Characterization of aquatic injury in Escherichia coli and Salmonella typhimurium
by Susan Kay Zaske

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University
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Abstract:
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Although catalase added to DLA did not significantly improve enumeration on that selective medium, TSYA overlayed with DLA allowed colony formation by injured cells and yet retained the selectivity of DLA. Dilution blanks made with 1% non-fat dry milk or 1% peptone proved to be the best in terms of keeping the differential bacterial counts between media to a minimum. Injured cells recovered in 2 3/4 hours after being placed in TSY broth.

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SUSAN KAY ZASKE

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Microbiology

Approved:

Gordon A. McFeters by N. D. Reed
Chairperson, Graduate Committee

Norman D. Reed
Head, Major Department

Henry L Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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ABSTRACT

The quality of water used for injuring bacteria affected the degree of injury, the rate of damage, and the survival properties of the cells. Low resistance and periodic stirring of water in the injury vessel increased the rate and degree of injury while high cell populations prolonged the exposure time necessary for damage.

Lag time, percent spheroplast formation and the percent injury increased as aquatic exposure of E. coli increased. Salmonella typhimurium was sensitive to lysozyme before stress so that the spheroplast formation did not increase with exposure time, although the lag time, as calculated by the slide culture technique did increase.

Cell envelope damage appeared to be the primary site of aquatic injury as evidenced by the extreme sensitivities of these cells to sodium desoxycholate and lysozyme even though the bacterial particle counts remained high. Cell envelope involvement was also indicated by blebs seen protruding from the cell surface on electron micrographs. An extended lag in oxygen uptake of injured cells placed in TSY broth was seen. No oxygen uptake when these cells were placed in TSY broth supplemented with 0.15% sodium desoxycholate indicated membrane damage, as aerobic functions are part of an intact healthy cell membrane.

Although catalase added to DLA did not significantly improve enumeration on that selective medium, TSYA overlayed with DLA allowed colony formation by injured cells and yet retained the selectivity of DLA. Dilution blanks made with 1% non-fat dry milk or 1% peptone proved to be the best in terms of keeping the differential bacterial counts between media to a minimum. Injured cells recovered in 2 3/4 hours after being placed in TSY broth.

INTRODUCTION

Bacterial injury or sublethal damage has been recognized in a large number of microbial genera including Pseudomonas, Salmonellae, Vibrio, Azotobacter, Aerobacter, Klebsiella and Escherichia. A growing body of literature including reviews by Hurst (28) and Beuchat (6) have expressed the importance of this phenomenon when interpreting bacterial enumeration data. The impairment of bacterial metabolic or divisional processes in damaged cells hinder the effectiveness of common analytical methods used to qualitatively and quantitatively detect their presence (16).

In 1954, Heinmets (23) described the use of the term "killed" as the loss of viability when bacteria were incapable of multiplying. This incapacity is now interpreted to mean that the synthetic or metabolic processes have not necessarily failed and that such cells could be resuscitated. These organisms still could be capable of growth under suitable conditions following recovery or repair. Return to a normal uninjured state can occur once recovery has taken place.

The term injury is now being used to describe the temporary condition in which an organism has lost its ability to reproduce but still has some metabolic capacities. An organism is said to be metabolically injured when it multiplies in a nonselective complete medium but not in minimal media and structurally injured when it divides in nonselective but not in selective media (6).

Many stress factors have been studied as sources of bacterial injury. They include heat, (5,11,16,22,30,50), cold (46), exposure to sanitizers (58,59), starvation (20,35,37,47,48,57,66), freezing (54), freeze-drying (53,61), chlorination (9), and aquatic systems (12,37). Bissonnette et al. (4) have shown varying degrees of injury from exposure of indicator bacteria to different natural waters.

Bacteria in the injured state have been identified by a number of unique characteristics. An increased lag time and unusual sensitivities to selective or surface active agents, such as sodium desoxycholate, sodium chloride and lysozyme are found in damaged cells. In addition, injured organisms are frequently sensitive to antimicrobial drugs and develop increased permeability characteristics, loss of enzyme activity, an increased rate of glucose uptake, degradation of RNA and single and double-strand breakage of DNA (16).

The effect of injury upon enumeration of stressed indicator organisms has public health significance. The failure to detect significant numbers of stressed but viable indicator organisms in an aquatic system is a problem since it has been demonstrated (3) that approximately 90% of the indicator bacteria present in natural waters were not detected on selective media.

Since there is a problem with enumeration of indicator bacteria one must consider the possibility of similar problems in the detection

of enteric pathogens and determine if their pathogenicity has been altered because of injury. Sorrells et al. (62) have found that differences between healthy and freeze injured population of Salmonella gallinarum in terms of their viability were very small indeed and metabolic injury did not alter their pathogenicity. Evidently repair took place within the peritoneal cavity following introduction of the stressed bacterium into susceptible chicks. This phenomenon could also take place in aquatic systems as Schillinger and Stuart (60) hypothesize a "regrowth" downstream from a sewage plant. They believe organisms used nutrients in sewage to regain their metabolic and reproductive capacities. Short flow time and cold water made multiplication unlikely.

Little work has been completed to determine the physiological consequences of aquatic stress. Further studies to characterize this injury need to be accomplished so that better recovery methods for detecting these water-borne organisms can be implemented.

Statement of purpose

Because of the importance of injured indicator organisms to public health, a characterization and study of the physiological aspects of aquatic injury was deemed worthy of further investigation. One of the primary objectives of this study is to characterize aquatic injury in terms of metabolic and/or structural features. The cause of this

injury along with the mechanisms of recovery is also important. Another aspect of this study is to compare the characteristics of injury in indicator bacteria to potential enteric pathogens.

MATERIALS AND METHODS

Experimental Organisms

A fecal coliform (Escherichia coli) from the culture collection at Montana State University was used in these studies and was originally a stream isolate from the East Gallatin River. It had a ++-- IMViC reaction and produced gas at 44.5 C in FC medium (Difco). Cultures were streaked periodically on Levine eosin methylene blue (EMB) agar (Difco) and colonies with a metalic sheen were picked to maintain cultural purity. Stock slants were maintained on Tryptic soy yeast agar (TSYA) (Difco) and transferred each month to insure a healthy viable population.

Salmonella typhimurium number 14028 from the American Type Culture Collection, with a -+-+ IMViC reaction was the pathogen used for laboratory injury studies.

Media used for the Determination of Injury

TSYA, which was prepared with Tryptic soy agar (Difco), was supplemented with 0.5% glucose (Difco) and 0.3% yeast extract (BBL). This rich medium was used to determine the total viable population. Minimal agar (MA), which was used to indicate metabolic injury, was prepared according to Scheusner, et al. (58). This medium required

three separate solutions which were made ahead of time, the agar was melted prior to use and the solutions were tempered before combining.

The sample was also plated on TSYA supplemented with 0.15% sodium desoxycholate (Difco), desoxycholate lactose agar (DLA), and MA supplemented with 0.15% sodium desoxycholate. The 15% solution of sodium desoxycholate was made and 1 ml was added to 99 ml of molten agar prior to pouring plates. These media were used as an indication of the very healthy non-injured population of cells, since only those cells which were not structurally damaged were capable of growth. Because Salmonella typhimurium does not ferment lactose, glucose was substituted for lactose in desoxycholate lactose agar. This agar was used only for Salmonella typhimurium.

Samples were surface plated and spread for about 15 seconds on each medium. These spreaded plates were then overlayed with a small amount of the same called medium to insure that no further injury to the cells would occur. In this way surface spreading of colonies was also eliminated so that accurate counting could take place. At least three replicates were placed for each dilution.

Preparation of Cells and Determination of Injury

Cells were grown in TSY broth for all experiments. Eighteen hour stationary phase organisms were harvested at 3000 X G in the refri-

gerated Sorvall RC2-B centrifuge and washed two times with sterile reagent grade water (Milli-Q Reagent Grade Water Systems, Millipore). The cells were resuspended in sterile Milli-Q water and placed in acid washed 2 l crystallization dishes. The resistance of this Milli-Q water was carefully monitored and the cartridges changed periodically when the resistance of the water decreased and the conductivity increased, signalling an increase of free ions. Unless specified all work was carried out using approximately 10^6 bacteria/ml suspended in reagent grade water which had a resistance of 16 Megohms-Cm.

Aliquots from the injury vessel were removed at intervals and plated directly or secondarily stressed with hydrogen peroxide. Sterile Milli-Q water was used as the diluent unless otherwise noted. Percent injury was assessed as the $\frac{\text{TSYA count - DLA count}}{\text{TSYA count}} \times 100\%$.

Diluents

Sterile Milli-Q water was used for all dilutions unless experiments to examine the effect of different diluents were being conducted. In those experiments 1% solutions of peptone, non-fat dry milk and $MgSO_4 \cdot 7H_2O$ were used. Phosphate buffer, prepared according to Standard Methods (1) was also used as one of these diluents. The bacteria were placed in these solutions for a total of 15 minutes before 0.1 or 0.2 ml aliquots were spread on DLA and TSYA.

Preparation of Hydrogen Peroxide used for Secondary Stress

One hundred mg/l of hydrogen peroxide (H_2O_2) was used for all secondary stress experiments. H_2O_2 solutions were made with standard PO_4 buffer (1).

A one ml aliquot of cells was taken from the injury vessel and placed in 8 ml of the H_2O_2 solution. After 20 minutes, 260 units/ml of catalase (Sigma) was added to neutralize the H_2O_2. Since catalase is an enzyme that is inactivated by an excess of the substrate (80) the amount of catalase used here was calculated to decompose the amount of H_2O_2 present. Catalase was left in the bacterial suspension for 10 minutes. Further dilutions were made and the cells plated. Standard PO_4 buffer, to which catalase was added after 20 minutes, served as the control.

Membrane Filter Chambers

In order to examine the bacterial suspensions in a natural situation, membrane chambers, which were developed by McFeters and Stuart (41), were positioned in Rocky Creek 2 miles east of Bozeman, MT. Large chambers which hold up to 150 ml of sample were modified using 12 bolts instead of 6 to hold the pieces tightly together. Stop-cock grease was applied to portions of the chamber which came

in contact with the membrane so that a water-tight seal was formed. Tear-resistant microweb membranes with a pore size of 0.45 µm (WHWP 304, Fl Millipore) were used as faces of the chamber to allow a flow of water through the sample. Chambers were assembled, steamed and the bolts retightened before loading with bacterial suspensions. Loading of the sample was done in the laboratory and the chambers carried to the creek in a bucket of single distilled water. The caps were held securely in place with plastic tape so that no leakage into the chamber would occur. Chambers were tied to a float or anchored with a rock and immersed in water. A recording thermograph was also placed in the creek beside the chambers. A chamber was sacrificed for each day of sampling.

Preparation of Spheroplasts

After 40 ml of cells were centrifuged for 10 minutes at 3000 X G, the supernatant was poured off and 2 ml of lysozyme (1 µg/ml) (Sigma) was used to resuspend the organisms. Resuspended cells were then placed on a shaker for 1 hour and 45 minutes after which they were again centrifuged at 3000 X G for 15 minutes in a small plastic centrifuge tube. Most of the supernatant was poured off leaving a small drop of liquid in which to resuspend the cells. A drop of this material was placed on a slide and observed with phase contrast microscopy.

Viewing took place 45 minutes after shaking, thus 2½ hours after lysozyme was added. Percentages of spheroplasts were estimated after looking at several fields and approximately 20-80 cells were counted.

Slide Culture

Two sterile microscope slides were immersed in molten TSA at 45 C three times to obtain a thin layer of agar. These slides were then placed in a dessicator for one hour. Cells were concentrated by centrifugation and resuspended in a small amount of Milli-Q water after which 0.05 ml of the concentrate was placed on the dried slides with a micropipette and later spread. The slides were allowed to dry for 10 additional minutes before being dipped once more in molten TSA at 45 C. Once the overlay medium had hardened a sterile coverslip was placed over the agar so that no air bubbles could be seen. One of these slides was sealed with agar and allowed to incubate at 37 C until the first divisions were observed. This lag period was recorded and compared with the % injury. Another slide was sealed with molten paraffin (32), incubated at 37 C, and observed 24 hours later. Colonies and single cells were counted and the index of % viability determined by the following equation: $\frac{\text{Colonies}}{\text{Total Cells}} \times 100\%$

Bacterial Particle Counts

Bacterial particle counts were used to determine the total number of bacteria. These studies were done using black Nuclepore nitrocellulose membranes and epi-fluorescent microscopy. A 0.1% acridine orange stock solution was prepared which contained 2% glutaraldehyde. This was done as recommended by Hobbie, et al. (25) to prevent daily filtration. Cell dilutions of 9 ml were treated with a 9:1 toluene: acetone solution and stained for five minutes with one ml of the acridine orange stock solution. Thus, the final acridine orange solution was 0.01%. For a better vacuum distribution the 0.45 µm pore size cellulose filter was placed on a thick glass fiber membrane pad in a stainless steel filter head. This membrane was then washed with at least 10 ml of filtered reagent grade water (33). At least 10 ml of the stained bacterial solution was then filtered with a vacuum of five inches of mercury. A small section of the filter was cut out and placed in oil on a clean microscope slide. Oil was then spread on top of the piece of filter followed by a coverslip and additional oil.

The slide was viewed under oil phase with a fluorescent vertical illuminator (Ploemopak 2.3, Leitz). At least 20 fields of bacteria per filter were counted and the number of total bacteria/ml was established according to the following equation:

$$\frac{62475 \times \text{average number of organisms/field} \times \text{dilution factor}}{\text{number of ml filtered}}$$

The constant (62475) was found by calculating the area of the field, the total area of the filter on which bacteria were deposited, and the number of fields per filter.

Protein Determination

Liter portions of injured bacteria samples were centrifuged at 3000 X G for 10 minutes and the supernatant was poured off into an acid washed flash evaporator vessel. The supernatant was then evaporated with the Buchi Rotavapor R7 to approximately 20 ml and brought up to 25 ml with sterile Milli-Q water. This insured that an equal volume from each sampling day was recorded. The A_{260}/A_{280} of the supernatant was taken as a measure of the protein/nucleic acid content and the Lowry protein procedure (39) was also used to determine the protein content.

Respirometry Experiments

Cells used in these experiments were harvested after 18 hours of growth in TSY broth by centrifugation at 3000 X G, washed two times and resuspended in equal volumes of sterile reagent grade water. Injured cells were harvested from membrane filter chambers and resuspended in sterile reagent grade water. These cells were enumerated and the per-

cent injury calculated by plating on TSYA and DLA. Flasks prepared and the respirometer operated aerobically according to Dockins and McFeters (14).

Electron Microscopy

The Zeiss EM 9S-2 electron microscope was used to view the morphology of injured cells. Cultures of E. coli, which were grown in TSY broth, were harvested after 18 hours of incubation. These cells were washed three times in sterile Milli-Q water and resuspended to approximately 10^6 organisms/ml in a large glass acid washed carboy filled with Milli-Q water. These containers were then placed at room temperature for several days to insure bacterial injury. The water was stirred, sampled and plated on TSYA and DLA at timed intervals to monitor injury. Once injury had occurred the cells were harvested with the aid of the continuous flow centrifuge assembly (Sorvall).

The pellet was fixed for one hour at 4 C with 3% glutaraldehyde (Polysciences, Inc.) in 0.1 M PO_4 buffer, pH 7.2 to kill the cells and stabilize the protein. The sample was then vacuum infiltrated for an additional 20 minutes and washed three times, for 10 minutes each, with 0.1 M PO_4 buffer, pH 7.2. These steps were followed by 3-4 hours of further fixation with 1-2% OsO_4 (Polysciences, Inc.) in 0.1 M PO_4

buffer, pH 7.2 at room temperature.

Since Spurr's, the embedding medium, was not miscible with water the specimen was dehydrated with a series of increasing concentrations of acetone. The progressive dehydration series consisted of washing the sample two times, for 5 minutes each, with 30%, 50%, 70%, 85%, 95% and then 100% acetone. Propylene oxide (P.O.) (Eastman) was then used as a transitional solvent and the speciman was washed in it two times, for 10 minutes each.

Infiltration was carried out on a shaker with a solution of P.O.:Spurr's (2:1) for 15 minutes, 1:1 P.O.:Spurr's for 1½ hours, pure Spurr's under vacuum for 15 minutes and finally with pure Spurr's on the shaker for 1 hour. The sample was held in pure Spurr's overnight and then positioned in a polyethylene capsule (BEEM) which was filled with pure Spurr's. The capsule was then set in a 70 C oven for 9-12 hours while polymerization took place.

When the Spurr's hardened, the polyethylene capsule was removed. The block was then trimmed prior to thick and then thin sectioning on a Reichert ultramicrotome with glass knives, which were made with an LKB Knifemaker.

Thin sections were positioned on an uncoated grid and post-section stained in uranyl acetate (Matheson Colemann and Bell). Grids were immersed in saturated uranyl acetate in absolute methanol for five

minutes and rinsed with absolute methanol. To prevent the sections from curling, the grids were rinsed with 100%, 90%, 70%, 50%, 30% and 15% methanol followed by distilled water. Grids were further stained with Reynold's lead citrate for 2-3 minutes and rinsed with 0.02 N NaOH followed by distilled water.

Negatives taken with the Zeiss EM 9S-2 were enlarged, printed on Kodak ektamatic paper via an ektamatic processor, fixed, washed and dried.

Recovery Experiments

Injured cells were diluted 1/10 in TSY broth, TSY broth supplemented with 0.2% sodium citrate (J.T. Baker Chemical Co.) and TSY broth supplemented with pantoyl lactone (ICN Pharmaceuticals, Inc.). Recovery of the cells or the converging differential enumeration between TSYA and DLA was monitored at intervals by plating on both media.

Since it would be convenient for injured cells to recover and grow on some type of selective agar without being transferred from preenrichment broth in which division must be monitored, the effects of catalase added to DLA and TSYA overlayed with the selective medium were observed. A small aliquot of catalase, 0.1 ml, which contained 400 units of active ingredient, was spread on DLA along with the

bacterial inoculum and the same amount of catalase was also added to TSYA to determine if the number of colonies on this medium could be increased. TSYA plates were also spread with the bacterial sample and overlayed with DLA. A 0.1 ml aliquot of lysozyme which contained 5 µg was spread on TSYA along with the microbial inoculum to determine if it would prevent recovery and growth of the damaged cells.

RESULTS

The Effect of Population Size and Water Characteristics on Injury

Water with different characteristics and various cell population sizes was placed in vessels to observe the effect of these variables on cellular damage. The effect of constant or periodic stirring, prior to sampling, on cell debilitation was also of interest. Higher cell populations showed very little injury throughout the experimental period, and the time required to achieve this damage was lengthy as can be seen in Fig. 1. These two vessels were stirred constantly with magnetic stirrers over which styrofoam pads were situated to protect the sample from additional damage due to heat released from the stirrers. New Milli-Q water, with a resistance of 18 Megohms-Cm, was used as the medium of injury.

