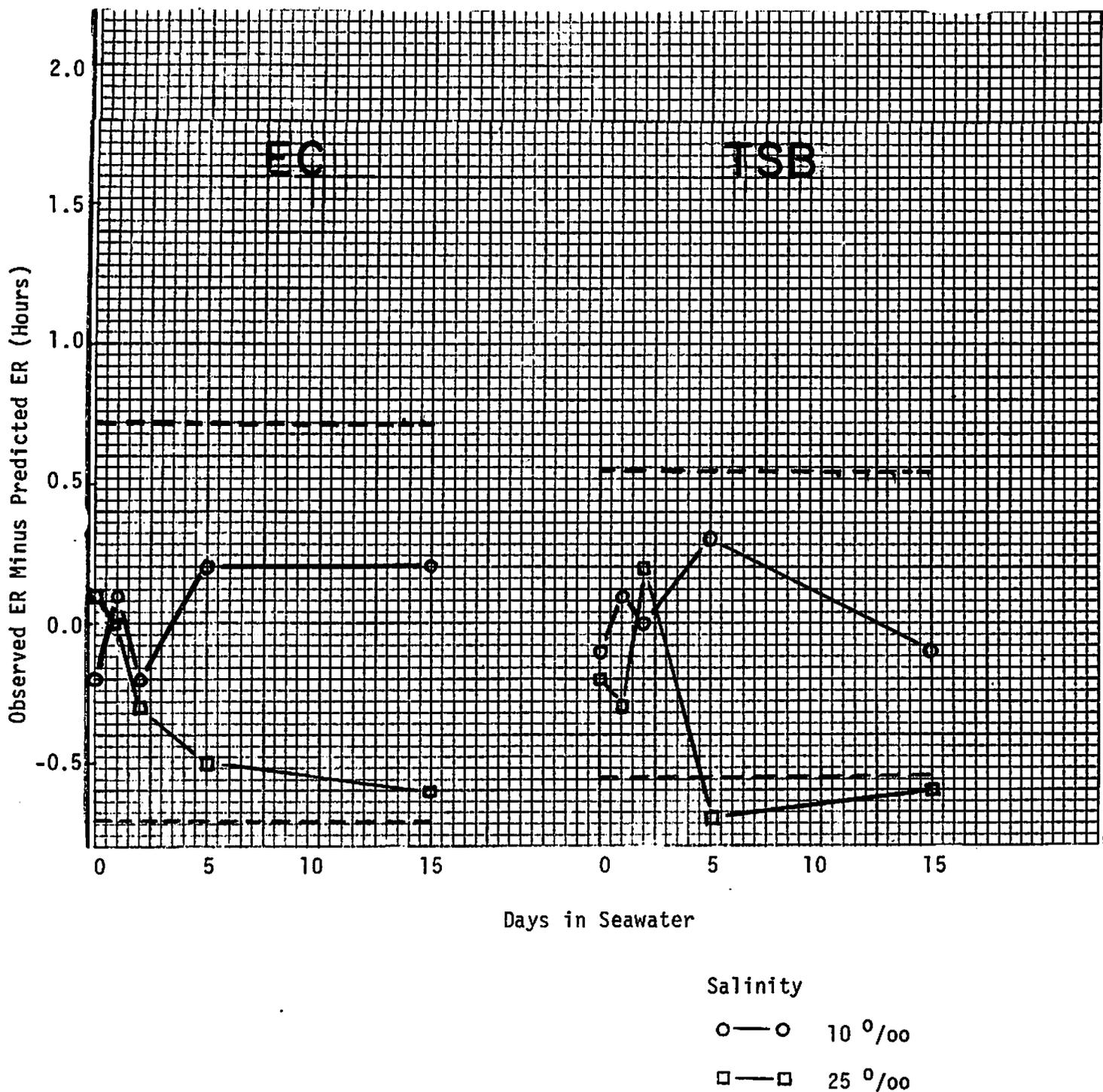
Figure 8. Effect of starvation at 20°C in seawater (10 °/oo and 25 °/oo) on the endpoint response (ER) of E. coli inoculated into either EC or TSB at 44.5°C. Prior to starvation the isolate (estuarine water) was pre-grown in M-9 medium at 35°C. Viable counts determined using PBS diluent at room temperature.
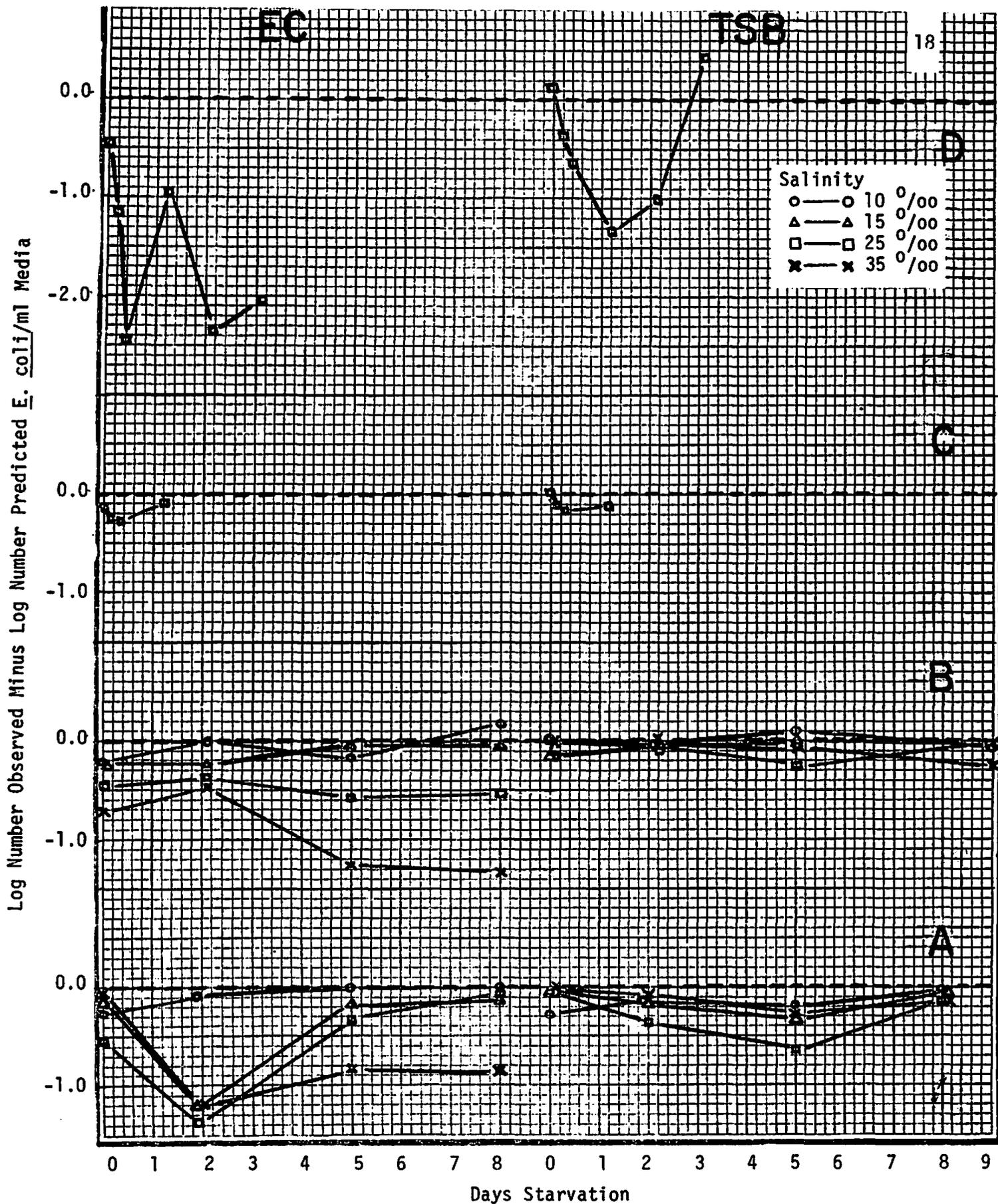
Figure 9. Effect of starvation at different temperatures in seawater of various salinities on the enumeration of E. coli (urinary tract isolate) immediately following a ten-fold dilution into either EC or TSB at 44.5°C. Bacteria pregrown in M-9: A. Seawater temperature = 20°C, PBS diluent at room temperature and B. Seawater temperature = 30°C, PBS diluent at room temperature. Bacteria pregrown in TSB: C. Seawater temperature = 35°C, TSB diluent at 35°C and D. Seawater temperature = 20°C, PBS diluent at room temperature.