Reagent grade water with resistances of 18 and 7 Megohms-Cm were also compared as sources of debilitation. The difference between the two can be seen in Fig. 2. Water with lower resistance caused a quicker and more dramatic increase in injury. A very rapid decrease in the viable population occurred after the third day of aquatic exposure. Cells in new Milli-Q water, which had the higher resistance, showed a much lower percentage of injury and a greater viability throughout the experimental period. Both of these vessels were stirred constantly with the magnetic stirrer and were protected from heat.

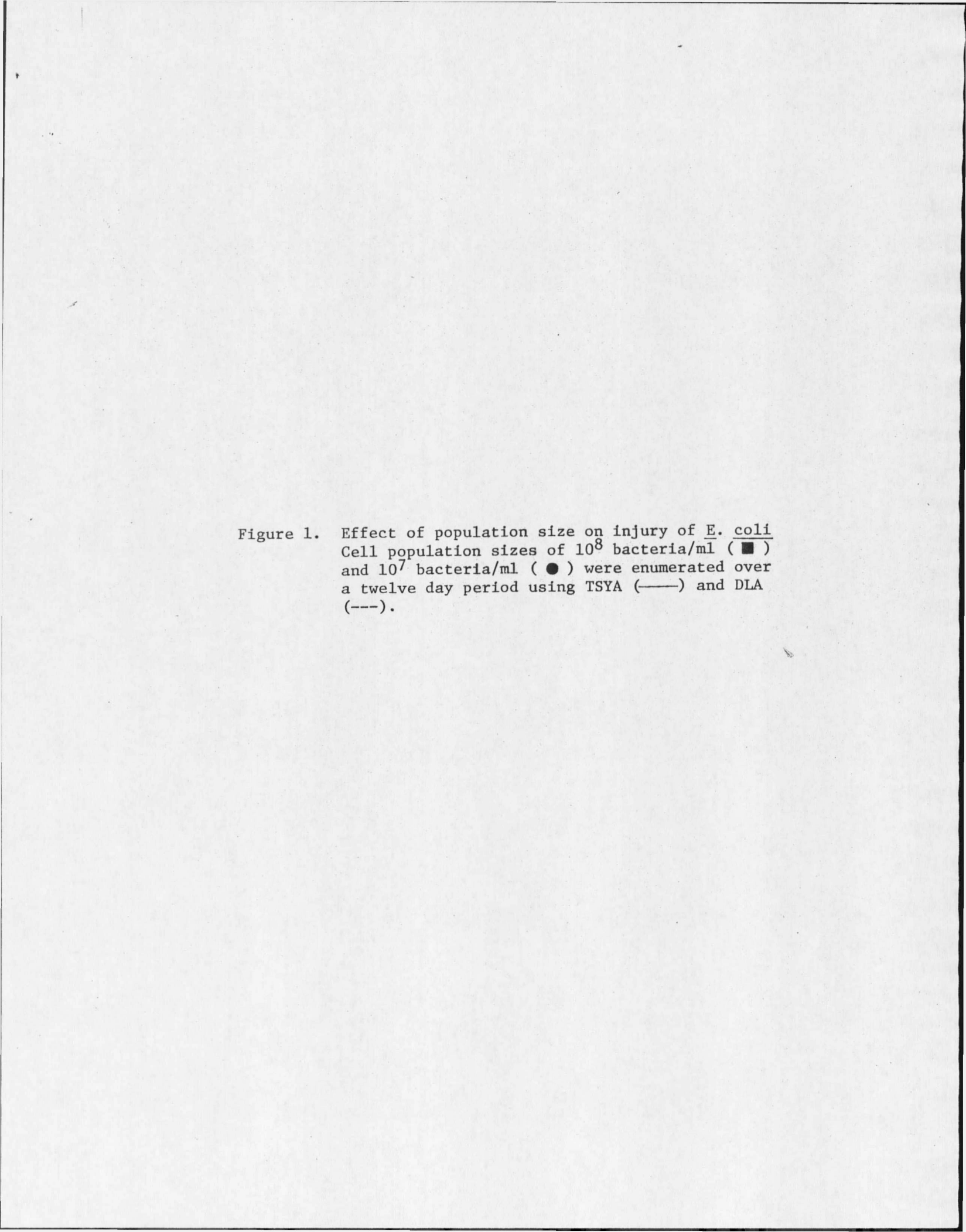


Figure 1. Effect of population size on injury of E. coli Cell population sizes of 10^8 bacteria/ml (■) and 10^7 bacteria/ml (●) were enumerated over a twelve day period using TSYA (——) and DLA (---).

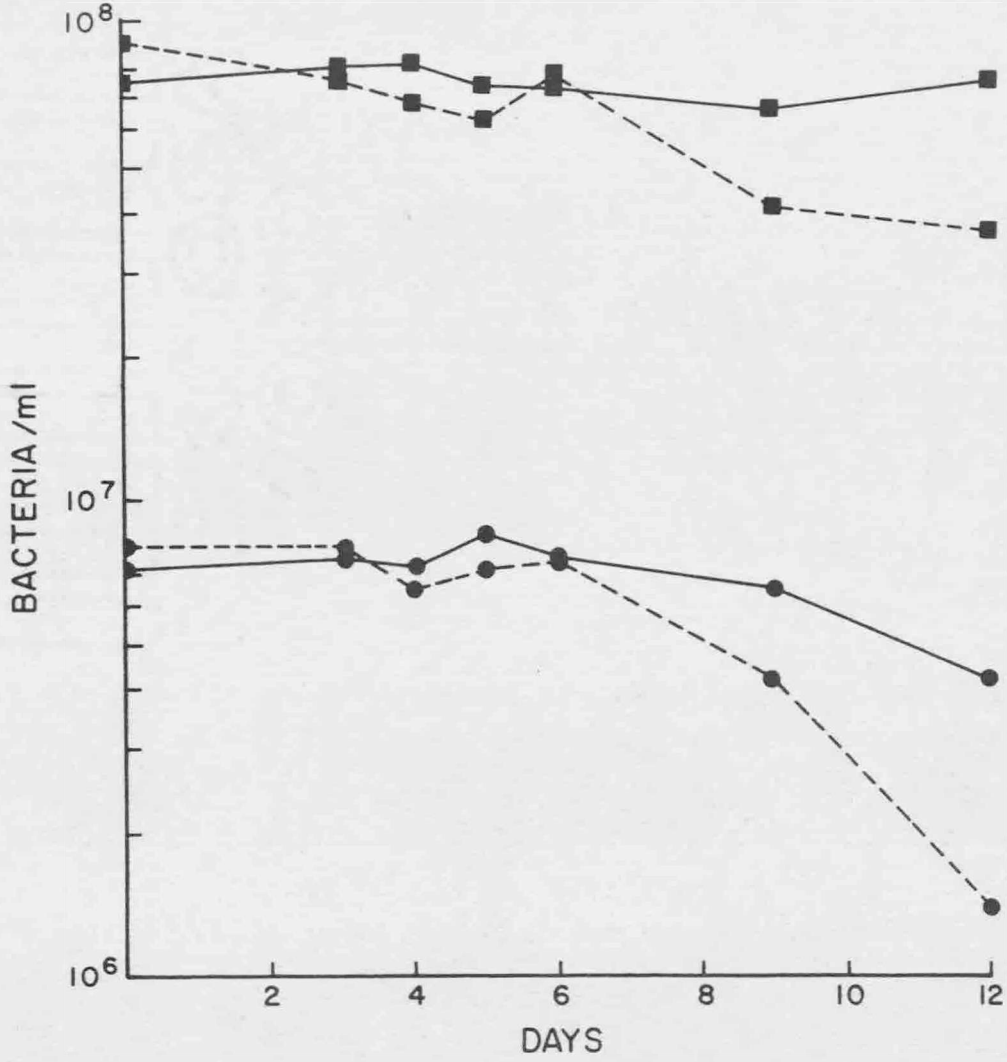

10^8
10^7
10^6
BACTERIA/ml
2
4
6
8
10
12
DAYS

Figure 2. Effect of resistance and constant stirring on *E. coli* injury. Cells placed in reagent grade water with resistances of 18 Megohms-Cm (■) and 7 Megohms-Cm (●) were enumerated over a twelve day period using TSYA (——) and DLA (----).

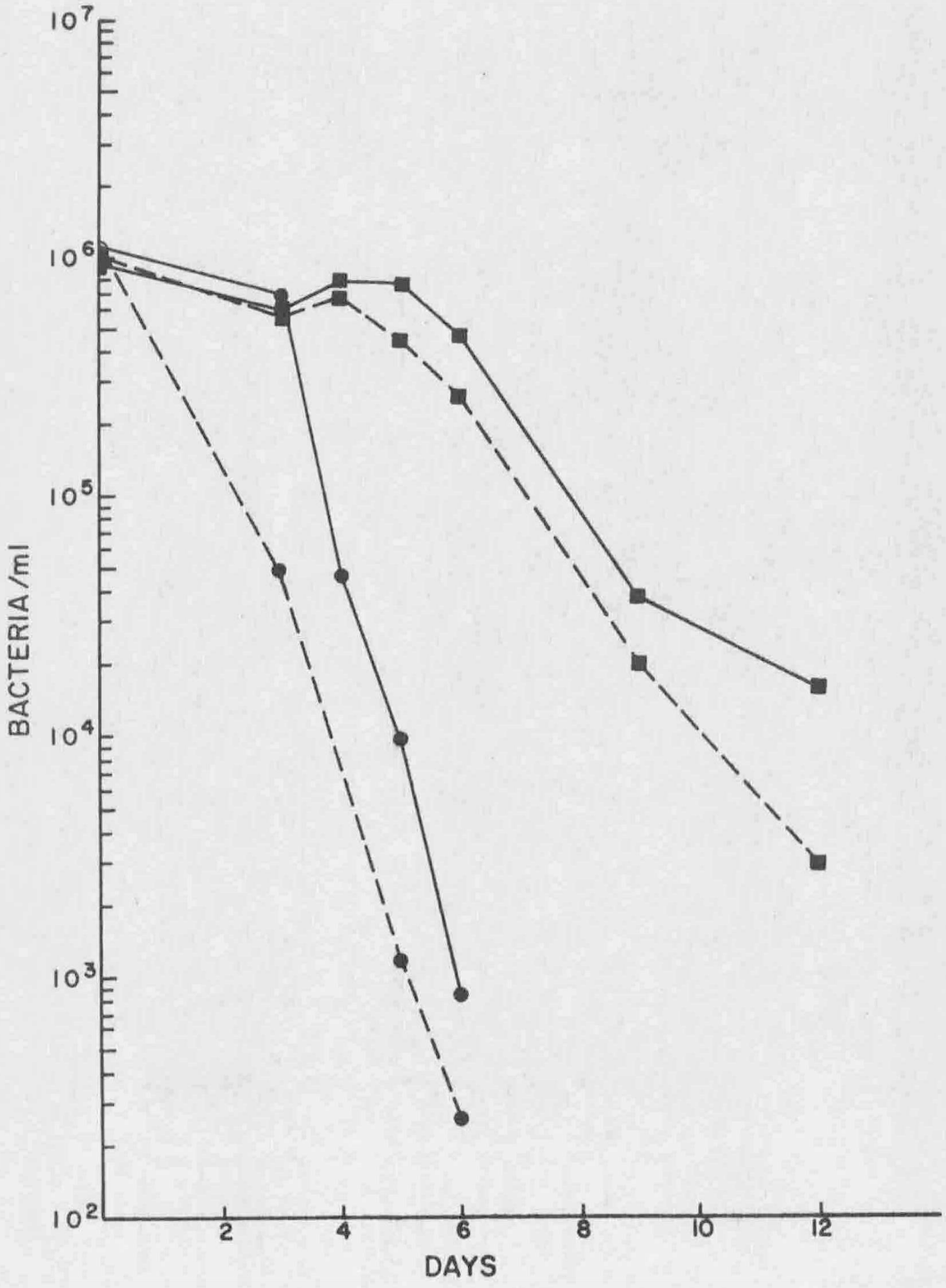
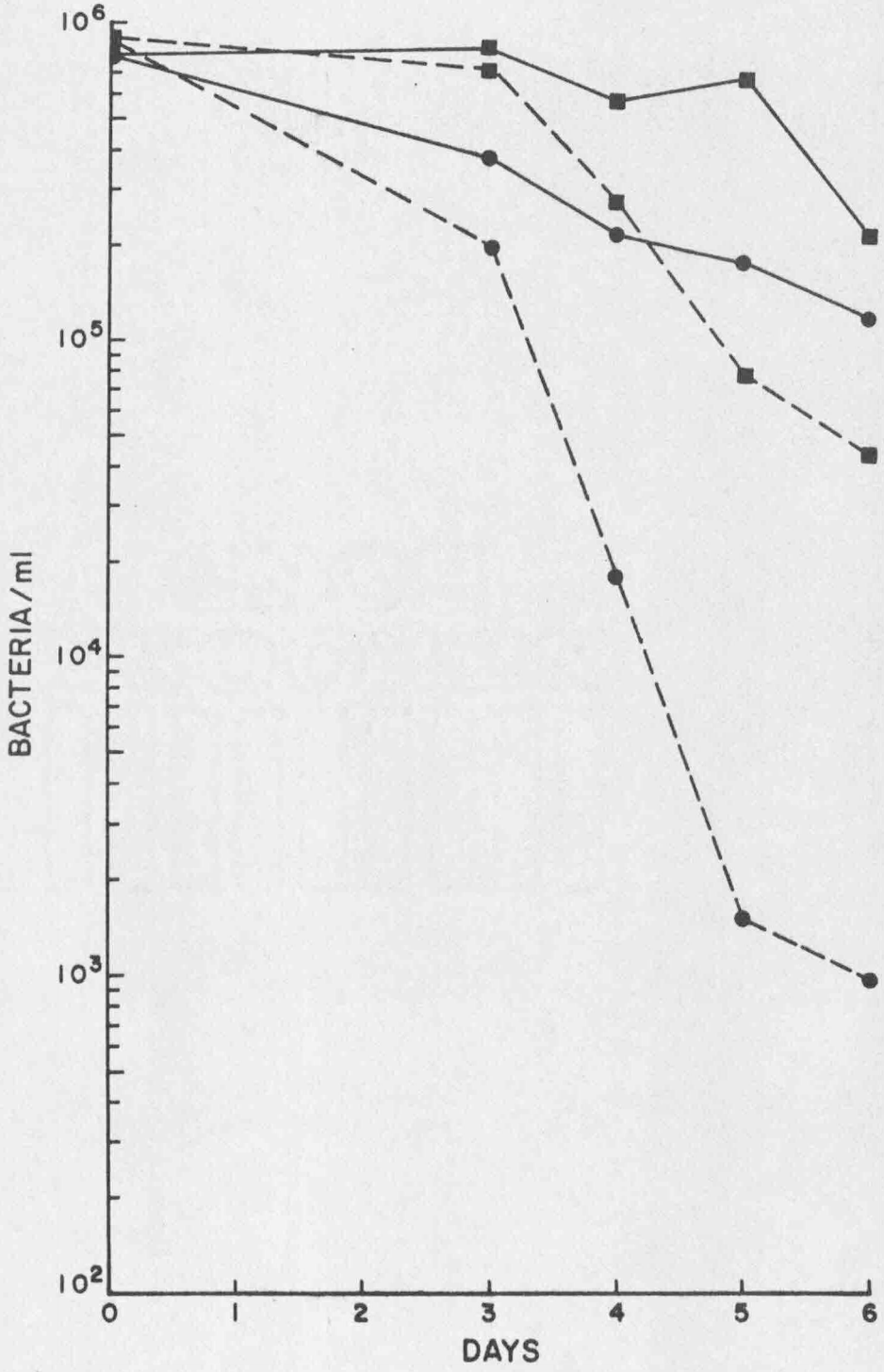

10^7
10^6
10^5
10^4
10^3
10^2
BACTERIA/ml
2
4
6
8
10
12
DAYS

Fig. 3 shows cellular injury when standard PO_4 buffer made with new Milli-Q water was used to induce damage. This figure also shows extensive cellular debilitation after three days of exposure when high quality reagent grade water was used. This water was stirred prior to sampling only. Of the two injury vessels which contained reagent grade water with high resistances, the vessel which was stirred constantly had a greater population of viable cells and fewer damaged cells than did the vessel which was stirred only before sampling. The percentage of injury in these cell populations can be seen and compared in Fig. 4.

Rocky Creek water was brought into the laboratory and used as another source of debilitation for the bacterial cells. One batch of this water was autoclaved while another was filter sterilized using a 0.22 μm membrane filter. No measurable bacterial damage occurred in autoclaved water after six days of exposure but there was a loss of viability and considerable injury in the filter sterilized creek water (Fig. 5). Preliminary tests to determine the presence of virus in the filtered water were negative. The extent of this injury is shown in Fig. 6.

Cell volumes measured during aquatic laboratory exposure dropped one cubic micrometer in the initial three days of injury and then remained stable with a range of 2.66-1.89 μm^3 throughout the next

Figure 3. Effect of periodic stirring and PO_4 buffer on *E. coli* damage. Organisms placed in periodically stirred reagent grade water with a resistance of 18 Megohms-Cm (■) and others placed in periodically stirred PO_4 buffer (●) were enumerated using TSYA (——) and DLA (----).