Figure-10. Effect of starvation at 20°C in seawater (10 °/oo and 25 °/oo) on the enumeration of E. coli immediately following dilution into either EC or TSB at 44.5°C. Prior to starvation the isolate (estuarine water) was pregrown in M-9 medium at 35°C. Viable counts determined using PBS diluent at room temperature.
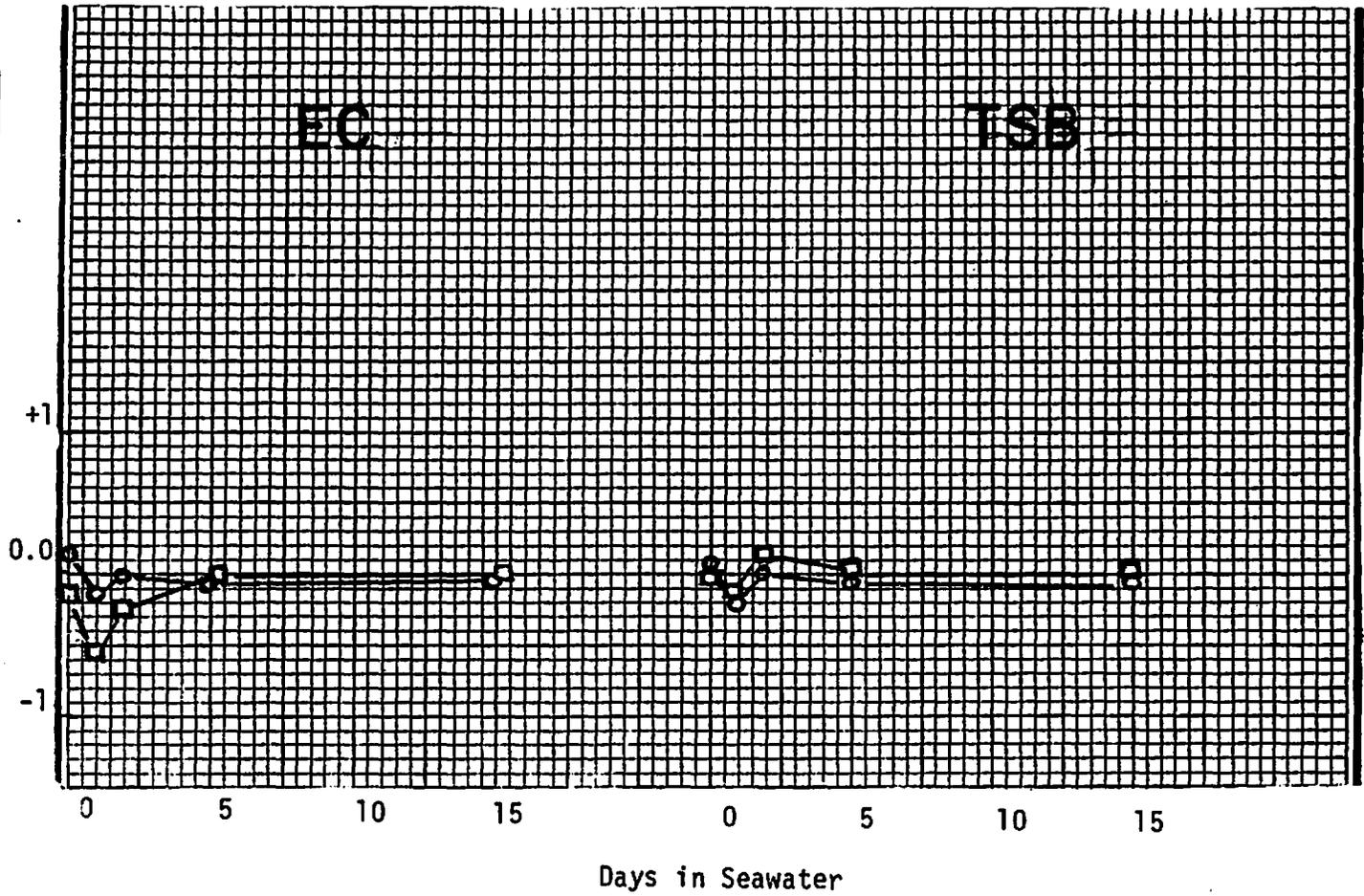
in EC and TSB was examined after various exposure periods to 25 °/oo seawater at 20°C. Figure 11 illustrates that upon inoculation into EC, starved cells evidenced a decline in viable count for approximately 1 h with the lag period extended an additional hour after 2 da but not after 8 da starvation. Similar effects were not observed in TSB nor was there an apparent effect on growth rate in either medium.

A series of experiments were initiated to evaluate the impact of pre-starvation history of the inoculum on viability in seawater and on the post-starvation behavior in test media at 44.5°C. The urinary tract isolate was grown under the following set of conditions: (a) exponential phase in minimal medium (M-9), (b) stationary phase in a rich medium (TSB), and (c) exponential phase in a rich medium (TSB). Treated cultures were then inoculated into 25 °/oo seawater at different temperatures. Exponential phase cells from M-9 and stationary phase cells from TSB showed similar viability profiles (Fig. 12). Maximum cell death occurred during the first 3 da of starvation and ranged from 0.7 to 1.2 log units. In contrast, exponentially grown TSB cells were more sensitive to starvation with a reduction of 2.3 log units over a 3 da period at 20°C and 4.2 log units after 1 da at 35°C (Fig. 12C).

Similarly, cells grown exponentially in TSB, followed by starvation in seawater, showed an aberrant post-starvation behavior in test media at 44.5°C. After starvation in 25 °/oo seawater at 20°C, TSB grown cells evidenced greater die-off when introducted into either TSB or EC at 44.5°C than was previously observed with M-9 grown bacteria (Figs. 9A and 9D). Use of PBS at room temperature as diluent for serial dilution and plating may have contributed to this apparent die-off. For the cells starved at 35°C, TSB prewarmed to 35°C was used as diluent, and this apparent cell die-off was not observed (Fig. 9C). Stress as measured by an increase in observed minus predicted ER time was more
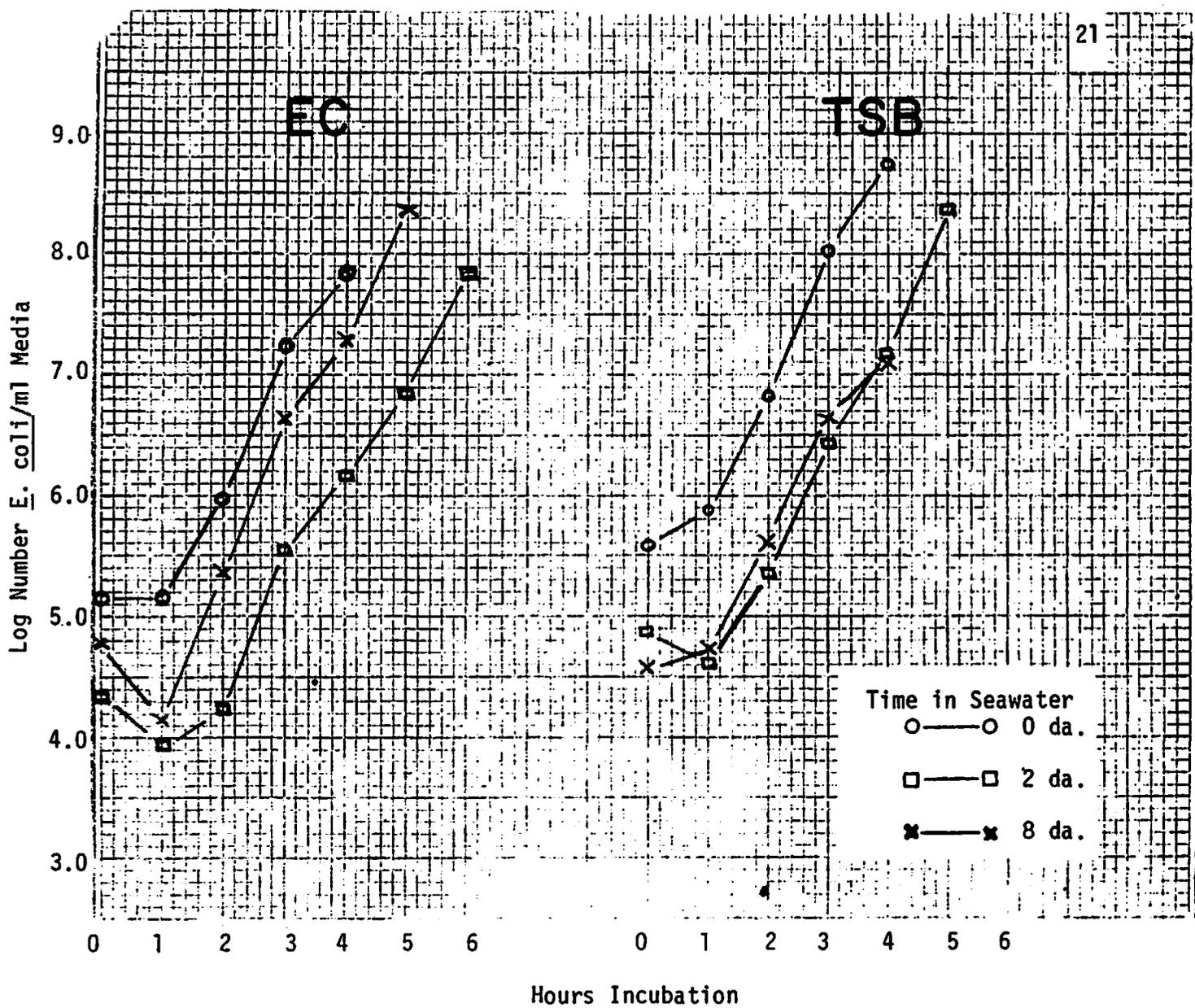
Figure 11. Growth of E. coli in EC and TSB, 44.5°C after 0, 2 and 8 days
starvation at 20°C in seawater (25 °/oo). Prior to starvation
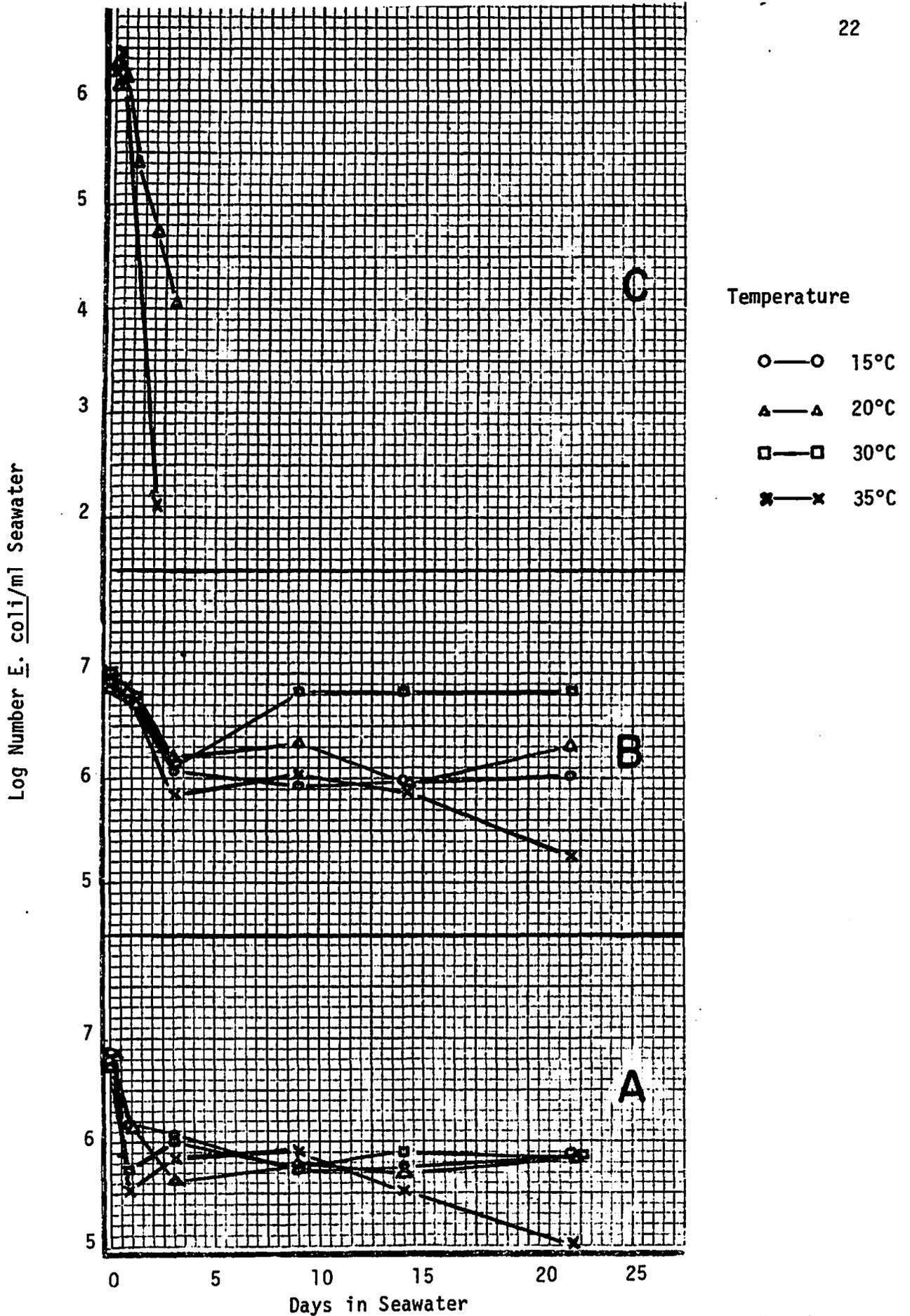the isolate (urinary tract) was pregrown in M-9 medium at 35°C.

Figure 12. Viability of E. coli at various temperatures in seawater (25 °/oo).
(A) Exponential phase bacteria pregrown in M-9 medium at 35°C.
(B) Stationary phase bacteria pregrown in TSB at 35°C.
(C) Exponential phase bacteria pregrown in TSB at 35°C.

obvious in the TSB pregrown culture starved at 20°C than at 35°C (Figs. 13 and 14). This stress occurred earlier than in the M-9 pregrown cultures, as soon as 5 h after exposure to seawater. The degree of sublethal stress at 35°C was probably obscured by the rapid loss of viability.