10^6
10^5
10^4
10^3
10^2
BACTERIA/ml
0
1
2
3
4
5
6
DAYS

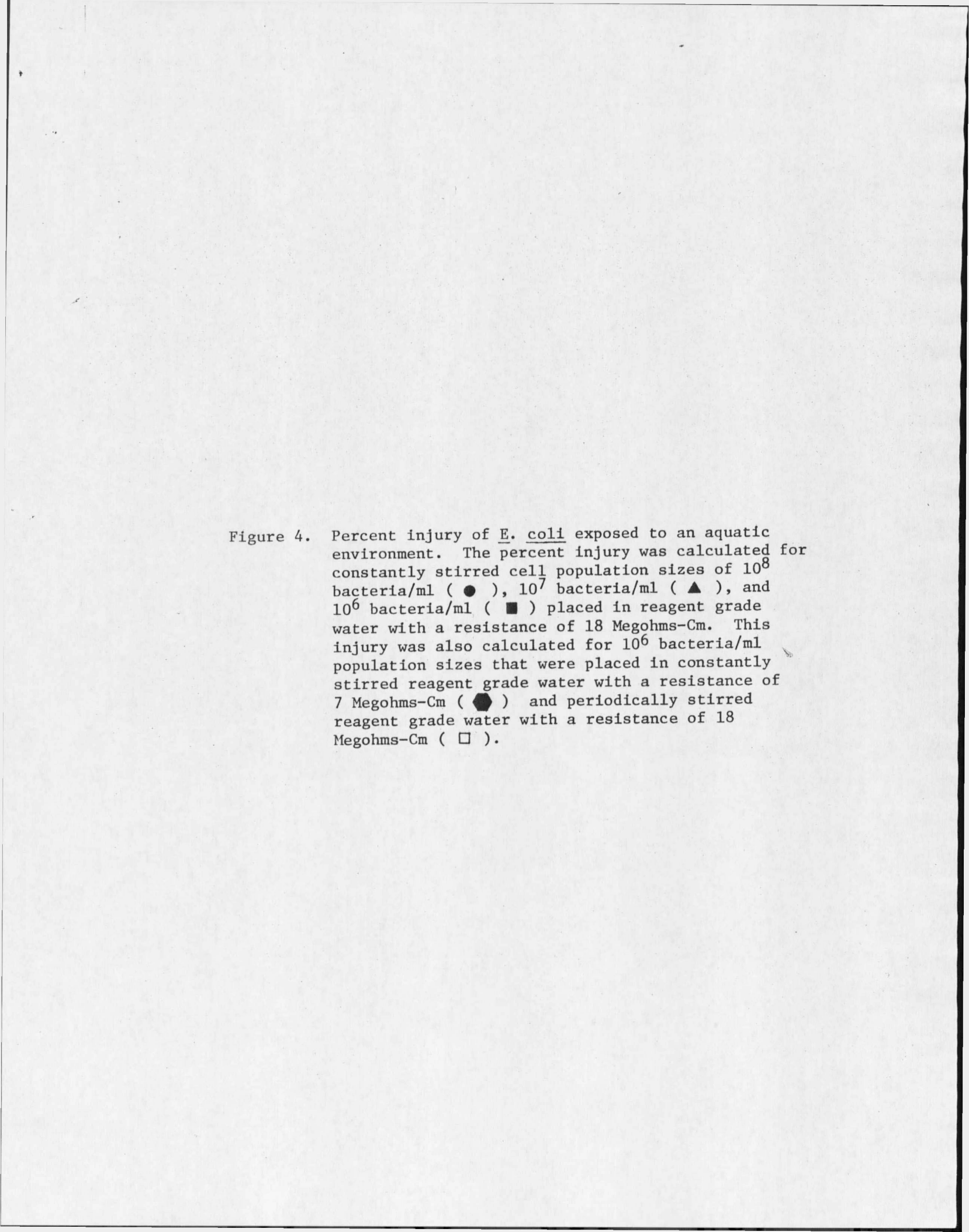


Figure 4. Percent injury of E. coli exposed to an aquatic environment. The percent injury was calculated for constantly stirred cell population sizes of 10^8 bacteria/ml (●), 10^7 bacteria/ml (▲), and 10^6 bacteria/ml (■) placed in reagent grade water with a resistance of 18 Megohms-Cm. This injury was also calculated for 10^6 bacteria/ml population sizes that were placed in constantly stirred reagent grade water with a resistance of 7 Megohms-Cm (⬢) and periodically stirred reagent grade water with a resistance of 18 Megohms-Cm (□).

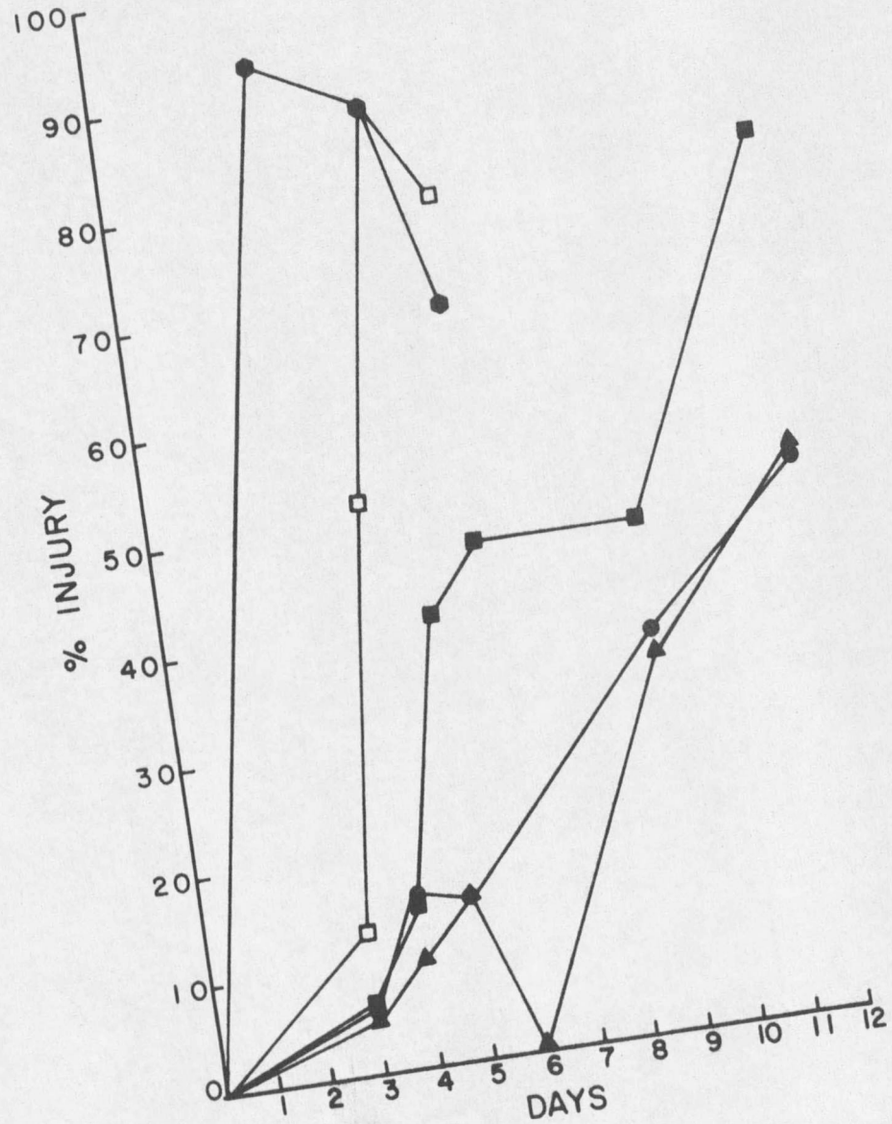
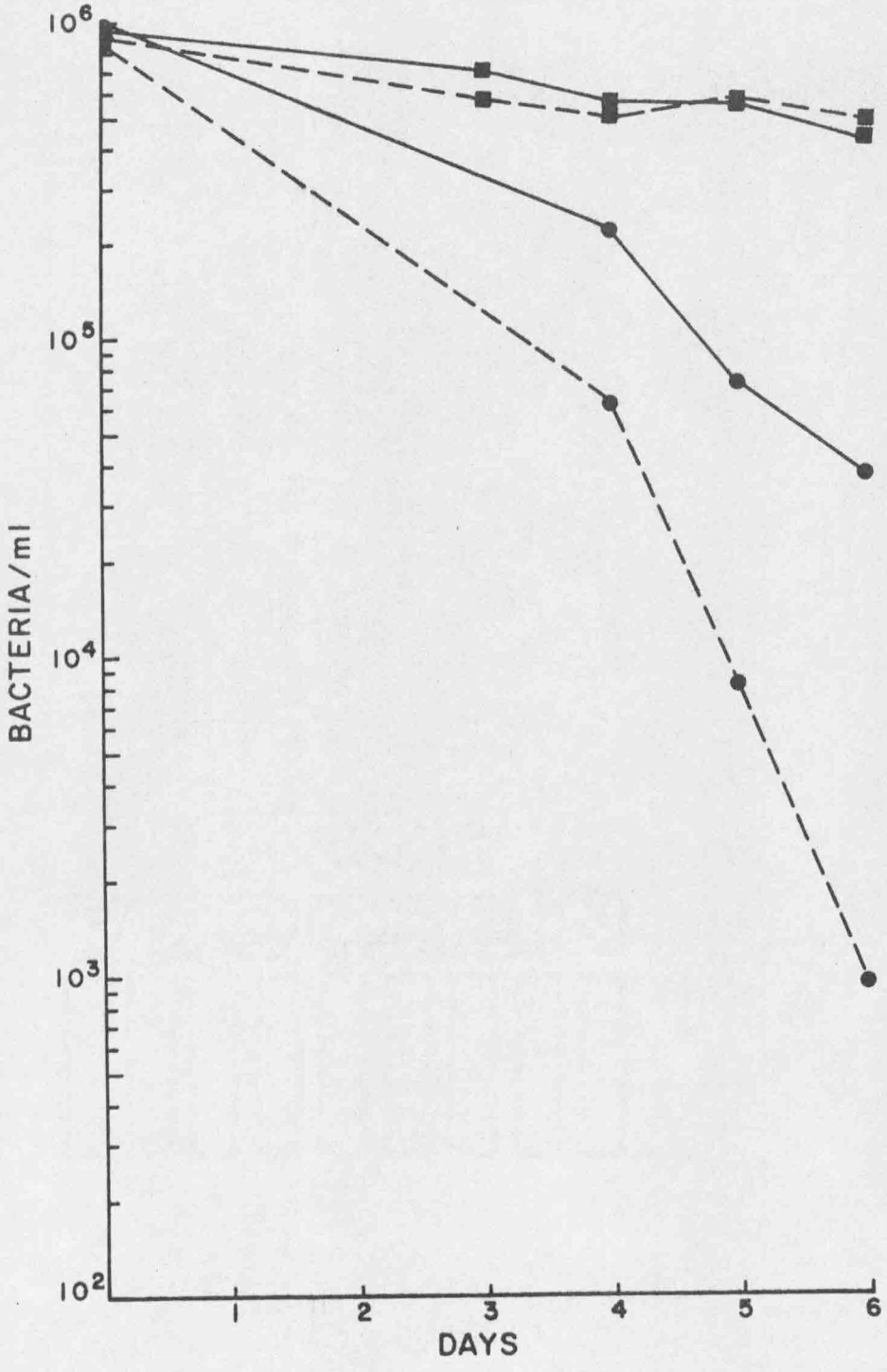
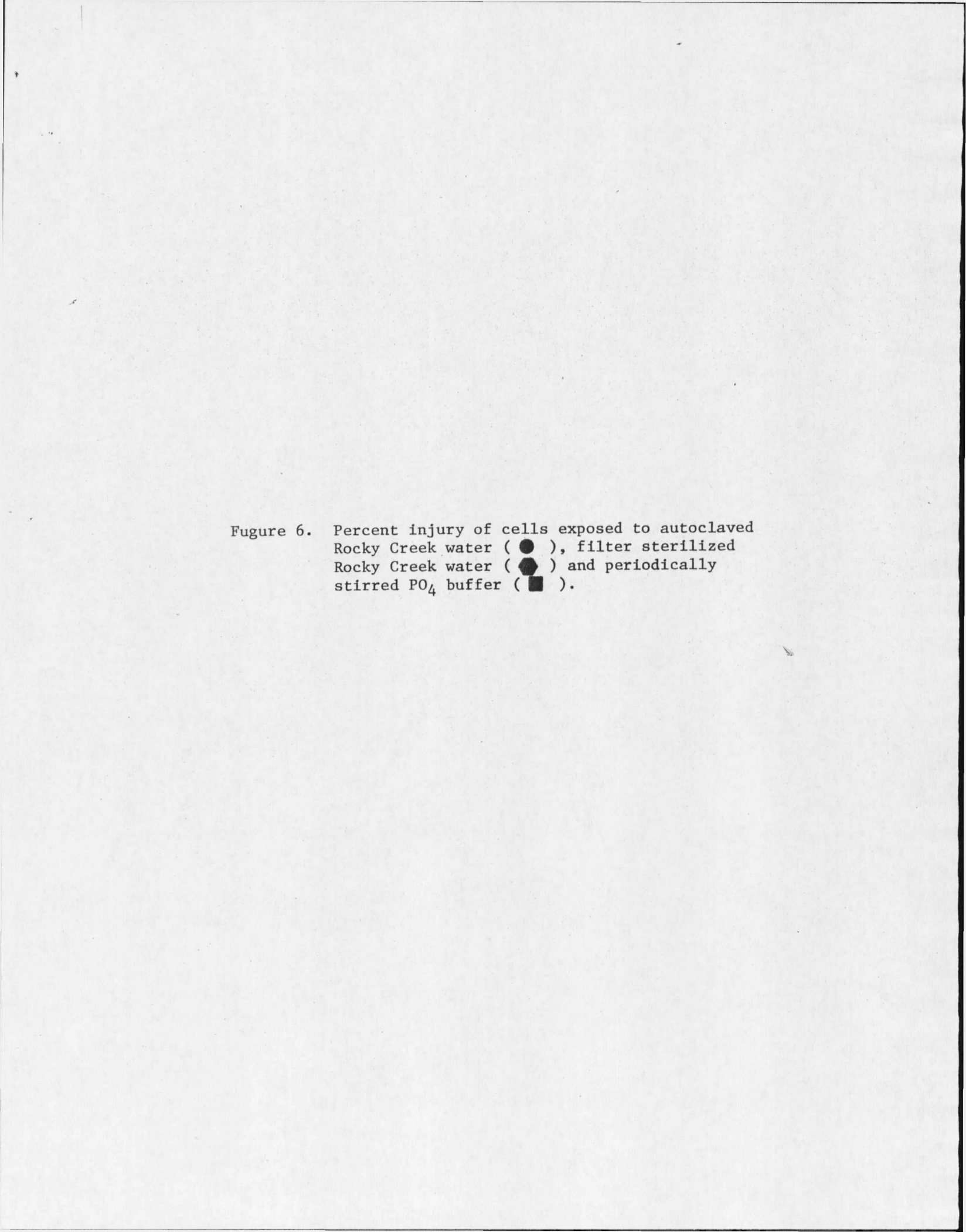
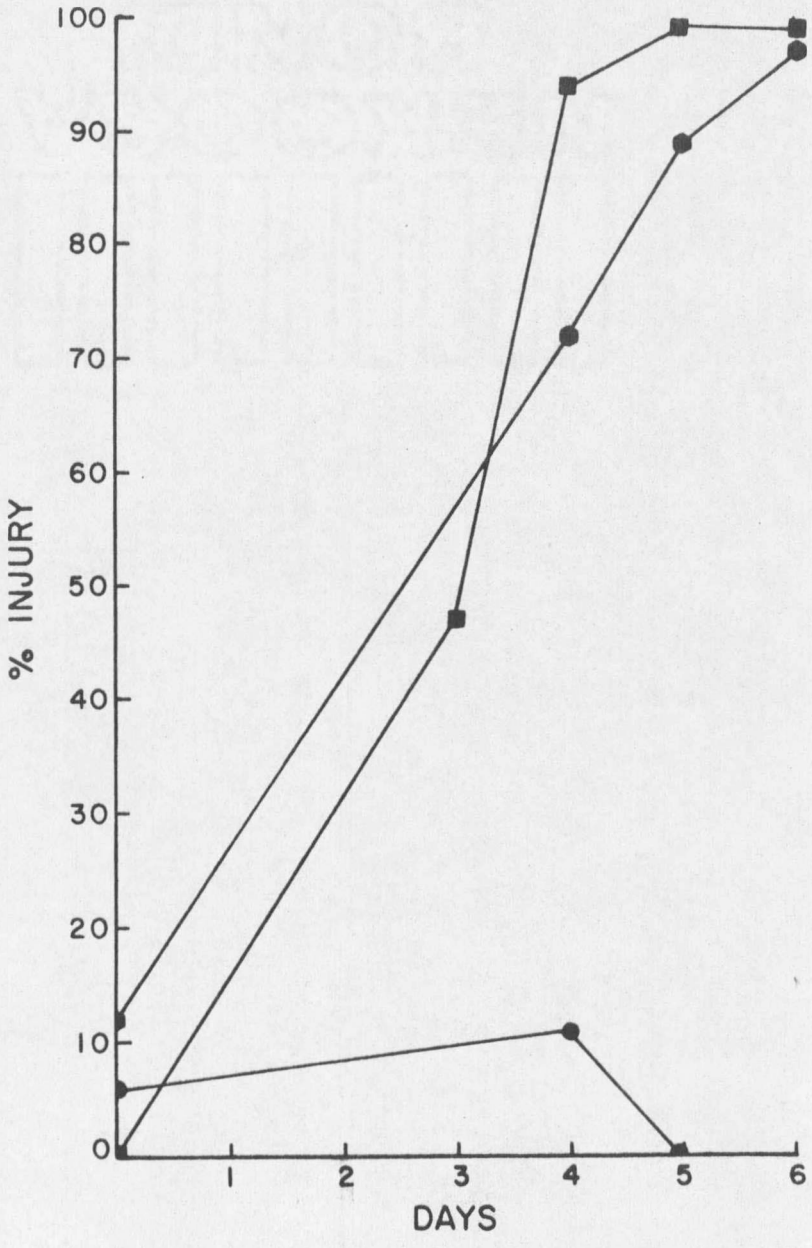

100
90
80
70
60
50
40
30
20
10
0
% INJURY
1
2
3
4
5
6
7
8
9
10
11
12
DAYS

Figure 5. Effect of natural water on cellular injury. Cells placed in autoclaved Rocky Creek water (■) and filter sterilized Rocky Creek water (●) were enumerated using TSYA (——) and DLA (----).


10^6
10^5
10^4
10^3
10^2
BACTERIA/ml
1
2
3
4
5
6
DAYS



Fugure 6. Percent injury of cells exposed to autoclaved Rocky Creek water (●), filter sterilized Rocky Creek water (⬢) and periodically stirred PO_4 buffer (■).