The effects of cold temperature starvation on viability and sublethal stress were determined for an E. coli isolate obtained from human feces. It should be noted that this culture differed from the other isolates in having been maintained on artificial media approximately six months prior to experimentation while other isolates were used immediately after isolation with laboratory maintenance not exceeding two months. The fecal isolate was grown in M-9 and inoculated in 25 °/oo seawater at 2°C. After 3 wk starvation, no die-off was evident with the maximum reduction of 0.4 log units occurring by 12 wk. However, ER times increased dramatically, especially in TSB indicating the existence of sublethal stress (Fig. 15). The increase in ER represented increased lag time and/or decreased growth rate since very little cell die-off occurred when the starved cells were introduced into test media at 44.5°C (Fig. 16). Growth of this same fecal isolate in EC and TSB following starvation in 25 °/oo seawater at 20°C revealed a longer lag time in TSB as starvation progressed (Fig. 17). In contrast, growth curves of the urinary tract isolate following starvation revealed increased lag times in EC (Fig. 11). These data illustrated that length of exposure to artificial media was an important aspect to be considered in conducting seawater survival experiments.

Standard curves were developed for use in detecting stress in field isolates (Figs. 18 and 19). For these studies one part TSB pregrown inoculum was added to one part single strength Medium A-1 or EC at 44.5°C. The ER times in Medium A-1 were shorter than those in EC for similar inoculum levels with time differences decreasing as the inoculum size increased. Introduction

Figure 13. Effect of starvation at 20°C in seawater (25 °/oo) on the endpoint response (ER) of E. coli inoculated into either EC or TSB at 44.5°C. Prior to starvation the isolate (urinary tract) was pregrown in TSB at 35°C. Viable counts in both experiments determined using PBS at room temperature. Expt. 1 □——□ and Expt. 2 ■——■ .

Figure 14. Effect of starvation at 35°C in seawater (25 °/oo) on the endpoint response (ER) of E. coli inoculated into either EC or TSB at 44.5°C. Prior to starvation the isolate (urinary tract) was pregrown in TSB at 35°C. Viable counts determined using PBS (□——□) at room temperature or TSB (■——■) at 35°C.
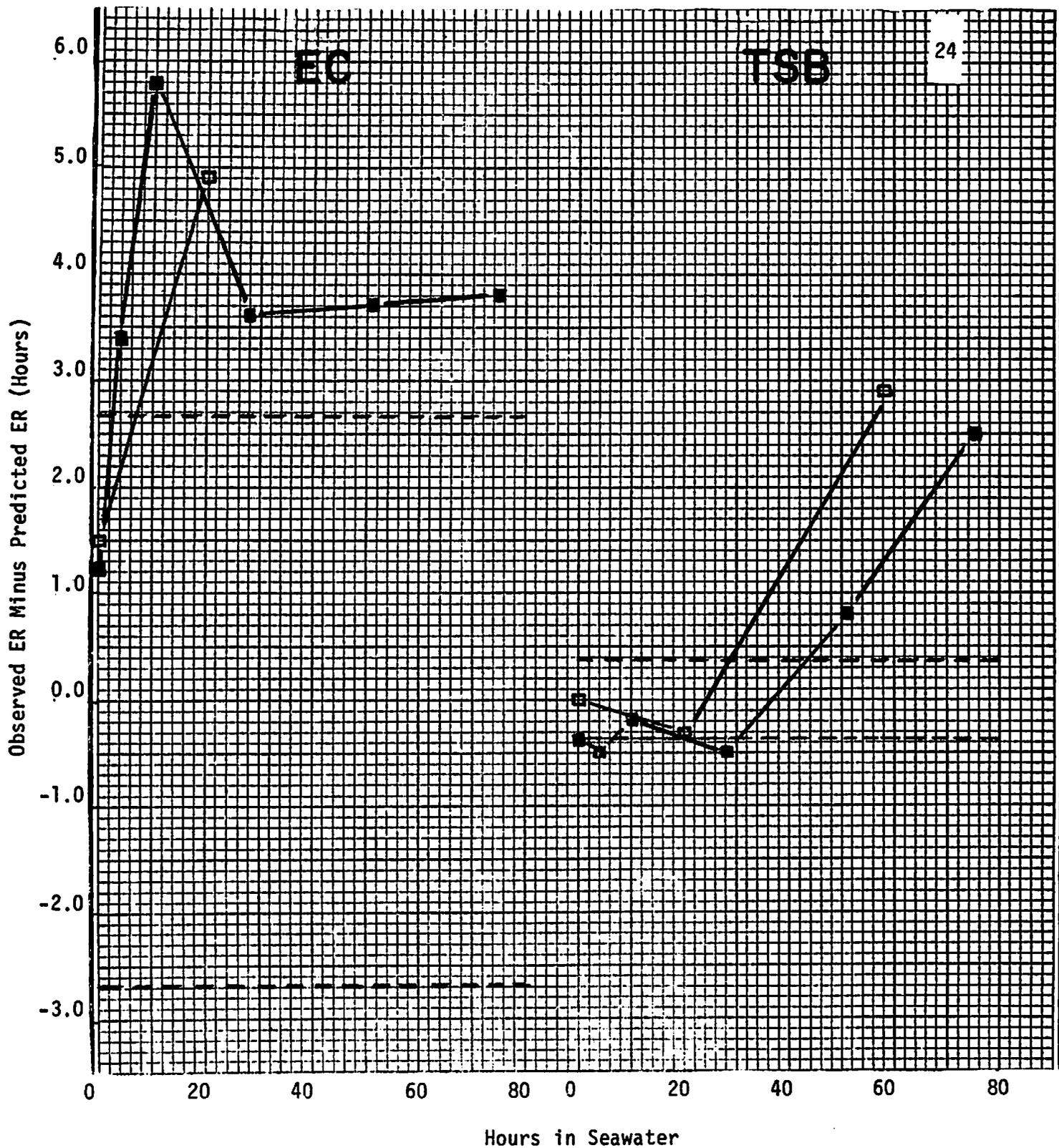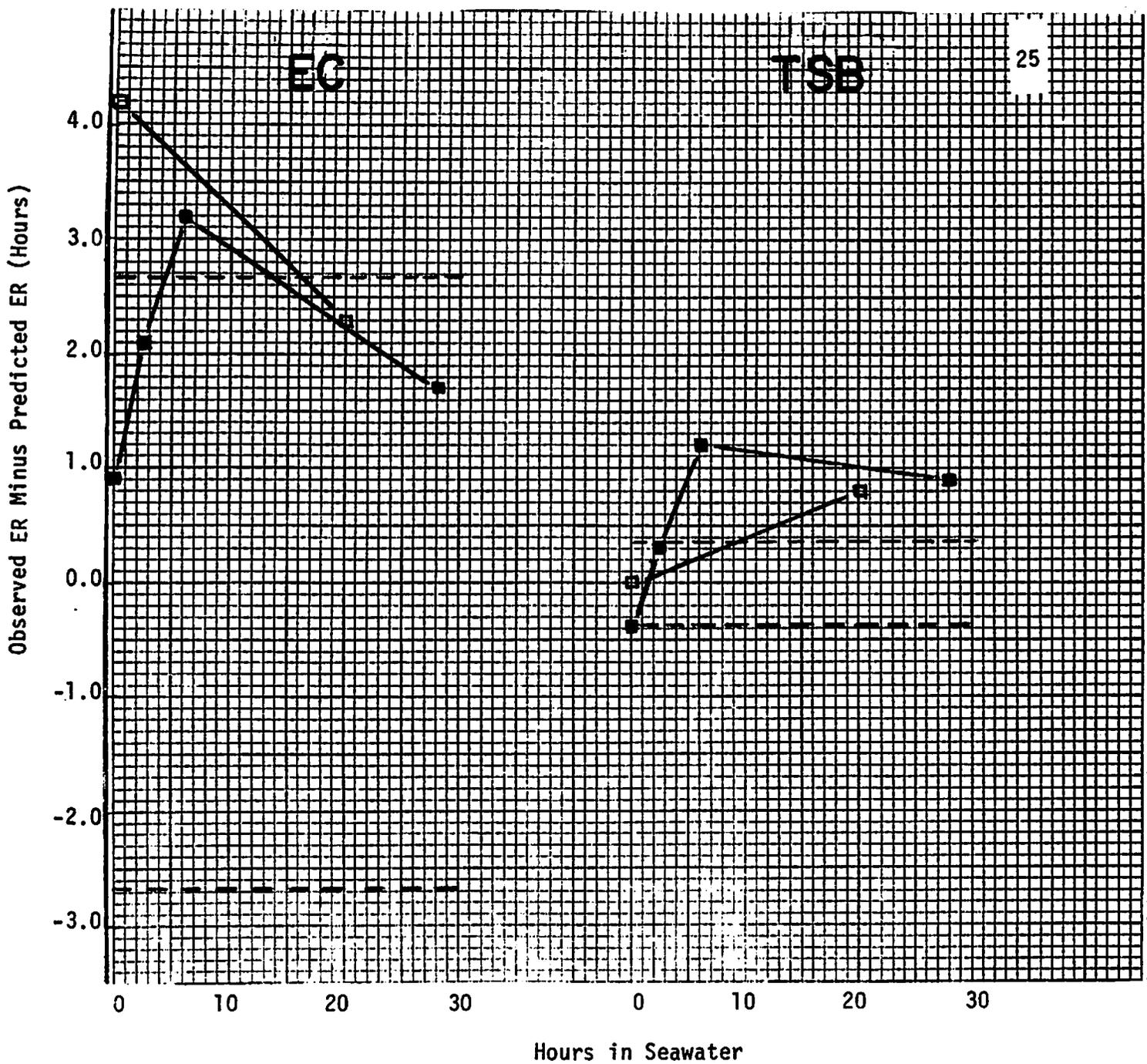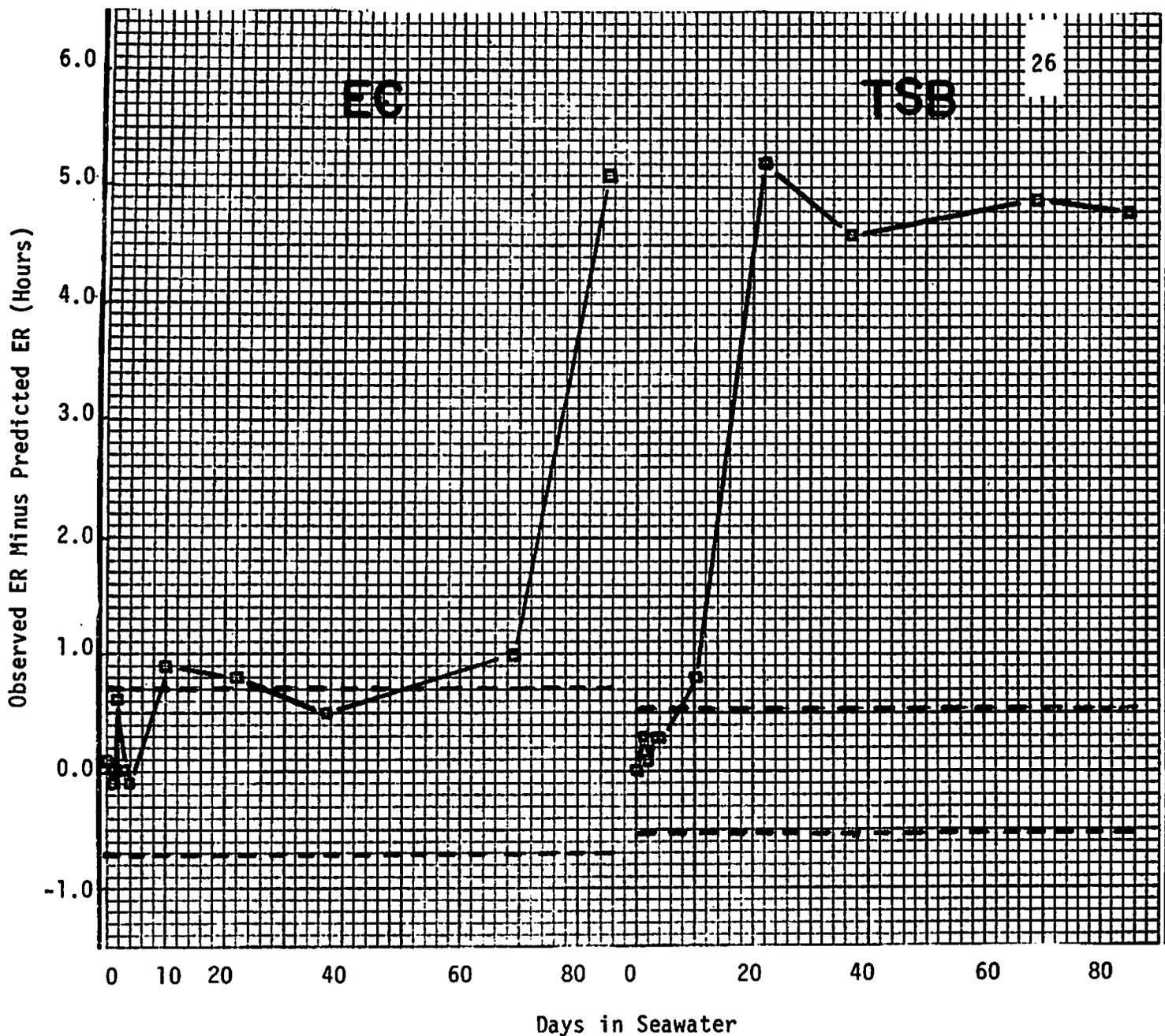
Figure 15. Effect of starvation at 2°C in seawater (25 ^{O}/oo) on the endpoint response (ER) of E. coli inoculated into either EC or TSB at 44.5°C. Prior to starvation the isolate (feces) was pregrown in M-9 medium at 35°C. Viable counts determined using PBS diluent at room temperature.