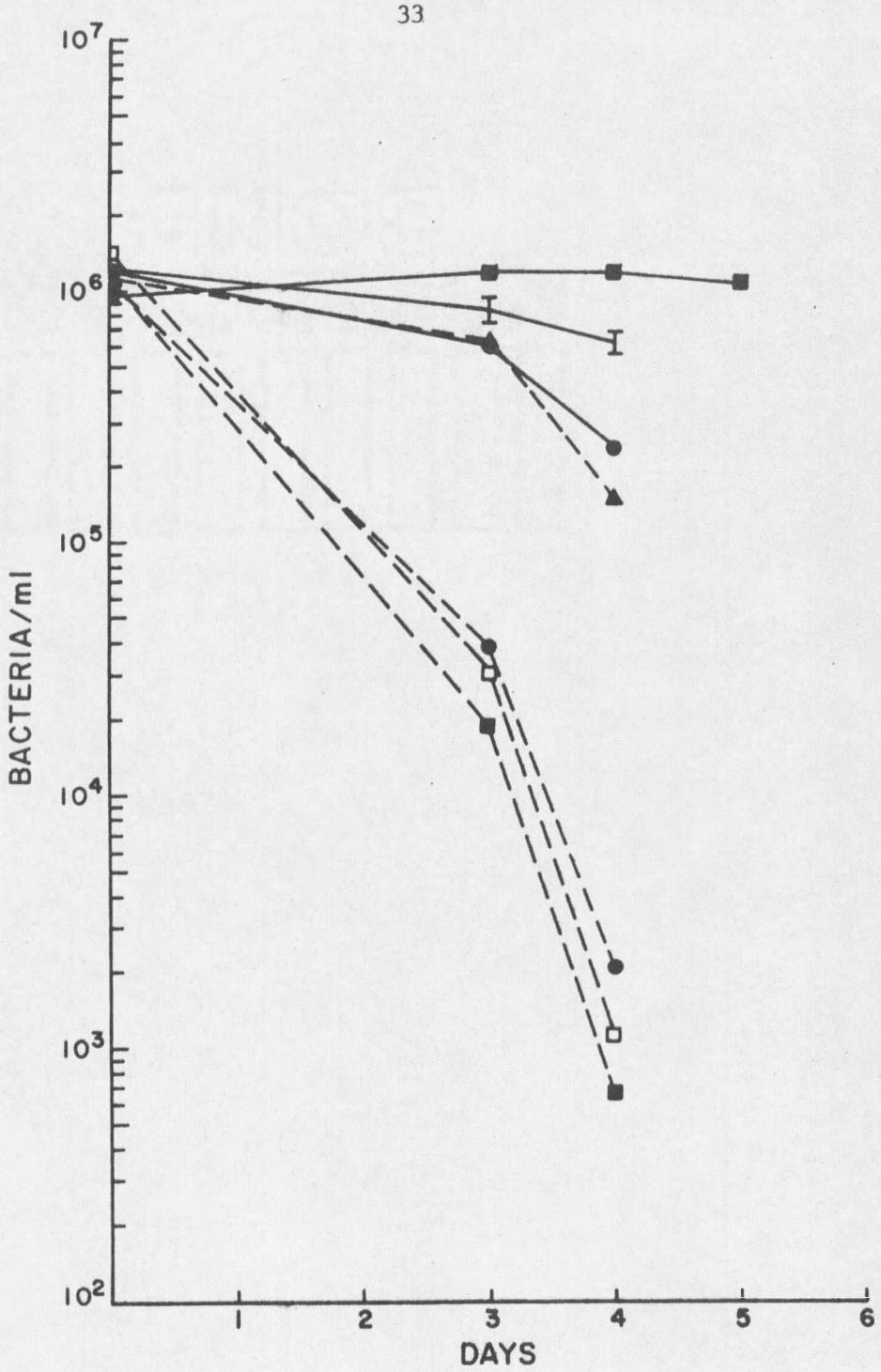

100
90
80
70
60
50
40
30
20
10
0
% INJURY
1
2
3
4
5
6
DAYS

three days. The temperature during the time of exposure ranged from 20-23.3 C and the pH of these waters were seen to vary from 5.4-7.8 with a pH of 8.2 in the Rocky Creek water. Conductivity, measured after the last sample was taken, was 330 µmhos/cm and 430 µmhos/cm in creek water and the Milli-Q waters ranged from 2.4-5.9 µmhos/cm.

Laboratory Injury Studies

Characterization of cells stressed in vessels of water was completed. Plate counts, fluorescent counts, percentage of spheroplasts formed during exposure to lysozyme, recovery or lag time, and cellular protein leakage were monitored as indices of injury. Fig. 7 shows that the fluorescent counts remained constant and on the fourth day of injury were actually 42% above the TSYA counts. The most injurious medium was the TSYA supplemented with 0.15% sodium desoxycholate, followed by DLA and MA supplemented with 0.15% sodium desoxycholate. These three media had similar counts throughout the span of exposure (Fig. 7). The percent injury as determined with these media varied from 96-98% on day three and all showed 100% injury by day four. Hydrogen peroxide (H_2O_2) and MA counts remained near the TSYA counts until the third day, after which they diverged, and injuries of 78% and 65% respectively resulted. Lag time, as determined by slide culture, percent injury, and the percentage of spheroplasts formed

Figure 7. Enumeration of E. coli stressed in vessels of reagent grade water. Stressed cells were enumerated using TSYA and H_2O_2 controls (—⊥—) fluorescent particle counts (—■—), MA counts (—●—), MA supplemented with 0.15% sodium desoxycholate (-●--), TSYA supplemented with 0.15% sodium desoxycholate (-■--), DLA (-□--), and H_2O_2 counts (-▲-).


10^7
10^6
10^5
10^4
10^3
10^2
BACTERIA/ml
1
2
3
4
5
6
DAYS

following lysozyme treatment increased in a linear manner with exposure time as shown in Fig. 8. As the injury, percent spheroplasts, and lag time increased the viability decreased to 12% (Fig. 8, Table 1). A plunge in the viability was noted during the initial three days of starvation stress. The temperature remained stable throughout the experimental period ranging from 22.5-24 C. The absorbance at 260/280 nm stayed relatively constant with a range of 1.06-1.20. The protein level determined by the Lowry procedure (39) was very low.

Salmonella typhimurium was also studied in the laboratory to determine if a pathogen reacted to aquatic stress in the same manner as observed in E. coli. The viability of these organisms decreased to a greater extent than did E. coli and after three days a one and a half log drop in viability was observed although the fluorescent counts remained high (Fig. 9). TSYA and MA both supplemented with 0.15% sodium desoxycholate were detrimental and resulted in injuries of 84% and 80%. DLA ranked third in this group of damaging media with a maximum injury of 72% on day three. MA and H_2O_2 showed impairing influences of damaged organisms and injuries of 42% and 56% respectively were noted. Lag time and percent injury increased in proportion to the time of aquatic exposure but the percentage of spheroplasts began at 40% and remained constant for the entire six days of exposure

Figure 8. Comparison of the percent injury, percent spheroplast formation, and percent viability versus the time of aquatic laboratory exposure obtained using E. coli. Lag vs exposure (-●-) % spheroplasts vs exposure (-▲-), % injury vs exposure (-■-), and % viability vs exposure (-●-) were calculated and graphed.

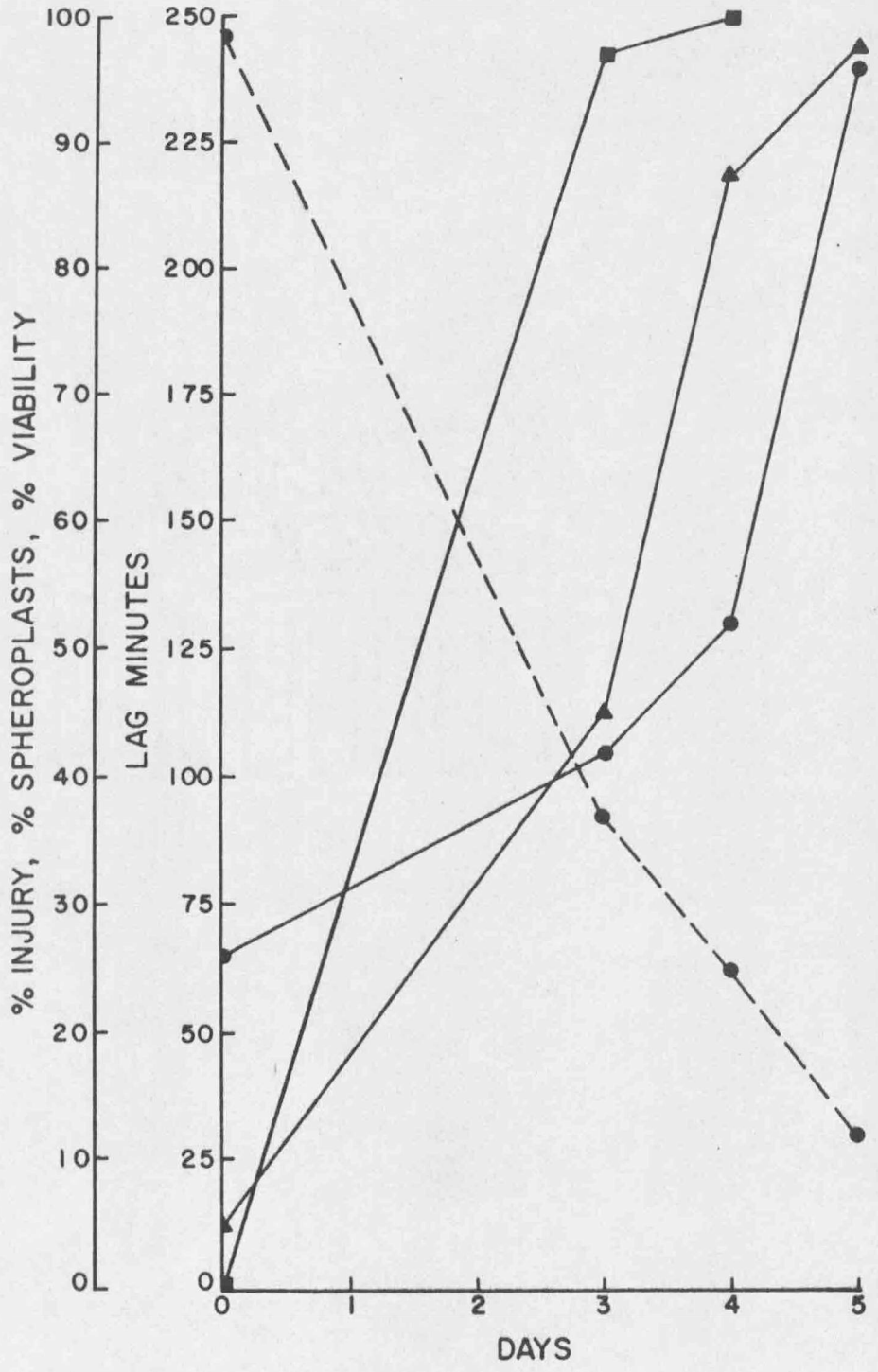
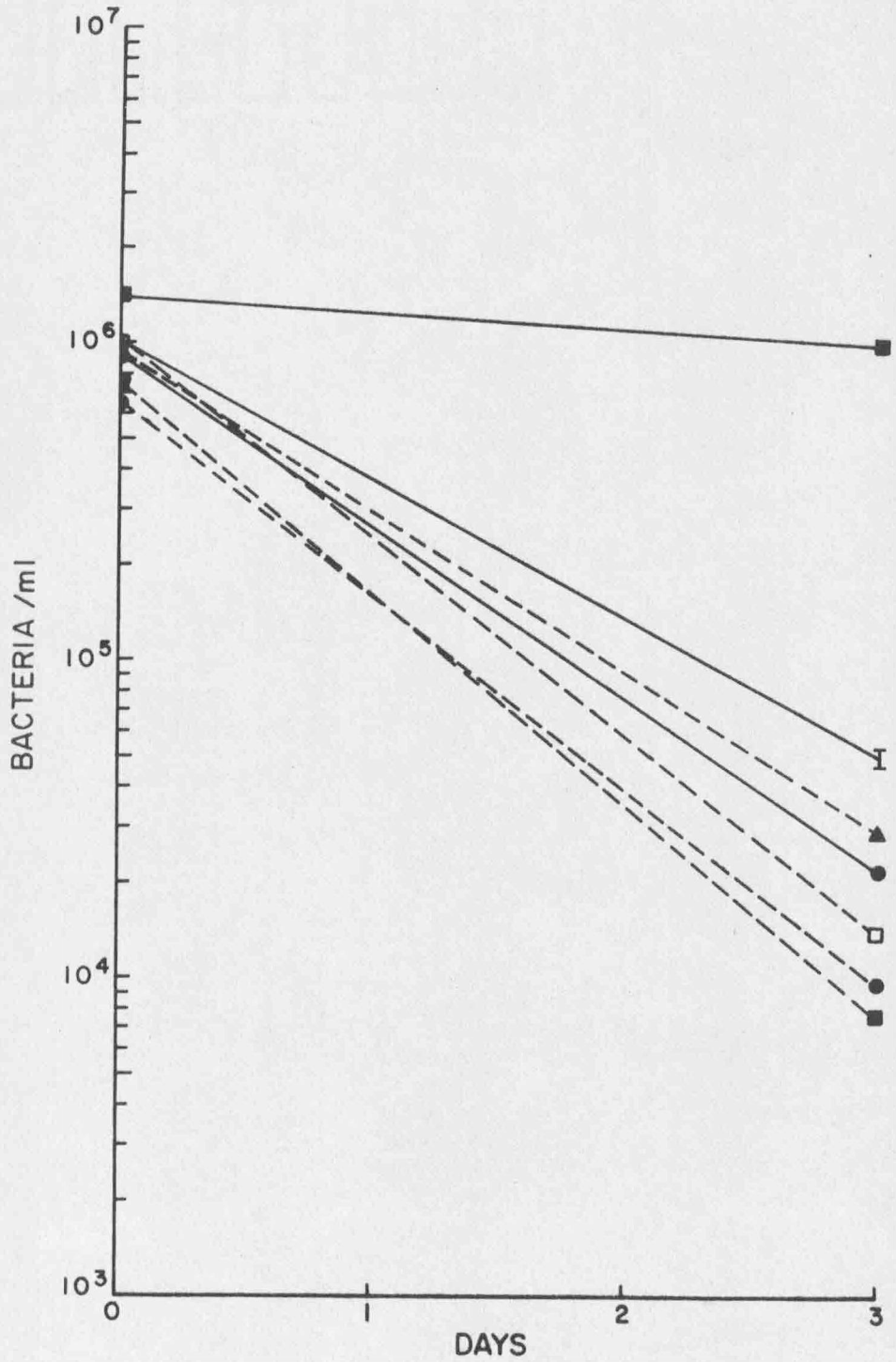

% INJURY, % SPHEROPLASTS, % VIABILITY
LAG MINUTES
DAYS
100
90
80
70
60
50
40
30
20
10
0
250
225
200
175
150
125
100
75
50
25
0
0
1
2
3
4
5

Table 1. Characteristics of aquatic laboratory stress using *E. coli*

Days of Exposure	Temperature (°C)	Absorbance of supernatant 260	280	260/280	Protein Concentration of supernatant (µg/ml)	Slide Culture Lag (minutes)	% Sphero-plasts	% Viability	% Injury
0	24	0.10	0.09	1.11	7.2	60-70	>5	98	0
3	23	0.165	0.155	1.06	5.2	100-110	40-50	37	97
4		0.075	0.063	1.19	1.2	125-135	85-90	25	100
5	22.5	0.138	0.115	1.20	12	230-250	95-99	12	

Figure 9. Enumeration of Salmonella typhimurium stressed in vessels of reagent grade water. Stressed cells were enumerated using TSYA and H_2O_2 controls (—┼—), fluorescent particle counts (—■—), MA counts (—●—), MA supplemented with 0.15% sodium desoxycholate (-●--), TSYA supplemented with 0.15% sodium desoxycholate (-■--), DLA counts (--□--), and H_2O_2 counts (-▲--).


10^7
10^6
10^5
10^4
10^3
BACTERIA /ml
0
1
2
3
DAYS

(Fig. 10, Table 2).

Electron Microscopy

Samples were prepared for electron microscopy when cells had reached 100% injury as determined by differential enumeration between TSY and DLA. Numerous blebs protruded from the outer membrane as seen in Figs. 11 and 12. Some of these blebs appear to have double membranes while some were recognized as small spheres. The interior of the cells looked quite normal and no unusual granules of cytoplasmic constrictions were noted. The plasma membrane seemed to be separated from the cell wall and the outer membrane. This phenomenon was also observed in undamaged control cells which were centrifuged and prepared for electron microscopy. Control cells showed no blebs and a continuous cell envelope.

Injury in Rocky Creek

Work similar to that done in the laboratory was performed with organisms that were placed in the chambers and immersed in Rocky Creek. TSYA supplemented with 0.15% sodium desoxycholate was again the most harmful agar in terms of injury and MA supplemented with 0.15% sodium desoxycholate and DLA followed close behind (Fig. 13). The injuries defined by these media were approximately 76% on day three and rose to

Figure 10. Comparison of the percent injury, percent spheroplast formation and percent viability versus the time of aquatic laboratory exposure obtained using Salmonella typhimurium. Lag vs exposure (—●—), % spheroplasts vs exposure (—▲—), % injury vs exposure (—■—), and % viability vs exposure (-●-) were calculated and graphed.

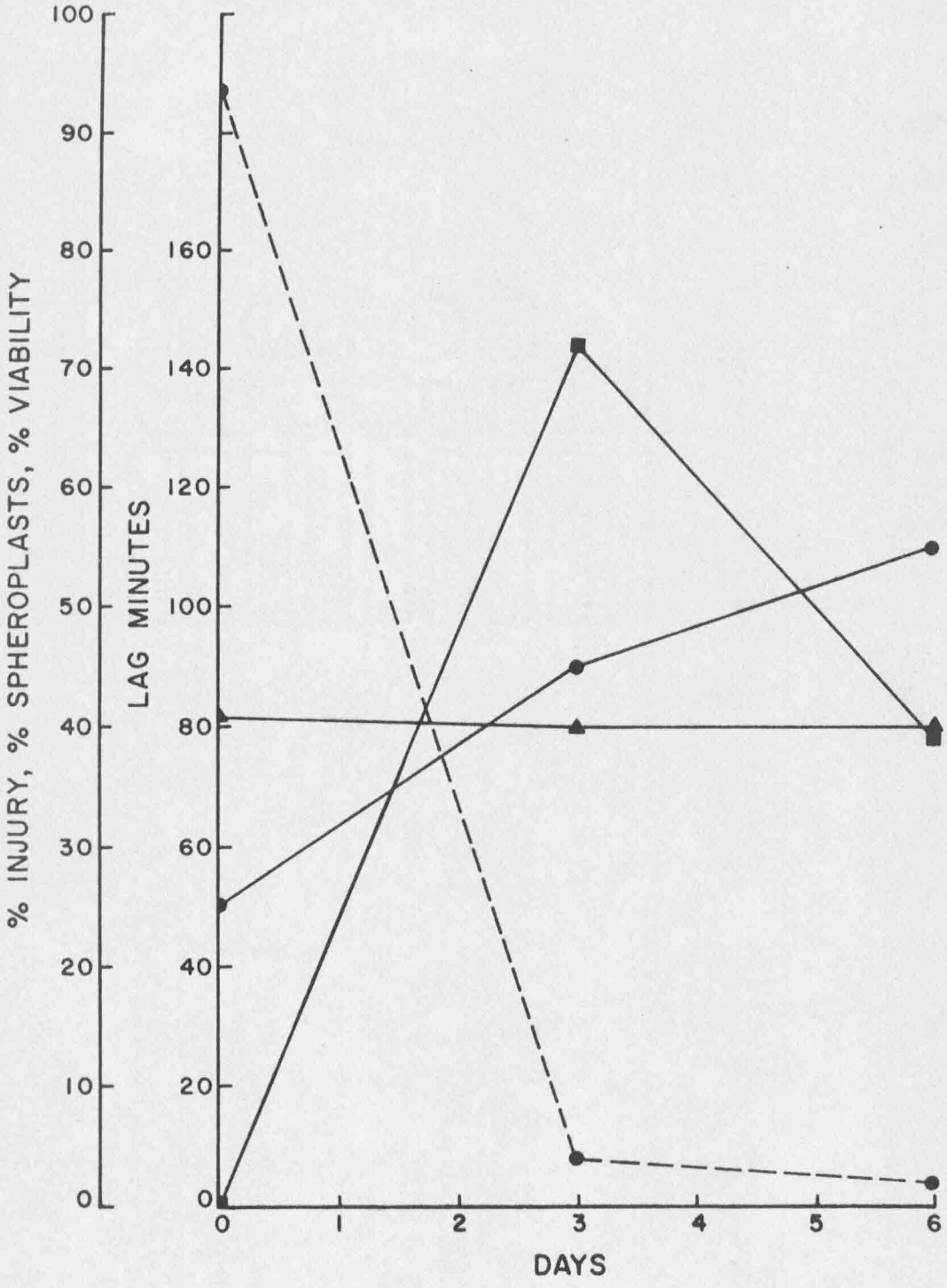

% INJURY, % SPHEROPLASTS, % VIABILITY
LAG MINUTES
DAYS
100
90
80
70
60
50
40
30
20
10
0
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6

Table 2. Characteristics of aquatic laboratory stress using S. typhimurium

Days of Exposure	Slide Culture lag (minutes)	% Spheroplasts	% Viability	% Injury
0	50	41	94	None
3	90	40	4	72
6	100	40	2	39

Figure 11. Transmission electron micrographs of 100% injured *E. coli* magnified 78,300X (bar = 0.4 μm).