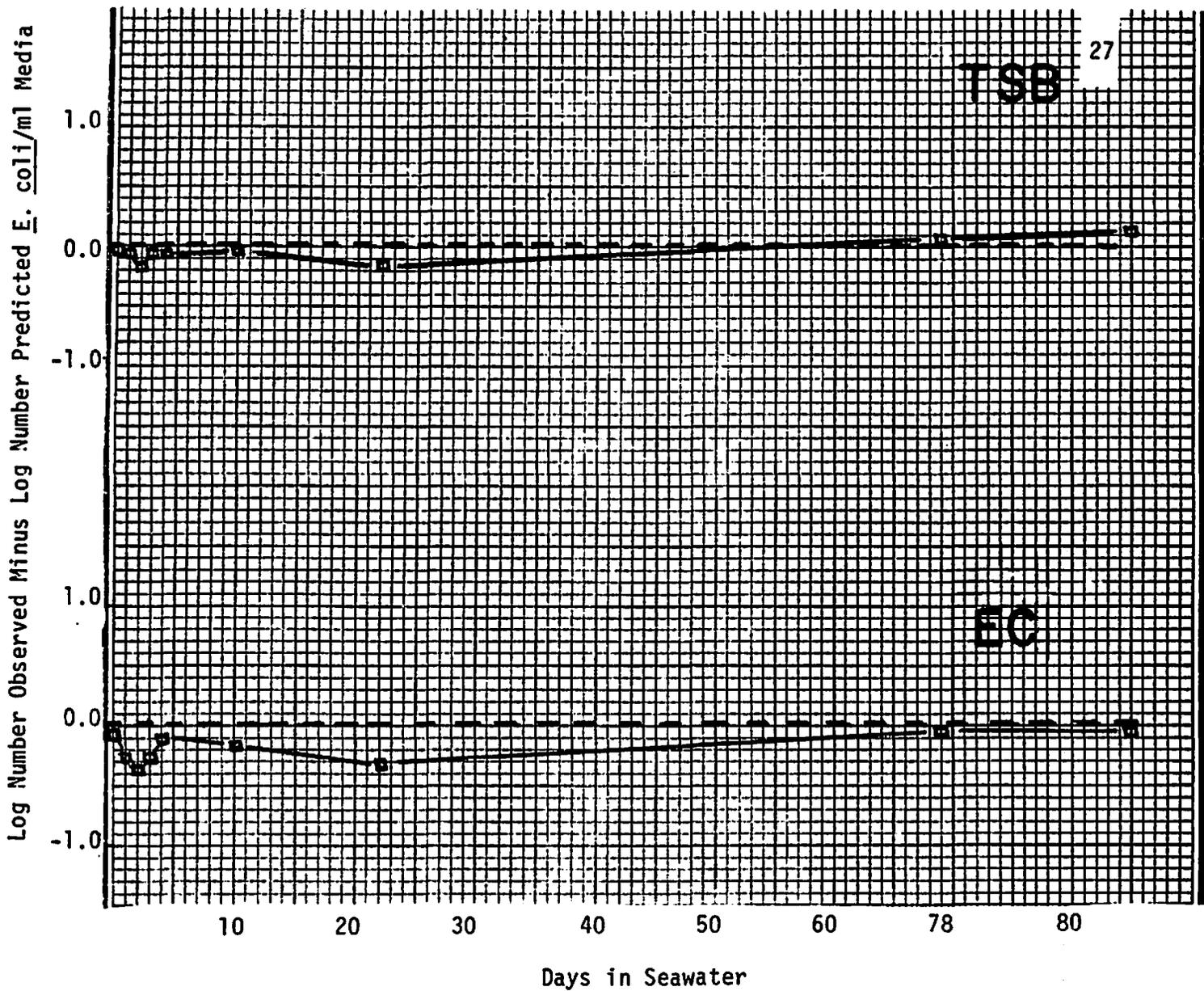
Figure 16. Effect of starvation at 2°C in seawater (25 °/oo) on the enumeration of E. coli immediately following a ten-fold dilution into either EC or TSB at 44.5°C. Prior to starvation the isolate (feces) was pregrown in M-9 medium at 35°C. Viable counts determined using PBS diluent at room temperature.
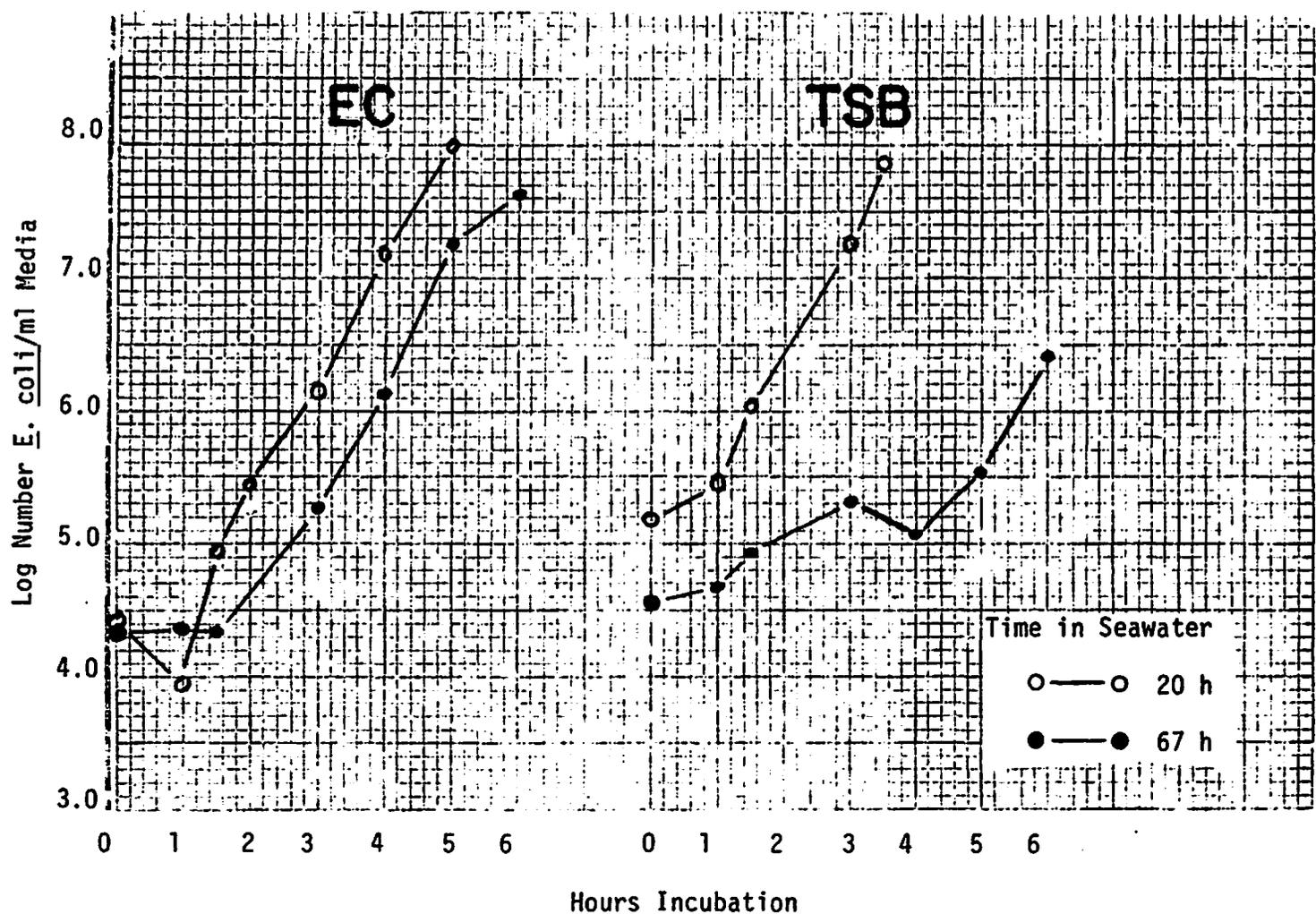
Figure 17. Growth of E. coli in EC and TSB, 44.5°C, after 20 and 67 hours
            starvation at 20°C in seawater (25 °/oo). Prior to starvation
            the isolate (feces) was pregrown in M-9 medium at 35°C.
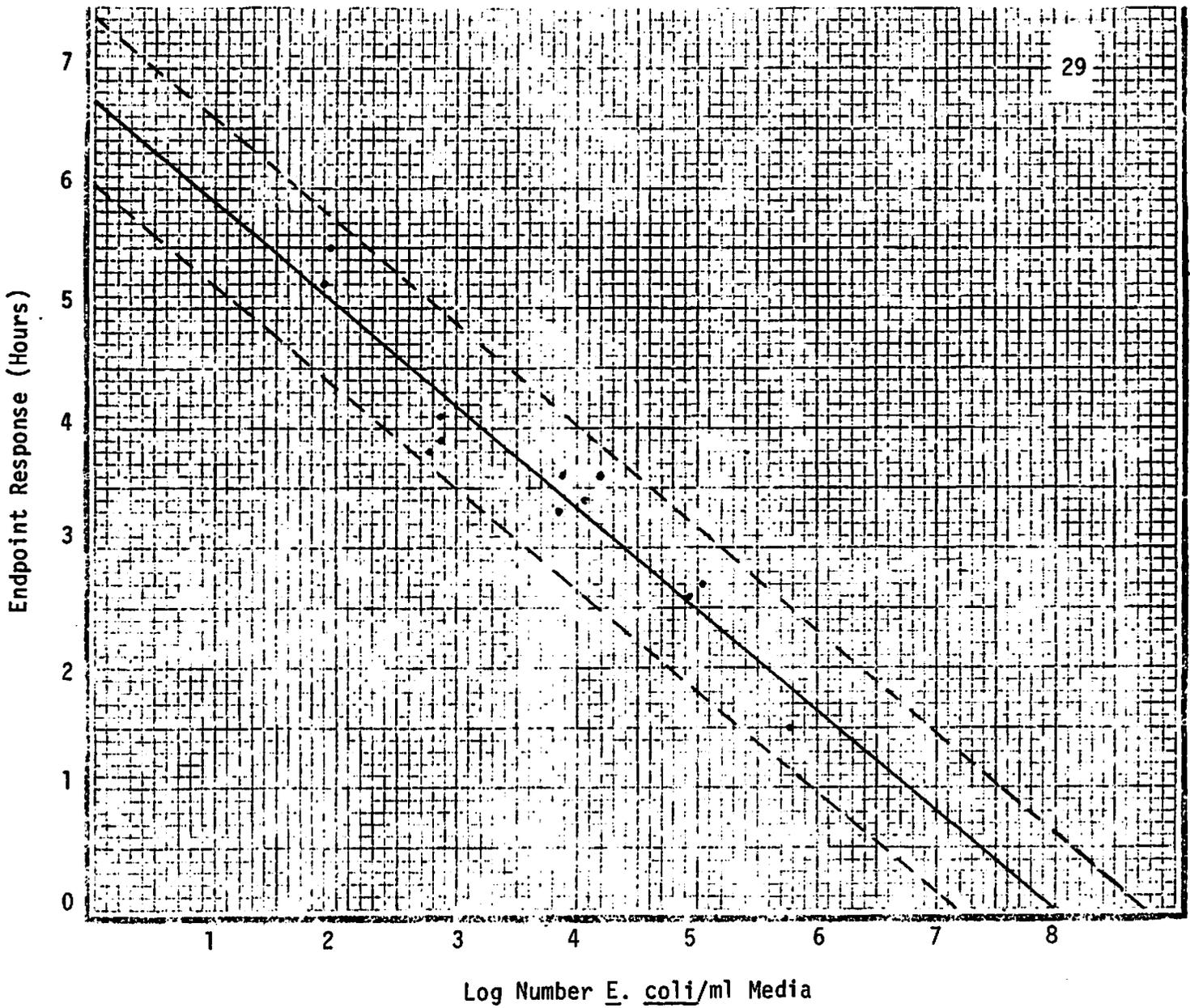
Figure 18. Relationship between inoculum size and the time of endpoint response in half-strength Medium A-1 when inocula were pregrown in TSB at 35°C. A linear least-squares regression calculation gave a correlation coefficient of -0.97 (N = 12) with an intercept of 6.71 and a slope of -0.84. The standard error of estimate at the 95 percent confidence level was ± 0.69.
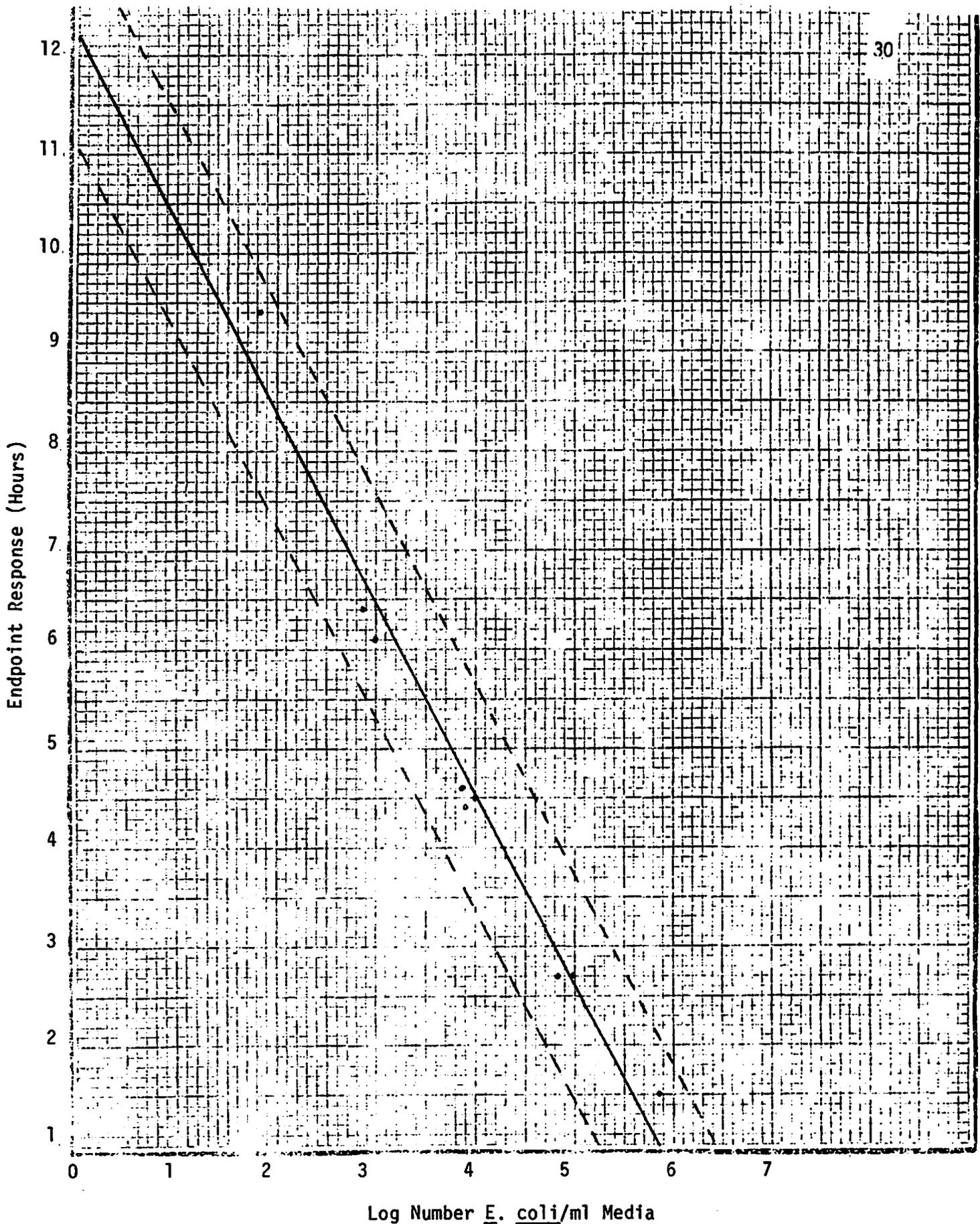
Figure 19. Relationship between inoculum size and the time of endpoint response in half-strength EC at 44.5°C when inocula (urinary tract isolate) were pregrown in TSB at 35°C. A linear least-squares regression calculation gave a correlation coefficient of -0.99 (N = 9) with an intercept of 12.17 and a slope of -1.91. The standard error of estimate at the 95 percent confidence level was $\pm$ 1.14.