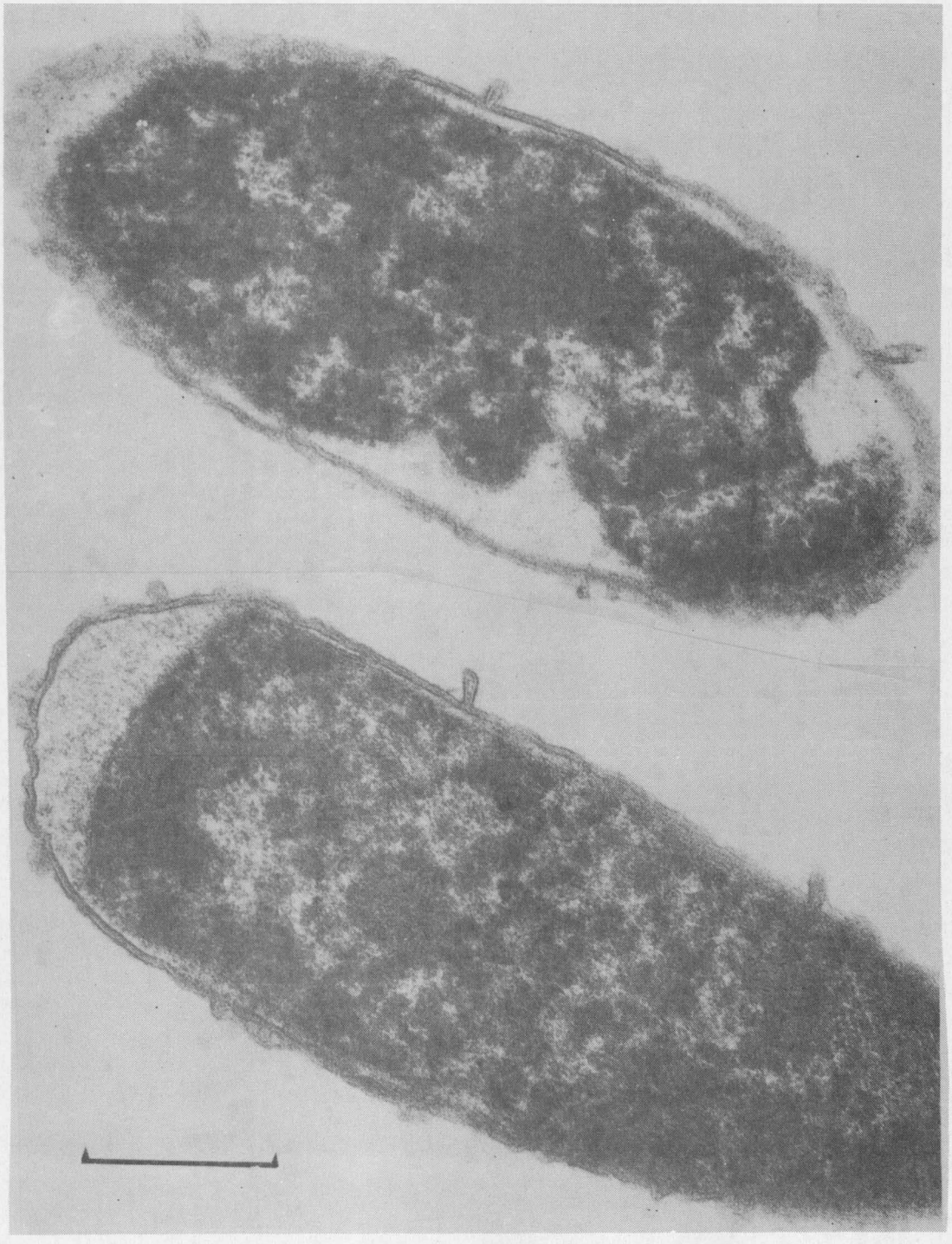


Figure 12. Transmission electron micrograph of 100% injured *E. coli* showing numerous blebs, magnified 43,500X (bar - 0.5 μm).

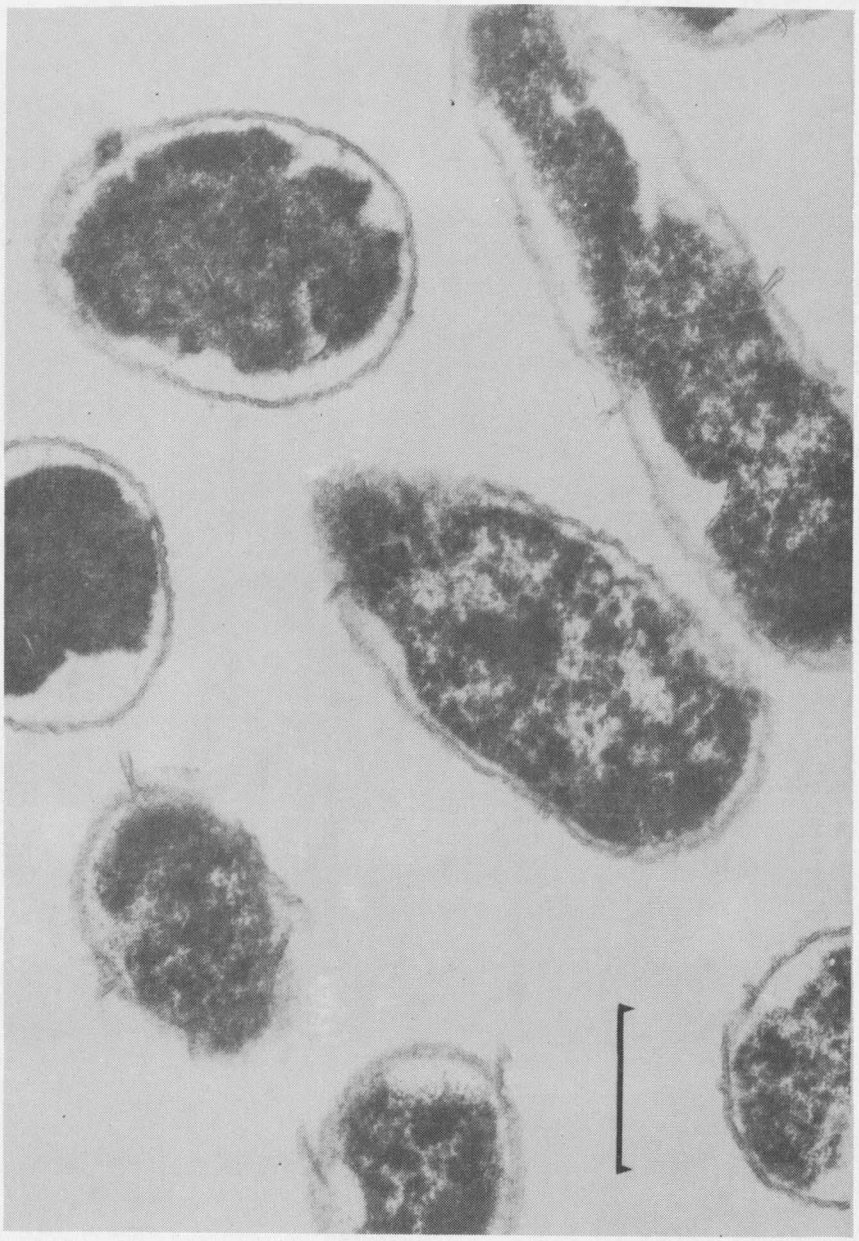
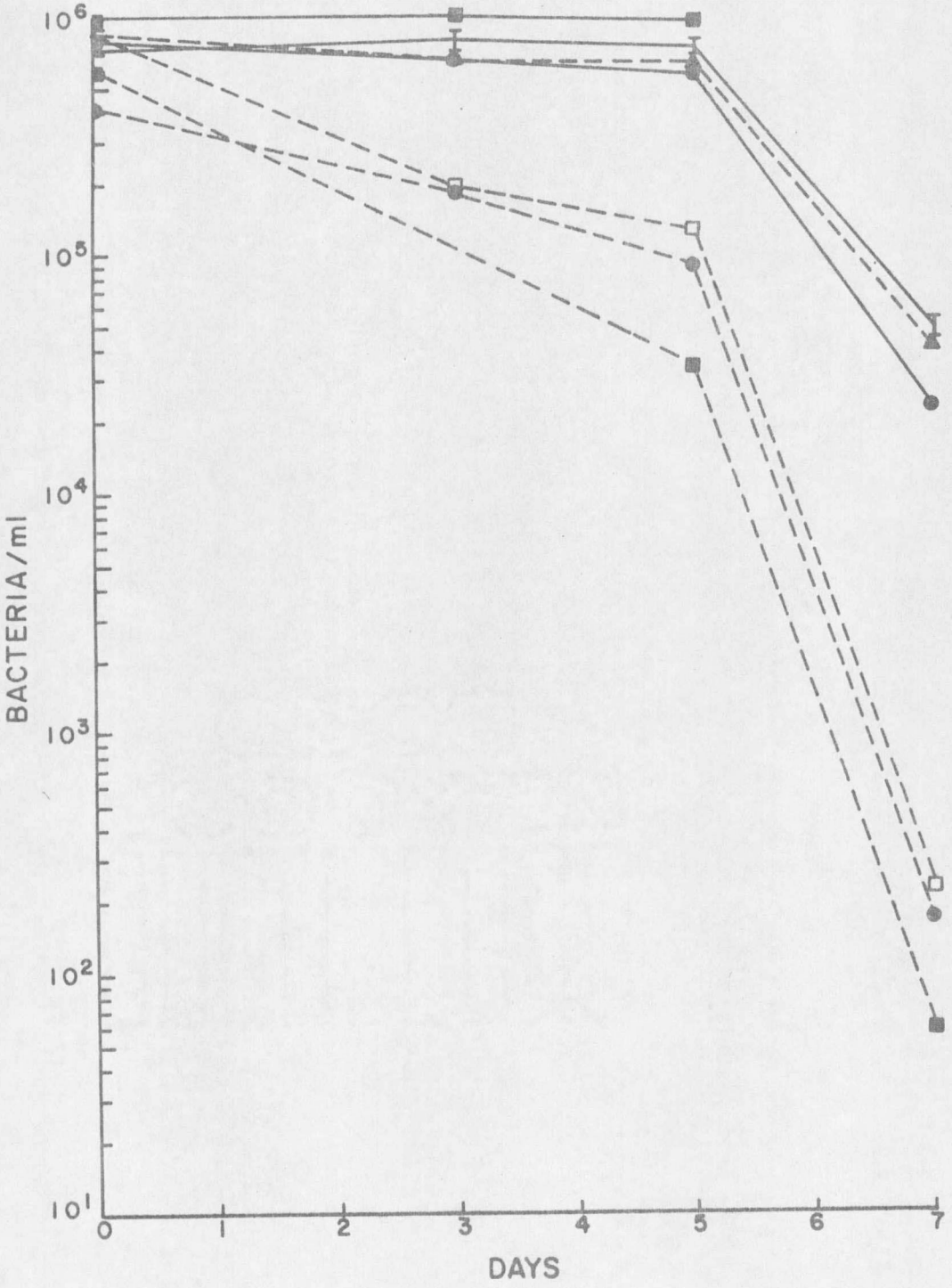


Figure 13. Enumeration of E. coli stressed in Rocky Creek. Stressed cells were enumerated using TSYA and H_2O_2 controls (—┼—), fluorescent particle counts (—■—), MA counts (—●—), MA supplemented with 0.15% sodium desoxycholate (-●--), TSYA supplemented with 0.15% sodium desoxycholate (-■--), DLA counts (-□--), and H_2O_2 counts (-▲-).


BACTERIA/ml
10^6
10^5
10^4
10^3
10^2
10^1
0
1
2
3
4
5
6
7
DAYS

100% by the seventh day. Minimal agar counts showed very little difference in comparison with TSYA counts until the seventh day when 58% injury was observed. Hydrogen peroxide showed little effect on these organisms throughout the injury period and only a 25% injury was demonstrated in this manner. The microscopic cell counts remained approximately 25% above the TSYA counts through the fifth day of exposure. In this case, as in the laboratory stressed cells, the percentage of spheroplasts increased as the injury and lag time increased (Fig. 14) but the viability remained quite high and there were 82% viable cells on the last day of sampling (Table 3). The temperature continued to be low because of cold weather during the experimental period and varied from about 4-10 C each day due to diurnal fluctuations.

Heavy snowfall followed by a warming trent in the weather occurred just before the next series of field experiments, as a consequence, the creek received a large amount of runoff from the surrounding area and the water appeared murky. The experimental results were much the same during this series of experiments as in the previous trials including MA, MA + 0.15% sodium desoxycholate, TSYA + 0.15% sodium desoxycholate and DLA counts as seen in Fig. 15. The level of debilitation, as determined with MA, was 53% and with the other media it ranged from 96-100% by the third day of the experimental period. The

Figure 14. Comparison of the percent injury, percent spheroplast formation, and percent viability versus the time of exposure for E. coli immersed in Rocky Creek. Lag vs exposure (—●—), % spheroplasts vs exposure (—▲—), % injury vs exposure (—■—), and % viability vs exposure (-●--) were calculated and graphed.

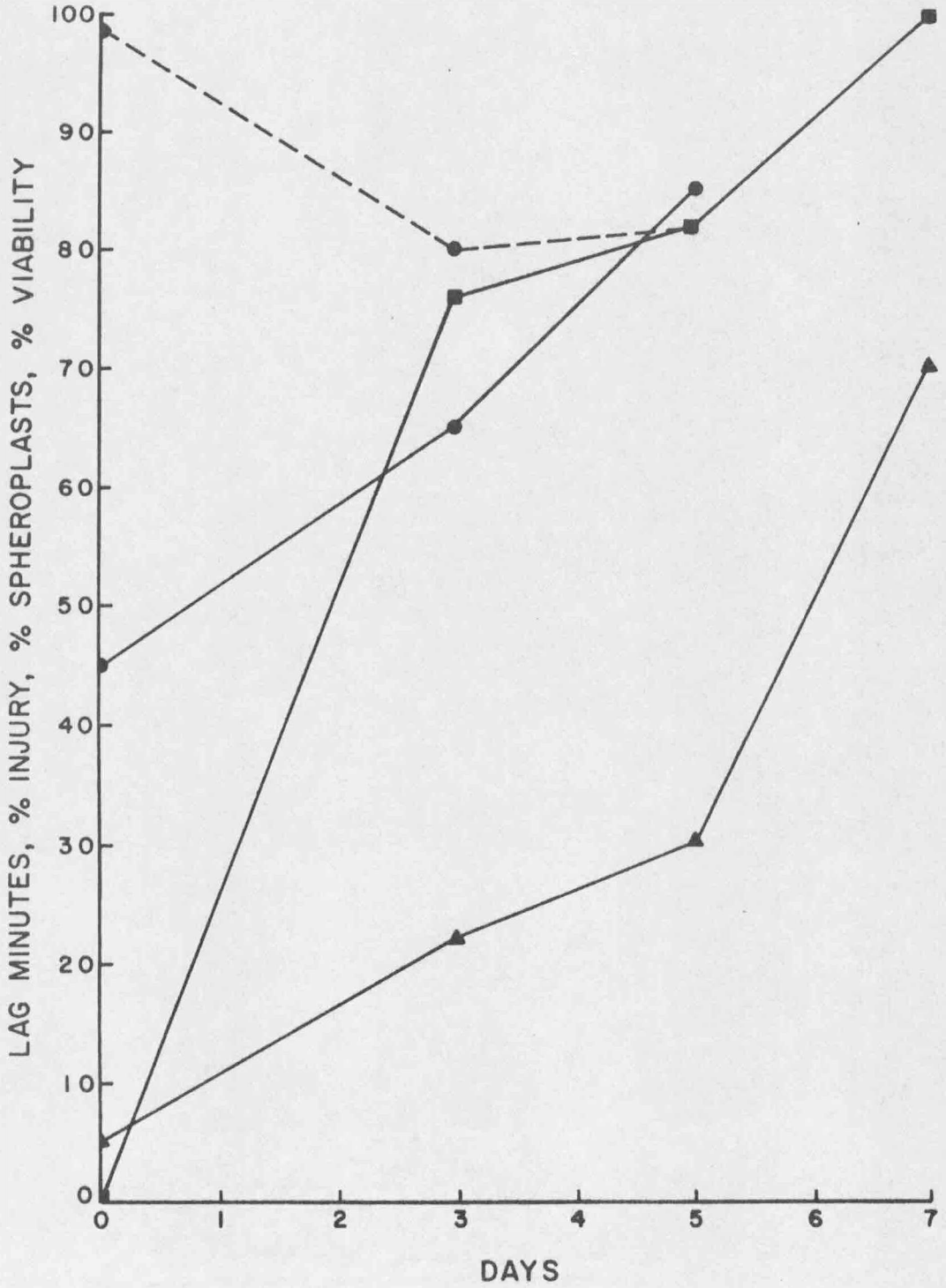
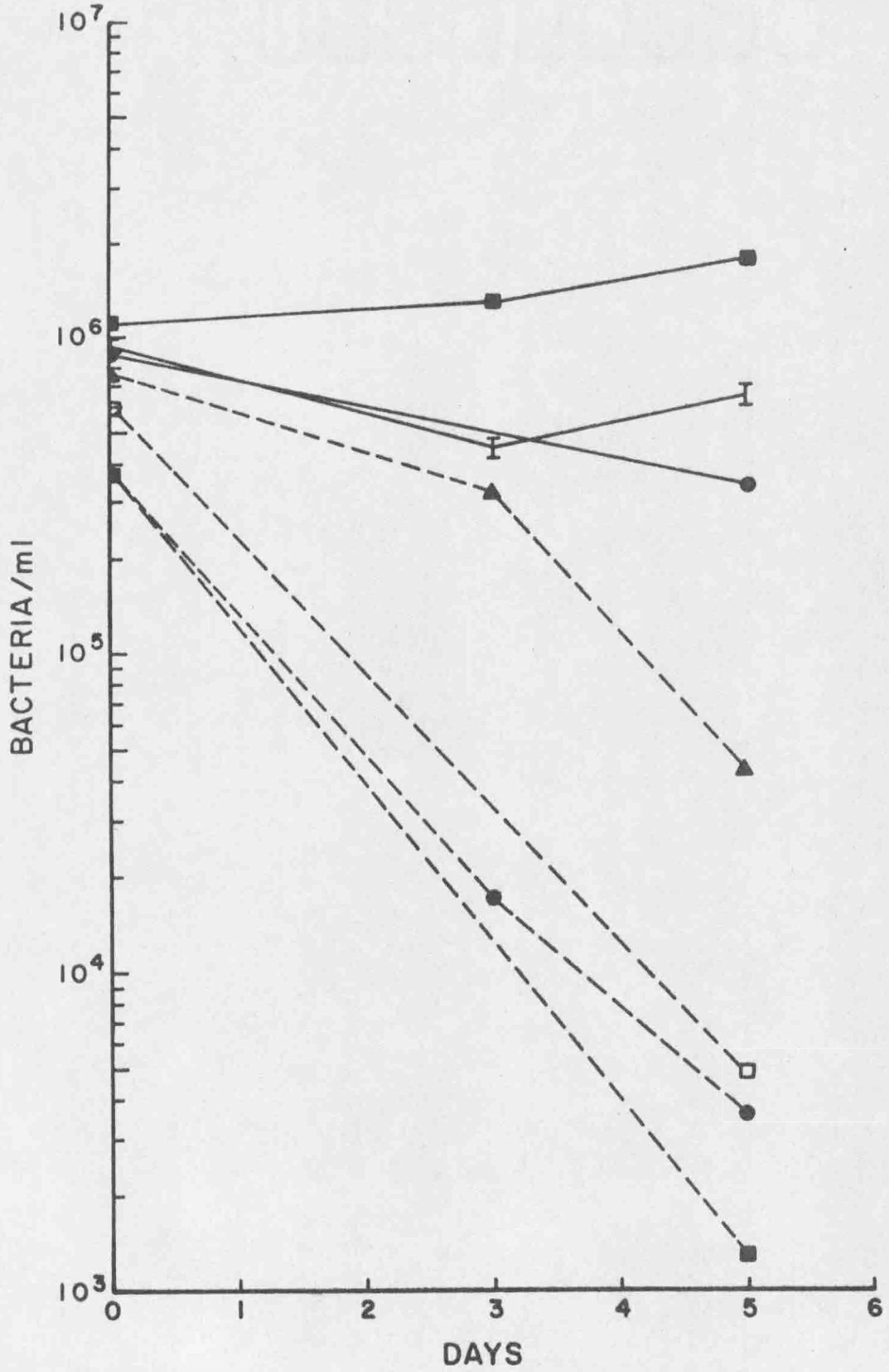

LAG MINUTES, % INJURY, % SPHEROPLASTS, % VIABILITY
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
DAYS

Table 3. Characteristics of aquatic stress in Rocky Creek using *E. coli*

Days of Exposure	Temperature (°C)	Slide Culture Lag (minutes)	% Spheroplasts	% Viability	% Injury
0	8-10	45	<5	99	0
3	5-10	65	21.9	80	76
5	6-10	85	28.8	82	82
7	6-10		70		100

Figure 15. Enumeration of E. coli stressed in Rocky Creek during a period of high runoff. Stressed cells were enumerated using TSYA and H_2O_2 controls (—┼—), fluorescent counts (—■—), MA counts (—●—), MA supplemented with 0.15% sodium desoxycholate (-●-), TSYA supplemented with 0.15% sodium desoxycholate (-■-), DLA counts (-□-), and H_2O_2 counts (-▲-).


10^7
10^6
10^5
10^4
10^3
BACTERIA/ml
0
1
2
3
4
5
6
DAYS

lag time increased with injury and the viability decreased sharply during the first few days of aquatic exposure (Fig. 16, Table 4). An interesting difference between this and prior experiments was the effect of hydrogen peroxide on injury. After the third day of exposure in water a much greater difference between TSYA counts and the H_2O_2 treated cell counts were found (Fig. 15). A 94% injury was observed after three days of exposure and the fluorescent particle counts which were initially comparable to the TSYA counts increased to 60% and 64% above the TSYA counts by day 5.

Oxygen Uptake

It was of interest to determine if oxygen or its radicals had a detrimental effect on injured cells and if this influence could be seen by plate counts, especially since it has been reported that anaerobic conditions enhance the growth of cell wall defective E. coli (27). Cells plated aerobically and anaerobically both showed 99.4% injury.

Oxygen uptake rates were determined with a non-injured healthy cell population of 1.0×10^9 bacteria/ml and can be seen in Fig. 17. A greater lag in oxygen uptake was seen when TSY + 0.15% sodium desoxycholate was used as the suspending medium.

Cells with 93.8% injury and a population of 7.9×10^8 bacteria/ml

Figure 16. Comparison of the percent injury, percent spheroplast formation and percent viability versus the time of exposure for E. coli immersed in Rocky Creek during a period of high runoff. Lag vs exposure (-●-), % spheroplasts vs exposure (-▲-), % injury vs exposure (-■-), and % viability vs exposure (-●-) were calculated and graphed for E. coli.

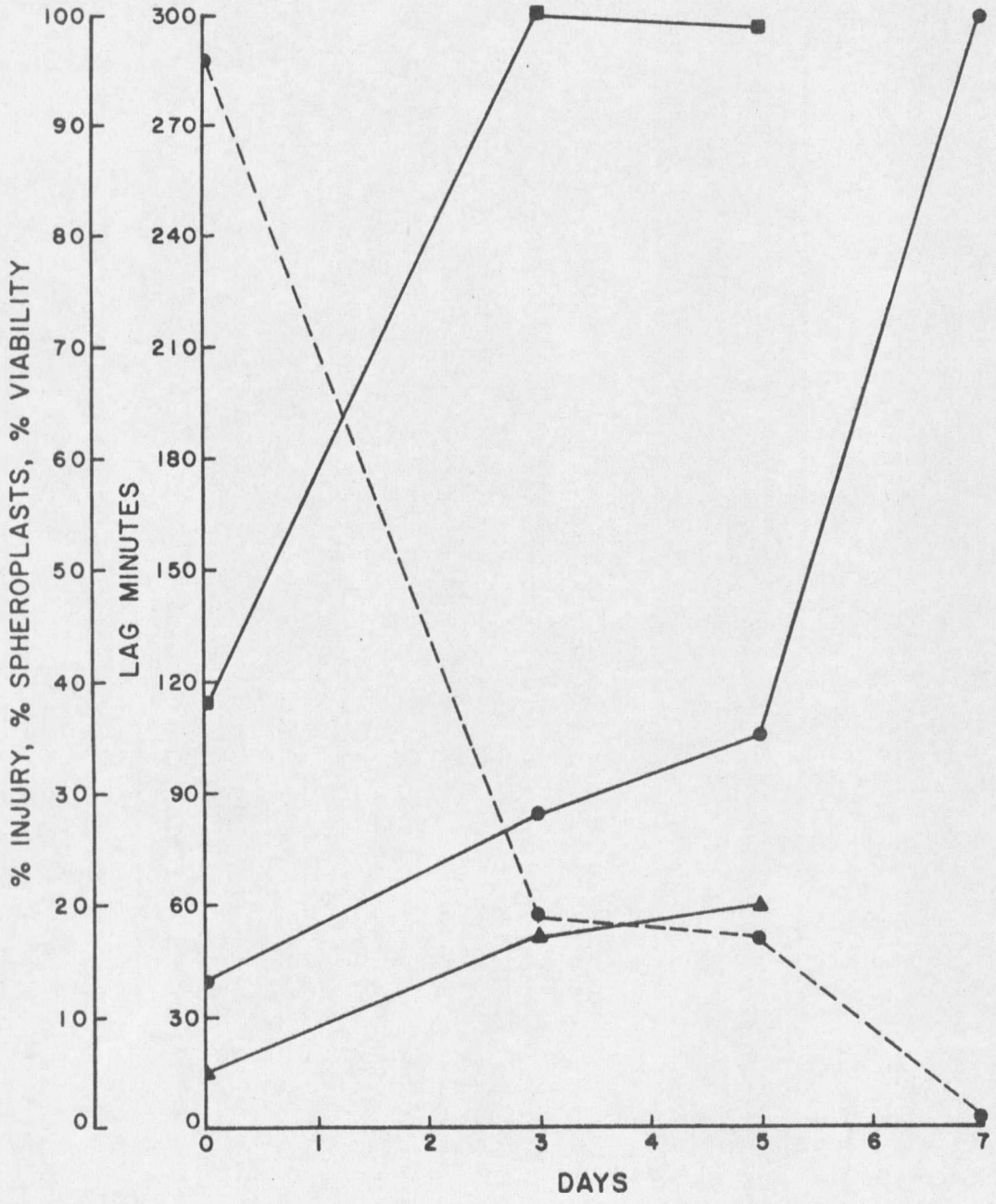
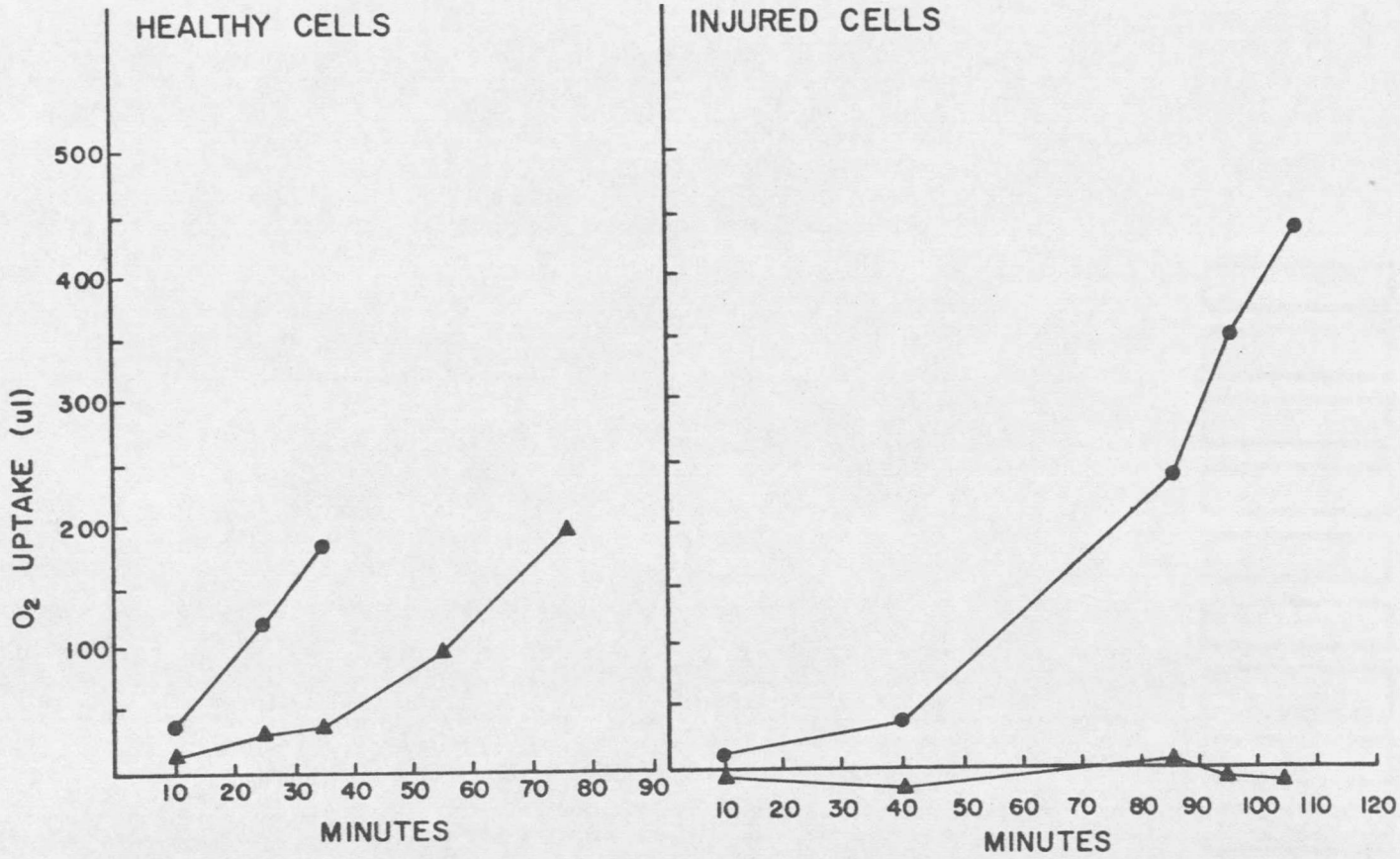

% INJURY, % SPHEROPLASTS, % VIABILITY
LAG MINUTES
DAYS
100
90
80
70
60
50
40
30
20
10
0
300
270
240
210
180
150
120
90
60
30
0
0
1
2
3
4
5
6
7

Table 4. Characteristics of aquatic stress in Rocky Creek during a period of high runoff using E. coli.

Days of Exposure	Temperature (°C)	Slide Culture Lag (minutes)	% Spheroplasts	% Viability	% Injury
0	7 - 11.5	40	<5	96	38
3	9 - 13	85	17	19	100
5		105	19.3	17	99
7		300		1	

Figure 17. Oxygen uptake rates in injured and healthy *E. coli*.


HEALTHY CELLS
INJURED CELLS
O_2 UPTAKE (ul)
500
400
300
200
100
10 20 30 40 50 60 70 80 90
MINUTES
10 20 30 40 50 60 70 80 90 100 110 120
MINUTES

can also be seen on Fig. 17. A greater lag in oxygen uptake was seen when damaged cells were placed in TSY broth. These same damaged cells showed no oxygen uptake at all during 18 hours of incubation when placed in TSY broth + 0.15% sodium desoxycholate.

Effect of Diluent and Media on the Evaluation of Injury

Studies were conducted to identify the effect of various diluents on the assessment of injury. Samples damaged with reagent grade water in the laboratory were placed in these diluents for a total time of 15 minutes and plated. The results are presented on Fig. 18 and Table 5. Injury was greatest when 1% $MgSO_4 \cdot 7H_2O$ was used as the diluent. Damage was first observed on the third day of exposure and increased to 97% on day six. Results with standard PO_4 buffer and Milli-Q water were much the same and the damage obtained was not as dramatic but reacted 94% by day six. Peptone (1%) and 1% non-fat dry milk were the best diluents in terms of the least injury obtained. The debilitation seen when peptone buffer was used began on day four and reached 63% by day six. The non-fat dry milk exhibited very little injury until the sixth day of the experimental period. On that day the injury was not excessive and reached only 37%. The pH of the diluents ranged from approximately 6.0-7.3.

Experiments using TSYA and DLA plus additives were completed to

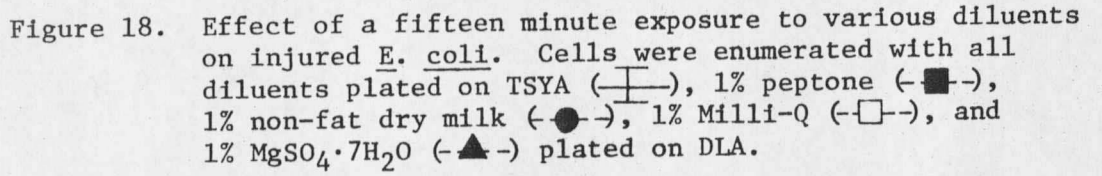


Figure 18. Effect of a fifteen minute exposure to various diluents on injured E. coli. Cells were enumerated with all diluents plated on TSYA (-┼-), 1% peptone (-■-), 1% non-fat dry milk (-●-), 1% Milli-Q (-□-), and 1% $MgSO_4 \cdot 7H_2O$ (-▲-) plated on DLA.