of a larger volume of inoculum, resulting in dilution of selective agents as well as reduction in the medium temperature probably contributed to more consistent results and earlier ER values as compared to previous standard curves in EC using TSB grown inocula (Fig. 3).

Similar results were noted when field samples were diluted 1:1 into single strength test media. The ER times occurred 2 to 11 h sooner in A-1 than in EC with a mean difference of ca. 5 h. When compared to the standard curve, field samples inoculated into A-1 gave ER values extended by mean values of 0.6 and 0.9 h for samples collected from waters with mean temperatures of 14°C and 29°C respectively. Since the standard error of the estimated y ($S\hat{y}$) at $t_{0.05}$ for the A-1 standard curve was $\pm$ 0.7 h these results did not indicate that the cells had been greatly stressed at either temperature range.

Corresponding field samples processed in EC produced ER times with mean deviations from the predicted times of + 1.65 h for samples taken from cooler waters ($\overline{X}$ = 14°C) and - 1.78 h for samples taken from warmer ($\overline{X}$ = 29°C) waters. Since the standard error of the estimated y for the standard curve in EC was $\pm$ 1.14, and since the number of sampling points was limited, the question as to whether or not fecal coliforms in our environmental samples were stressed remains unresolved.

DISCUSSION

Fecal coliforms introduced into the estuarine environment are exposed to various physico-chemical and biological factors which contribute to viability losses. For example, dilution, adsorption to particulates (9), sedimentation, sunlight (3, 10), organics (11, 21, 23, 27, 32), salinity (6), temperature (9, 22, 33), dissolved oxygen (9), heavy metal toxicity (14), algae (18), bacteria (15, 19, 20, 26), bacteriophage (7), and protozoa (8, 19), have all been discussed. In addition, researchers have suggested that a portion of the coliform population may be sublethally stressed following exposure to the aquatic environment (4, 36).

Our study has been limited to the effects of three variables -- salinity, temperature, and previous cultural history on viability and stress of fecal coliforms. Although the data showed that viability of E. coli was inversely related to salinity, a similar relationship with respect to temperature was not as evident. Cells at 15, 20, 30 and 35°C showed similar viability profiles, although at 35°C there was a continued loss of viability after the initial sharp decline in bacterial numbers. A negligible loss of viability was demonstrated after 85 da of exposure to 2°C seawater (25 °/oo). Cryptic growth may have occurred thus obscuring viability losses since the initial bacterial levels in seawater in all of these experiments ranged from ca. 10^6 - 5 X 10^7 cells/ml.

The literature suggests that Enterobacter (as Aerobacter) aerogenes exposed to either distilled water or buffer solutions at low temperatures, displays loss of permeability control which results in leakage of cellular constituents with a progressive and rapid loss of viability (30, 31). In the absence of pronounced cell death in our experiment at 2°C, it could be

hypothesized that the hyperosmotic environment provided by 25 $^o/oo$ seawater protected chilled cells by decreasing the diffusion rate of cellular constituents.

The most dramatic differences between viability profiles were observed to be a function of cultural history. Cells harvested during exponential phase from a rich medium (TSB) were more sensitive to seawater than either exponential cells from minimal media M-9, or stationary phase cells from TSB. Although it is tenuous to extrapolate laboratory results to the estuarine environment, our findings are of value to those concerned with estuarine modeling studies involving the determination of coliform die-off coefficients. The previous history of coliforms introduced to the estuary could effect survival. For example, the physiological fate of E. coli derived from untreated sewage or other rich organic milieux as opposed to immediate storm water run-off may differ when discharged into the estuarine environment.

Little actually is known about the physiological changes that occur in E. coli in the estuarine environment. There is no doubt that the organisms develop different levels of sublethal stress depending upon the conditions encountered. Reports in the literature suggest that difficulties in analyzing water samples using membrane filter techniques may be due to the fragility of sublethally stressed organisms. These organisms may undergo mechanical destruction during the filtration process or be killed by selective media used in the enumeration procedure (12). New techniques have been devised to deal with sublethally stressed organisms -- some involved a period of resuscitation in a non-selective media (4, 34); others use a sandwich technique involving non-selective and selective media (25, 29).

The electrochemical detection method (ECDM) assays the time required for the inoculum to become metabolically active and reach a critical cell density.

Wilkins and Boykin (36) suggested that ECDM might provide a rapid and inexpensive method for monitoring coliform levels in the estuarine environment. However, it was not clear how the presence of stressed cells would affect the reliability of the ECDM results. Our study was concerned with what conditions of salinity, temperature, or previous cultural history would produce the highest levels of sublethal stress as determined by ECDM. In addition estuarine water samples were collected for analysis by ECDM and by standard MPN techniques to determine if stress in environmental coliforms could be detected.

It was obvious from the data collected that ECDM could detect stress induced in laboratory coliform populations. Stress was particularly evident in cells exposed to 25 and 30 °/oo seawater at 20°C. At 30°C stress was detected only in those cells exposed to the highest salinity (30 °/oo). When kept in seawater at 2°C (25 °/oo), cells developed significant stress only after a long exposure even though there was little loss in viability. Pronounced stress was also observed in samples of E. coli pregrown in a rich medium. The data suggested that prolonged ER times may be due to immediate cell die-off as the starved cells encounter the test media at 44.5°C and/or an increase in lag time.

When a limited number of environmental samples were analyzed by the ECDM method, stress was not apparent. All of our samples were collected in the immediate vicinity of a known point pollution source. The organisms sampled were likely to have had a short residence time in the estuarine environment and this could explain the absence of pronounced stress.

Analysis of our data suggested two possible applications for the ECDM. Firstly, the ECDM might be useful as a monitoring device in those areas where there is not likely to be a heavy load of highly stressed cells introduced

into the environment, for example, in areas free of industrial pollution or treated sewage. In order for ECDM to predict coliform levels accurately it would be advisable to perform additional experiments to determine selective media and temperatures most effective for the recovery of injured coliforms. Our data indicated that Medium A-1 would be preferential to EC at 44.5°C. Moreover, it is essential to have accurate standard curves developed using test media at the same nutrient and selective agent concentrations and temperatures as would be used in obtaining ER data from field samples. It is also imperative that low cell concentrations in the range 10^{-1} to 10^2 cells/ml be used in developing these standard curves. Standard curves developed during this study varied depending upon the media used, previous cultural history of the organisms, and nutrient and/or selective agent concentration in the media. In addition, there were suggestions that inocula of different volumes, although diluted to the same cell density could affect ECDM results.

A second application for the ECDM is as a research tool to determine the physiological condition of $\underline{E}$. $\underline{coli}$ (or other bacteria) exposed to the environment under a variety of conditions. The sensitivity of this method for monitoring stress should be examined. A comparison could be made with other techniques capable of detecting sublethal stress, i.e. adenylate charge, enzyme analyses, or growth on selective vs. non-selective media.

This investigation has posed additional questions. For example, what role does cryptic growth play in maintaining the apparent viability of starved cultures? Postgate and Hunter (24), demonstrated that denser populations of $\underline{E}$. $\underline{aerogenes}$ survived longer than sparser ones. Greater stress and viability losses might be observed if starvation experiments were conducted using cell inocula in the range 10^3 to 10^5 cells/ml. Such information would be valuable ·

in further assessing the ECDM as a method of monitoring stress. Viability and stress levels should be compared using cells starved under hypoosmotic vs. hyperosmotic conditions to evaluate whether or not the hyperosmotic environments encountered in these studies provided protection to the exposed cells. The degree of leakage of cell constituents under various conditions of salinity should be determined.

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