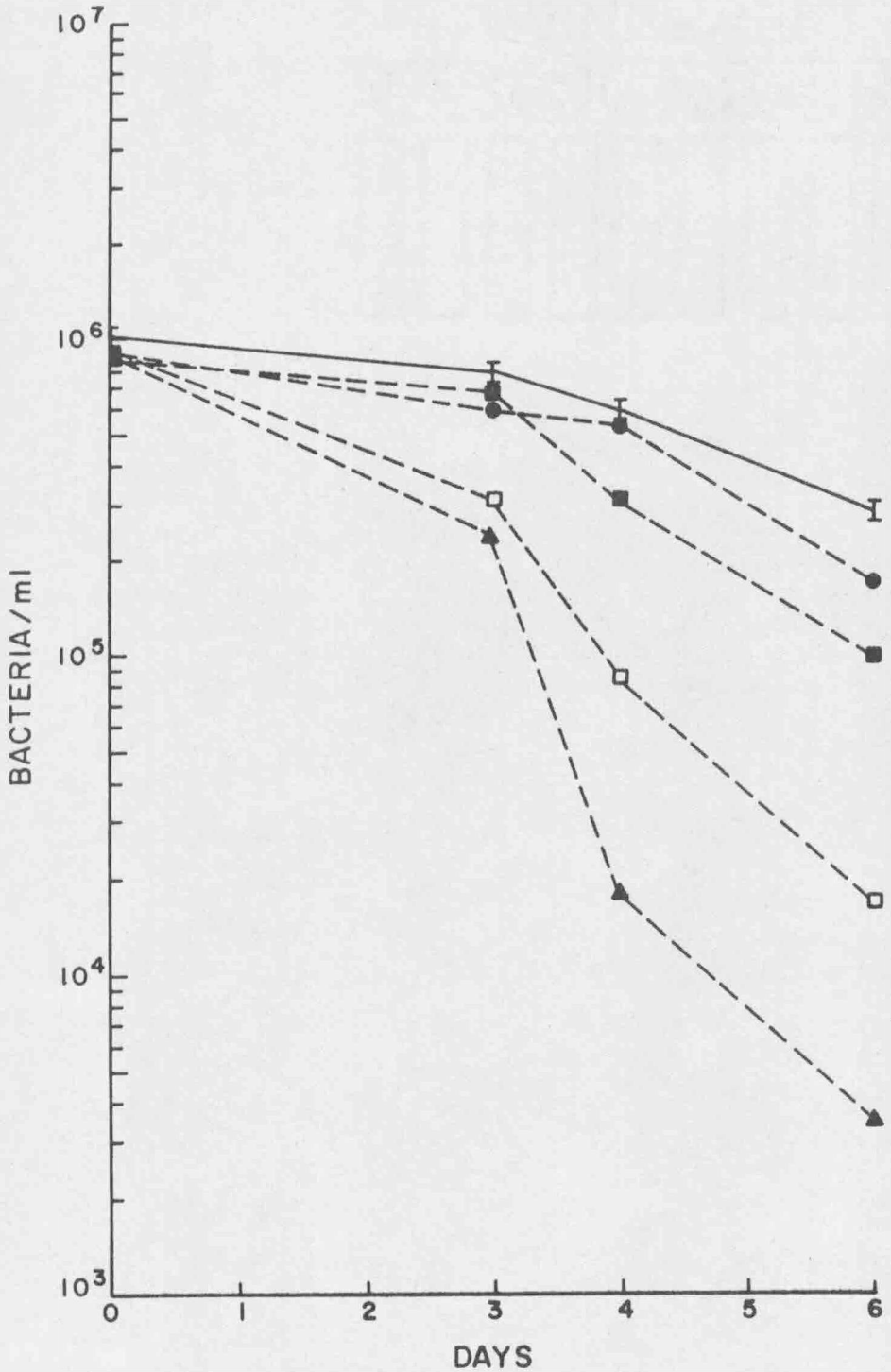

10^7
10^6
10^5
10^4
10^3
BACTERIA / ml
0
1
2
3
4
5
6
DAYS

Table 5. The effect of non-fat dry milk, peptone, Milli-Q (reagent grade) water and $MgSO_4 \cdot 7H_2O$ diluents on injury.

Days of Exposure	Diluent	pH	% Injury
0	1% peptone	7.1	7
	1% non-fat dry milk	6.9	18
	Milli-Q water	6.1	15
	1% $MgSO_4 \cdot 7H_2O$	5.9	14
3	1% peptone	7.1	13
	1% non-fat dry milk	7.0	18
	Milli-Q water	7.3	63
	1% $MgSO_4 \cdot 7H_2O$	6.0	68
4	1% peptone		45
	1% non-fat dry milk		0
	Milli-Q water		87
	1% $MgSO_4 \cdot 7H_2O$		97
6	1% peptone		63
	1% non-fat dry milk		37
	Milli-Q water		94
	1% $MgSO_4 \cdot 7H_2O$		99

determine their effect on injured cells and their influence on recovery. The effects of a DLA overlay on TSY plate counts and catalase added to TSY and DLA can be seen on Fig. 19. The percent injury reached its highest point on day four when 76% injury was noted on DLA. Injuries of 16%, 35%, and 52% were found on day four with TSYA + catalase, TSYA + lysozyme, and DLA + catalase respectively. The counts from TSYA, which were overlayed with DLA remained as high as the TSYA counts throughout the experimental period.

Recovery

The time frame for recovery of damaged cells with 86% injury was followed and presented graphically on Fig. 20. The recovery period ended when the counts on DLA and TSYA were equal. This occurred between 2 and 2 3/4 hours after the initial incubation of damaged cells in TSY broth, TSY broth supplemented with 0.2% pantoyl lactone and 0.2% sodium citrate.

Figure 19. Enumeration of *E. coli* using catalase, lysozyme, and DLA overlays to affect injury. Cells were enumerated using TSYA (—●—), DLA (--□--), TSYA + DLA overlay (—▲—), DLA + catalase (—■—), TSYA + lysozyme (--●--), and TSYA + catalase (—◆—).

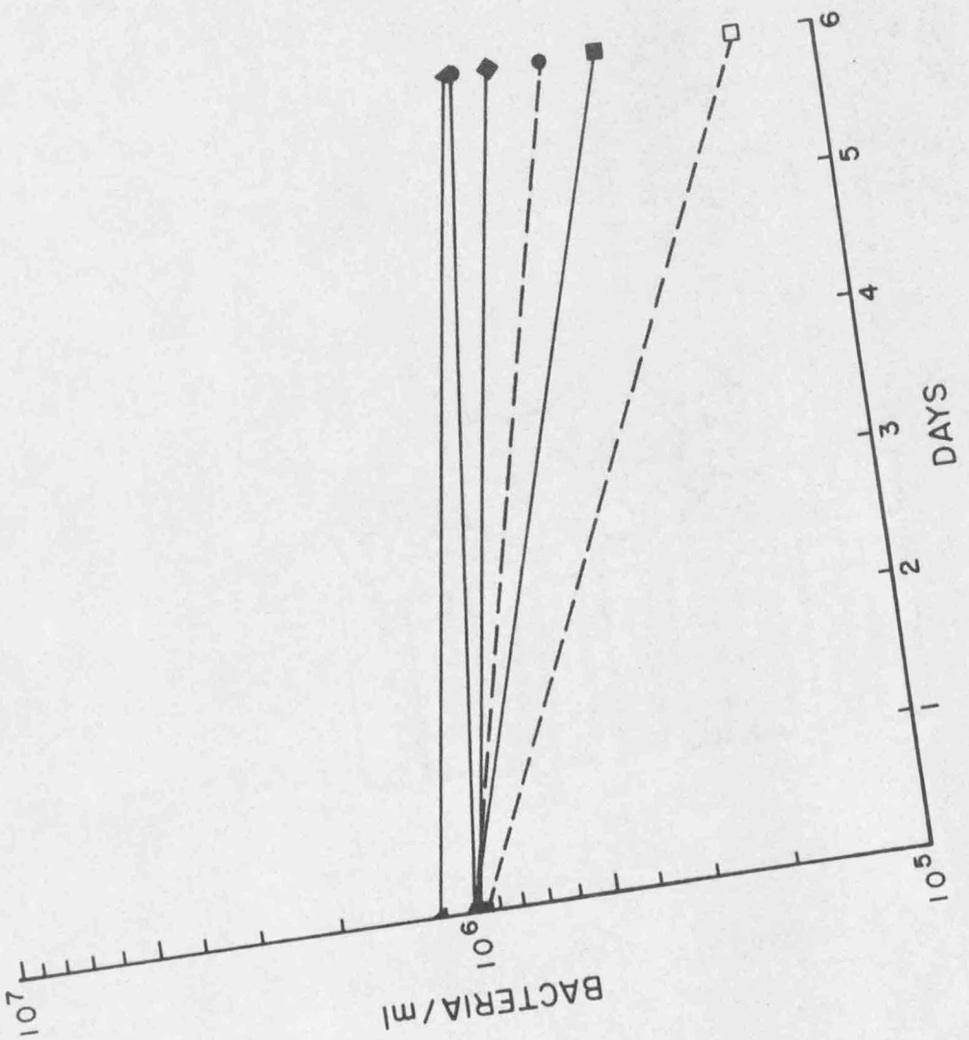
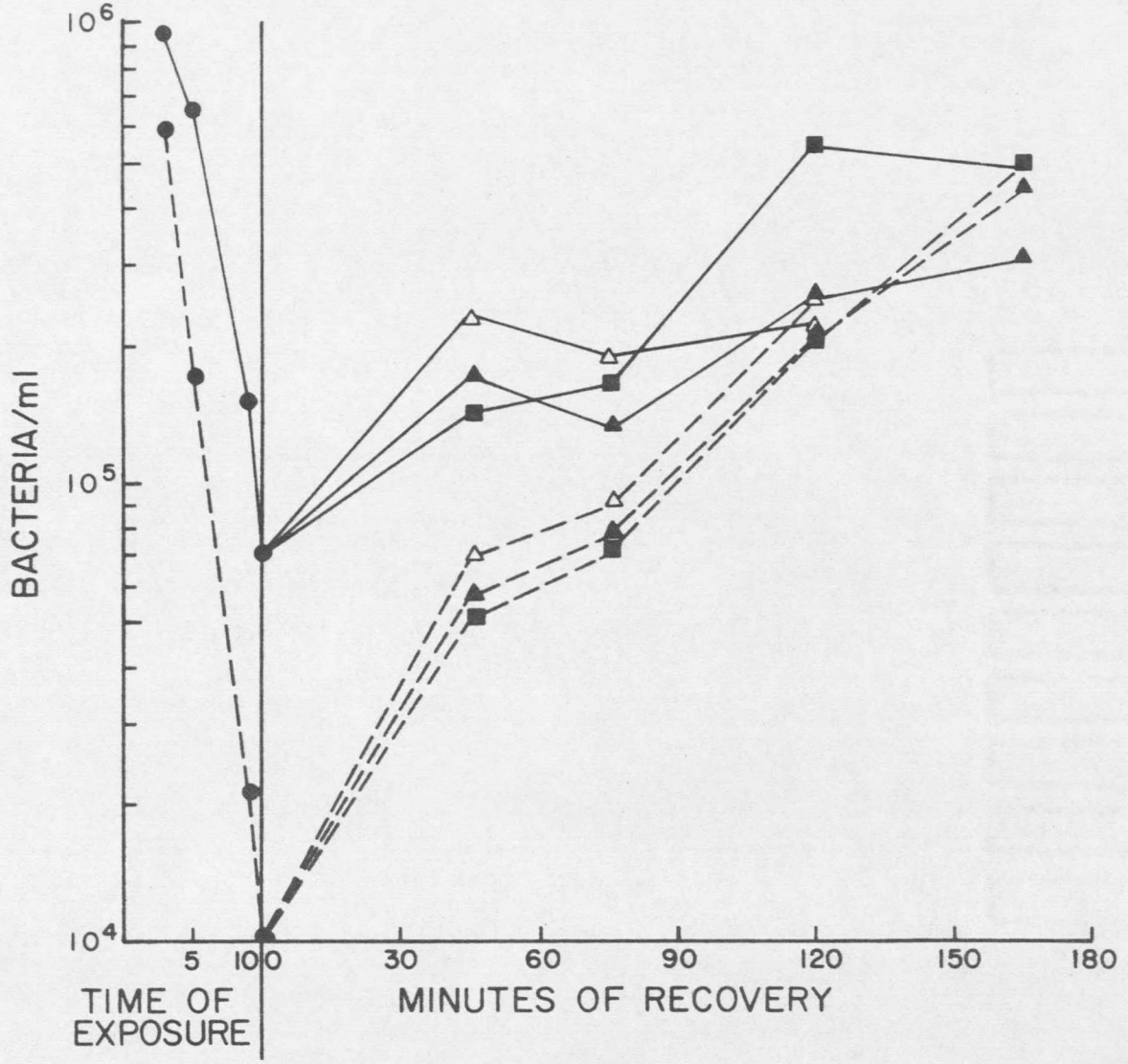

BACTERIA / ml
10^7
10^6
10^5
DAYS
1
2
3
4
5
6

Figure 20. Recovery of injured E. coli placed in TSY broth (■), TSY broth supplemented with an additional 0.2% sodium citrate (▲), and TSY broth supplemented with 0.2% pantoyl lactone (△) plated on TSYA (——) and DLA (---).


BACTERIA/ml
10^6
10^5
10^4
5
10
0
30
60
90
120
150
180
TIME OF EXPOSURE
MINUTES OF RECOVERY

DISCUSSION

Numerous studies have been reported which demonstrated degradation of RNA in cells undergoing carbon starvation (13,15,35,36). Dawes and Ribbons (13) have found immediate decomposition of RNA with an accompanying oxidation of ribose and the release of UV absorbing material. Other researchers (15) have discovered that rapid degradation of polyribosomes to single 70S ribosomes, as well as the loss of mRNA, contributed to a decreased capacity for protein synthesis. The most active adaptation was found to take place in the first 24 hours of nutrient starvation by Scherer and Boylen (57) who studied Arthrobacter. By the end of 28 days 75% of the RNA in the cell remained, and Arthrobacter was capable of maintaining a high energy level which presumably lengthened its activity and viability.

Kaplan and Apirion (35) have examined cellular RNA and believed its decay was dependent on the nuclease content within the cell, the cell's genotype in terms of ribonuclease activity, and the stress applied to those particular cells. Endonucleolytic attack in the degradation of RNA appeared to be the rate limiting step in the breakdown process. The attack was apparently due to the conformation of ribosomal subunits which became particularly susceptible to the nuclease. Ribosomal subunits broke apart and were decomposed to nucleotides by the exonuclease, RNase II, and the polynucleotide phosphorylase, PNPase. They have discovered the greatest RNA degradation

in strains which contained a periplasmic enzyme, RNase I, and suggest this enzyme entered the cell following membrane damage (36). Obinata and Mizuno (49) have also identified nucleases situated near the cell envelope that were released upon cell surface damage. Most of the decomposition occurred within organisms during the starvation for carbon and least in phosphate deprivation. Their viability studies have implied a correlation between the ability of cells to recover from starvation and their efficiency in decomposing RNA. Organisms which degraded RNA rapidly survived better during starvation stress than those which degraded the nucleic acid more slowly.

Scherer and Boylen (57) have observed a relatively stable protein concentration in Arthrobacter which underwent starvation stress for one day. A slow continuous proteolysis began after this period and in 28 days of starvation this organism lost 20-30% of its total protein composition. In E. coli a slow continuous degrading group of proteins was also identified by Nath and Koch (44,45) along with a limited amount of rapidly degrading protein whose synthesis increased as growth rate decreased. Up to 40% of the cellular protein was degraded during the first day of starvation. Intracellular proteolysis in E. coli is postulated by Pine (51) to be primarily regulated by active ribosomal function. Ammonium (NH_4^+) appeared to be the most effective regulator of this sytem and its depletion progressively

increased the basal proteolytic rate to a maximum when the doubling time was two hours. Although individual amino acids inhibited protein degradation when they were present or stimulated it when they were absent, proteolysis increased by a factor of two or less during starvation. Fan and Rodwell (17) studied Pseudomonas putida and discovered a type of proteolysis that required a divalent metal ion.

Transport and dehydrogenase activities remained unchanged in starved P. putida, but the oxidase, decarboxylase and hydrolase activities decreased (17). The activity loss in inducible enzymes and the decomposition of bulk protein seemed to be heterogeneous, although protein labeled during starvation was degraded at a faster rate than that labeled during log phase growth. Daubner (12) reported that oxygen consumption and dehydrogenase activity immediately decreased when E. coli were placed in an aquatic environment.

Studies conducted in our lab showed little protein leakage during aquatic injury. Because it was necessary to use a low cell density to achieve an acceptible level of cellular damage, flash evaporation was used to concentrate the cellular supernatant. The protein present in the supernatant may have been too limited in quantity and thus below the sensitivity limit of the Lowry technique (39) in determining the amount actually present. The A260/A280 ratio did not appreciably change throughout the experimental period indicating minimal protein

leakage (Table 1).

Intracellular polyglucose compounds (e.g. glycogen and poly-B-hydroxybutyrate) that are similar to animal glycogen could provide energy reserves for organisms in an unfavorable environment (64). Those cells rich in carbohydrate should survive better than cells with little reserve material, and it is believed that only microbes with comparably large amount of endogenous carbohydrate have long term survival characteristics. Dawes and Ribbons (13) found glycogen was rapidly utilized during starvation, and ammonia was released only after a lag period that paralleled the time needed for cellular glycogen to be nearly utilized. Proteolysis continued at a linear rate throughout glycogen consumption. They concluded that the presence of glycogen prevented net decomposition of nitrogenous material but did not hinder protein turnover. There was no significant lipid utilization during this starvation period and no loss of viability in 12 hours under aerobic or anaerobic conditions. A more rapid death rate appeared after 12 hours.

Although the E. coli studied in our lab did not undergo a drastic reduction in size like Ant 300, a psychrophillic marine vibrio (48), a decrease in volume during the initial few days of starvation was observed. This may have been due to consumption of some endogenous reserve materials. Daubner (12) found cell shrinkage in E. coli after

15-20 hours when the cells were placed in an aquatic environment. After 1-4 weeks the cell capsule and cytoplasm became reduced. In the electron micrographs processed in the present study, no cytoplasmic alterations were observed in E. coli which showed 100% injury. E.coli which are normally resistant to surface active agents and lysozyme become sensitive to these chemicals when their cell walls or surface structures are damaged or disorganized (7,38,68). LPS deficient mutants are extremely sensitive to lysozyme, and phosphate or glucose residues present in the polysaccharide portion of LPS were closely correlated with the sensitivity of cells to lysozyme (68). It was suggested (55,68) that part of the LPS on the cell surface forms a structure which make cells resistant to penetration by lysozyme as well as to low molecular weight antibiotics and surface active agents. Strains with reduced amounts of phosphate and glucose in LPS residues were more sensitive to lysozyme. Knox (38) reported that after treatment with lysozyme, these damaged cells could be identified by an excessive amount of extracellular material in the form of spheres and globules. Mutants defective in the structure of murein lipoprotein have also been characterized by numerous blebs (77). Circular regions of low electron density were also noticed along with abnormal permeability properties.

An autotroph which had been depleted of leucine for two hours (42)

was also burst by 10 µg/ml of lysozyme which suggested surface defects. Masses of stacked leaflets and globules, which may have been lipoplysaccharide (LPS), surrounded the organisms. Outer membrane blebs, which were sometimes still connected with that membrane, were distinguished as well as large aggregates and fiberous material within the cells. Normal appearance, which included a fiberous nuclear region and a multilayered cell wall recurred after addition of leucine.

The E. coli used in the present study were not sensitive to lysozyme in the initial stages of each experiment then healthy cells with a high eprcentage of viability were used (Figs. 8,14,16, Tables 1,3,4). As the time of aquatic exposure and injury progressed these cells became increasingly sensitive to lysozyme and a greater percentage of spheroplasts was observed. Cell wall damage or its disorganization became increasingly apparent as time of exposure continued and the possibility of LPS deficiency due to the release of this component from the cell exists. This could result in the formation of spheroplasts when such cells are treated with lysozyme.

Membrane blebs protruding from the injured E. coli were seen frequently in the electron microscopy preparation. Some of these blebs appeared as if they were bound by a double membrane and seemed to be shed from the cell, as may be evidenced by the numerous bleb structures seen throughout the preparation (Figs. 11,12). Although

no definite data on LPS release from the cell was collected because of the extreme delicacy of the lymulus lysate test (34,73), these blebs may have contained a large amount of LPS and periplasmic enzymes. These blebs may be released and result in lesions of the cellular envelope or erosion of its structure. On the other hand, it has been demonstrated that E. coli also release lipopolysaccharides, phospholipids, and proteins during normal growth (26,43), although probably not to the great extent they are released in stressed cells. These compounds seemed to be released as vesicles and membrane fragments that were practically unaltered outer membrane.

Regions of cells with less dense areas gave the bacteria and sometimes spheroplasts a mottled appearance late in the experimental period when the extent of injury was at its greatest. This phenomenon was also observed with Salmonella typhimurium, although its sensitivity to lysozyme was moderate even when healthy cells were examined (Fig. 10, Table 2).

Cells treated with EDTA lose LPS as well as some protein and phospholipid although no visible changes in cytology following observation by electron microscopy were detected by Bayer and Leive (2). Their treated coliforms became more sensitive to certain metabolites and developed an abnormal sensitivity to certain drugs. Ray et al. (54) whose freeze-dried cells were extremely sensitive to EDTA, also

indicated that freeze-drying may affect the LPS.

Hill (24) has indicated that some bile salts, such as desoxycholate, will partially dissolve cell walls of all strains he tested. Although there appeared to be some variation in the dissolvability of the cell wall which was dependent on the culture medium used for growth, there were no variations due to age. Muramic acid and mucopeptide were not solubilized to any detected level but teichoic acid and polysaccharide components were dissolved. Ribi et al. (56) have found endotoxin from three species of Gram-negative bacteria to be dissociated into nontoxic subunits by sodium desoxycholate while Woldringh (75) has treated organisms with 0.1% sodium desoxycholate in distilled water and discovered large areas of plasma membrane had dissolved as well as other structural alterations within the cell. The nucleoplasm was no longer distributed within the cell but had contracted to the center. Burman et al. (7) have found that cholate sensitivity of the outer membrane is an index of the damage of that membrane.

Growth on TSYA was used throughout the present study as an index of healthy and injured cells. Injury due to aquatic stress was seen earlier using media which were supplemented with sodium desoxycholate (Figs. 7,9,13,15). Sodium desoxycholate was probably harmful to the membrane since little injury was seen with MA until later in the

experimental periods. Therefore, damage to metabolic aspects of the cell occurred later and were not primarily involved in the first phase of cellular injury.

Hurst et al (30) have heated S. aureus to 52 C for 15 minutes in potassium phosphate and found a loss of salt tolerance which is usually used as an indication of injury. They used the K/N_a ratio as an index of membrane damage as ion transport is generally accepted as a membrane function. This ratio was 12.6 in the control cells and 3.4 in damaged cells. The aspartate uptake was only 10% of the control after injury had taken place. Once the cell had recovered, salt tolerance was regained, but a number of membrane functions such as aspartate uptake were still impaired. This suggests that salt tolerance was not a membrane function, although different damages may be repaired at different rates and the return of salt tolerance may be one of the early events. Hurst and Hughes (29) found that ribosome decomposition was not the initial effect of sublethal heating and had no correlation with the loss of salt tolerance.

According to the data presented, (Figs. 8,10,14,16) indices of injury including the difference in plate counts between TSY and DLA can be used to determine the extent of damage in stressed organisms. The lag time, percent spheroplasts, and percent injury increased as aquatic exposure time of E. coli increased. This was seen in cells

which were stressed in the laboratory as well as those stressed in stream environments. Although S. typhimurium did not show increased spheroplast formation, since its cell wall was quite sensitive to lysozyme even before stress, the lag time did increase as exposure time increased. The lag time calculated by the slide culture technique was an accurate and rapid determination of the extent of cell injury.

Daubner (12) has found that waters of different qualities do exert an influence upon indicator bacteria, not only on their regulatory mechanisms but also their basic physical properties and morphology. Cells became so altered that E. coli was observed to utilize citrate. In this case the membrane may have been damaged to the extent that leakage of citrate into the cell could occur. E. coli suspended in river water or distilled water for 24-48 hours had granules within the cytoplasm (possibly ribosomal degradation material), the cytoplasm became more dense and uniform, and the membrane separated from the cell wall.

In the present study dramatic changes in the interior of E. coli suspended in reagent grade water did not occur. These cells looked finely granular with intact cellular membranes (Figs. 11,12). After examining the cytology of these cells one would expect little metabolic damage and this was born out by the comparable counts on TSYA and

MA. The high counts that were observed with MA confirm that little metabolic injury occurred until later in the exposure period. MA is an index of the metabolic damage because the cells must produce necessary macromolecules not supplied in the medium, indicating little enzyme damage.

The characteristics of the water in which cells were injured have a great impact on the degree of injury and the survival rates (Fig. 2,3,5). Water with a lower resistance and thus a greater ion concentration consistantly showed an increased death and injury rate for cells placed in it. The ions present may act to destabilize the membrane as it is being damaged during aquatic stress and thus act to increase cellular damage. Periodically stirred vessels also resulted in a greater injury for cells than when placed in constantly stirred vessels (Fig. 4). Less injury was seen in autoclaved Rocky Creek water than when filter sterilized water from the same source was used (Figs. 5,6). Autoclaving may have changed the organic materials in the water and inactivated or precipitated harmful material.

Microbes previously exposed to water are especially susceptible to the stress of warm agar used in pour plates and starvation can increase this sensitivity (37,76). This caused significantly reduced recoveries when pour plates were compared with spread plates. Higher detection rates were found when selective agar was surface plated or

surface overlay plated and these techniques gave more reproducible results than MPN (54,72). The sample varience was numerically larger for spread plates than that for pour plates as found by VanSoestbergen and Lee (72), but the numerical value for the mean was significantly higher for spread plates than for pour plates.

The present experiments required that extreme care be taken in plating the injured organisms since little further stress due to warm agar could be tolerated. Spread plates were used rather than pour plates. A small amount of overlay was necessary to prevent the spread of surface colonies, but agar was cooled almost to the point of solidification before it was poured. The plates were spread for 15 seconds and the inoculum was allowed to absorb into the medium or evaporate. Clark (10) found that the volume of inoculum and a postponement in spreading plates had no measurable effect on the enumeration or distribution of colonies. His tests showed less than 4% of the total colony formers remaining on the spreader after 15 seconds of use.

Survival characteristics depend on many things including the medium used for growth (65). It was reported by Strange et al. (65) that cells grown in tryptic meat broth or tryptone glucose remained viable for longer periods of time than when grown in a simple medium with a limiting carbon source. Population density affected the

survival of cells and cryptic growth occurred at high population densities (65,67). According to the present studies population densities of 10^7 bacteria/ml and above showed no injury during aquatic laboratory stress. It is concluded that no injury occurred over a long period of time in this closed vessel because of cryptic growth and related phenomena. In populations of these sizes organic material, death and lysis, as well as a shielding population effect (i.e. aggregation) could buffer the cells from a harmful aquatic environment.

Harrison (20) proposed that slow growing log-phase cultures of Enterobacter survive better than normal log-phase cultures and that stationary-phase cells survive the best of all. The constant use of 18 hour stationary-phase populations in the present study insured minimal injury variation due to population age. Better survival occurred at suboptimal temperatures only when the growth rate was reduced by a limiting nutrient. Therefore, the concentration of nutrient at the time of harvest influenced the subsequent response to starvation more than the growth rate.

Scheusner et al. (58) have injured Staphylococcus, Streptococcus, and E. coli by exposure to a quaternary ammonium compound. Injury was measured by the inability of the cells to grow on a selective medium, metabolic injury was determined by plating on MA, and the number of injured organisms was the difference between counts on MA

and TSA. The number of dead organisms was determined by the difference in counts on TSA before and after treatment. A similar study by Scheusner et al. (59) showed that bile salts alone prevented as many injured cells from growth as did any combination of selective agents and that sodium desoxycholate was the bile salt most inhibitory to damaged cells. Those cells exposed to the quaternary ammonium compound and untreated cells both grew equally well on minimal media and thus no indication of metabolic injury was seen. Bile salts inhibited 65% of the surviving treated cells and similar inhibition was found using violet red bile agar. The number of bacteria that grew on the various media depended on the type of medium, on the treatment given to those microbes and their condition before plating. This group also studied the inhibitory properties of various dyes such as Eosin Y, brilliant green, neutral red and crystal violet. Varying results were found in this type of secondary injury.

Clark and Ordal (11) stressed S. typhimurium at 48 C for 30 minutes and found greater than 90% of these cells were unable to reproduce on EMB with 2% NaCl. Cells plated on desoxycholate agar also showed an extended lag period following the heat treatment, but re-recovered in a suitable medium such as nutrient broth or Tryptic soy broth. Variations in thermal inactivation rates were due to several factors such as the age of the organism at the time of heating, its

chemical makeup, and the pH of the heating medium (22).

When Aeromonas and E. coli were studied under starvation conditions by Klein and Wu (37) both of these organisms showed low basal endogenous oxygen uptake rates, but the addition of glucose at concentrations as low as 1 mg/1, increased oxygen consumption in E. coli. A slight increase in oxygen uptake which returned to a lower level within 10 minutes was observed in Aeromonas. E. coli, on the other hand, did not return to its original low level of oxygen consumption. They suggested that E. coli may be incapable of avoiding the utilization of substrate for respiration under conditions of nutrient limitation. It was further concluded that these cells must commit their energy and reserves to catabolism even though the energy released can not be utilized for growth under stressful starvation environments. Aeromonas, on the other hand, is endogenous to water and may have the selective advantage in that it has adapted to low nutrient conditions and can avoid the additional catabolism brought upon by the presence of glucose.

A psychrophilic marine vibrio studied by Novitsky and Morita (47) reduced its endogenous respiration rate over 80% during the first few days of survival and after the first week its respiration had been reduced to 0.0071% total carbon respired/hour. This lower respiration rate may prevent macromolecular decomposition and promote the long

term survival of these organisms which have successfully adapted to low nutrient concentrations. A type of endogenous dormancy or resting stage like that proposed by Stevenson (63) may have developed because this organism had prolonged exposure to starvation conditions.

In the present study, oxygen uptake rates of healthy E. coli showed a slight lag when 0.15% sodium desoxycholate was used as a supplement in TSY broth, while injured cells showed no oxygen uptake in the same supplemented broth (Fig. 17). A lag was seen even when these injured cells were placed in TSY broth. For a short period of time during this lag, cells must have been undergoing a repair process which could begin with little oxygen uptake. Cell membrane damage, which is apparently affected by sodium desoxycholate, can be related to the oxygen uptake, since many aerobic metabolic pathways are a function of an intact membrane. Since no oxygen uptake was seen, membranes may have been highly damaged and yet some cells were capable of growth on TSYA + 0.15% sodium desoxycholate. The number of cells capable of growth on this medium after several days of exposure is probably very small and the cells are able to grow anaerobically. Cells can recover on TSY anaerobically as evidenced by the data which show equal enumeration on TSYA and DLA both aerobically and anaerobically. Thus, oxygen radicals or the accumulation of H_2O_2 due to catalase inactivation does not seem to be an important aspect

of aquatic injury, at least during non-runoff conditions and when cells are plated.

ATP measurements, as well as energy charge $1/2(\frac{[ADP]+2[ATP]}{[AMP]+[ADP]+[ATP]})$, have been used as indices of cellular viability (8,71). The energy charge during growth is estimated to be above 0.8 by Chapman et al. (8), and although Walker-Simmons and Atkinson (71) believe that growth ceases at 0.8-0.9, both report that E. coli can maintain viability, ability to synthesize protein, and can also adapt to new substrates at that energy level and above. Chapman et al. (8) suggest starved cells are still capable of maintaining viability from 0.5-0.8. Both of these investigators propose that the loss of functional activities and death occur at energy charge values less than 0.5.

An indication of viability by some method other than plate counts and the determination of the length of time required for division to occur was used to complete these studies. Viability was calculated with the proportion of cells that were capable of division under optimal growth conditions. Postgate's slide culture technique (52) was modified and used to achieve the % viability. Postgate (52) found that in a population which contained a high percentage of viable cells all organisms that were capable of division had done so by the time the first dividers had undergone 2 or 3 divisions. Dying populations needed a longer incubation time and the loss of these

cells due to lysis with this particular method was rare. When large populations were used for these studies microcolonies tended to overgrow non-dividing members. A population size that gave approximately 100 cells/field using phase contrast microscopy prevented gross colony spreading when the slide was dipped in cool molten agar. Since populations in this study were often very debilitated, twenty four hours was used as an appropriate endpoint for an index of viable cells (i.e. if no microcolony formation was observed by this time, the cells were considered non-viable). To prevent the agar from drying during the twenty four hour incubation period, a further modification of Postgate's slide culture technique (52) was made by the use of molten parafilm around the outside of the coverslip.

Many factors seem to influence the catalase level in E. coli including the culture medium, growth phase, and temperature to which the cells are subjected (78). Flowers et al. (18) have added catalase to selective media and increased the enumeration of stressed cells with the most beneficial effect on the least efficient enumeration medium. The effects of catalase appeared to be due to the inability of stressed organisms to repair cellular damage in the absence of an exogenous decomposer of H_2O_2. They proposed that the intracellular catalase activity was reduced by stress which resulted in peroxide accumulation. The problem of deficient amounts of catalase was

resolved by addition of catalase to the agar plates. It had little effect on the non-stressed cells but greatly increased the enumeration of stressed cells with no effect on the selectivity of the medium. Martin et al. (40) have also added catalase to the surface of a growth medium and increased the enumeration of physically or chemically injured cells. They found that heated cells had decreased catalase activity. Catalase when attached to the intracellular membrane was in an active form and became inactive when released from the membrane. It is probable that the decrease in catalase activity and the corresponding injury, as found on TSAS were greatly different.

Brewer et al. (5) have added catalase or pyruvate to trypticase broth + 10% NaCl and have studied its effect on the MPN technique, which is considered a more efficient technique in the enumeration of low numbers of cells and is an estimate of the population rather that a precise enumeration. They have found increased enumeration of heat-stressed and unstressed cells with the addition of these chemicals. This is a practical technique and allows for recovery and growth in a selective medium.

Since catalase appeared to increase the enumeration of stressed microbes it was of interest to determine its effect on aquatically stressed cells. Various additions to media and overlays are listed according to their effectiveness in allowing greater enumeration:

TSY=TSY + DLA overlay > TSY + catalase > TSY + lysozyme > DLA + catalase > DLA (Fig. 19). Although catalase did increase the enumeration of cells somewhat on DLA, the increase was very little and suggested that the presence of H_2O_2 or reduced catalase activity had little effect on the growth of colonies from injured organisms. Lysozyme had little effect on the growth of cells on TSYA, and evidently cells on this rich medium can recover from the effects of lysozyme and form colonies. From the results presented here, TSYA with DLA overlay may be a solution to the problem of injured bacteria growth. Injured bacteria are plated on a rich medium which promotes the growth of damaged organisms and the overlay is selective enough to prevent the growth of non-coliforms.

In studies with Staphylococcus aureus, Gray (19) found a medium made with egg yolk was generally less inhibitory to injured cells but was usually less efficient as a selective medium. All media that were used were equally capable of normal cell enumeration, but as the heating time progressed variations in media productivity became more pronounced.

Depending upon the system used for damaging organisms, researchers (16,21,22,53,54,69) have found the need for cells to synthesize protein, ribonucleic acid, DNA, and membrane components for recovery. Energy also seems to be required as ATP facilitates the repair of

debilitated cells (53). Apparently large amounts of enzymes and structural proteins do not need to be synthesized for repair (16). Magnesium taken up during heating indicated the possible increased requirement for Mg^{++} to the system has lead to a reduced lag time for injured cells. Potassium was found to have little effect on unheated cells and may be detrimental to heat injured organisms (22).

Heinmets et al. (23) plated heat or chemically treated cells with 0.2% metabolites such as oxaloacetate, pyruvate, sodium citrate, and lactic acid. They observed an increase in viable cell counts only on occasions when treated samples were preincubated with metabolites.

Cells exposed to an aquatic stress recovered, as indicated by the converging plate counts using TSYA and DLA when the cells were incubated in TSY broth plus supplements for 105-165 minutes (Fig. 20).

Wu and Klein have reported the increased survival of stressed organisms which have been placed in peptone for one hour before plating. Improved detection has been found by using such diluents as 1% nonfat milk solids, 1% peptone, 1% $MgSO_4 \cdot 7H_2O$, and 0.1% trypticase (55,74). These dilution blanks were also used in the present study to determine their effects on organisms stressed in an aquatic environment. Each of these diluents, including milli-Q water and standard phosphate buffer, performed equally well on nonstressed cells (Fig. 18,

Table 5). As the time of exposure progressed greater enumeration on DLA was found when using 1% nonfat dry milk and 1% peptone dilution blanks. The order of diluents in terms of greater enumeration were as follows: 1% nonfat dry milk > peptone > milli-Q > phosphate buffer > 1% $MgSO_4 \cdot 7H_2O$. These injured organisms were placed in the dilution blanks for a period of only 15 minutes so it is unlikely that cell division occurred. Perhaps the protein and buffering action of the nonfat dry milk and peptone helped facilitate repair of the cellular membrane, so that when confronted with the sodium desoxycholate in DLA the bile salt was no longer harmful. It would be interesting to determine if DLA supplemented with these substances would allow damaged cells to divide and form colonies. It is unknown why $MgSO_4$ had such a detrimental effect on injured cells since Mg^{++} should help to stabilize the cell membrane. Perhaps additional buffering capacity was needed.

SUMMARY

The quality of water used for injuring organisms affected the degree of injury, the rate of that damage, and the survival properties of the cells. Low resistance and periodic stirring of water in the injury vessel increased the rate and degree of injury. High cell populations buffered the cells from injury and very little death or damage was found over long periods of time.

Indices of injury other than the difference in plate counts between TSY and DLA can be used to determine the extent of injury in aquatic stressed bacteria. Lag time and percent spheroplast formation, as well as the percent injury increased as exposure of E. coli increased. This occurred in the laboratory stressed cells and in the stream environments. S. typhimurium was sensitive to lysozyme before stress so that the spheroplast formation did not increase with exposure time, although the lag time, as calculated by the slide culture technique did increase.

Little metabolic damage occurred in the early stages of aquatic stress and only after several days did the cells show this type of injury by failing to grow on minimal agar. Cell envelope damage appeared to be the primary site of aquatic injury as evidenced by the extreme sensitivities of these cells to sodium desoxycholate and lysozyme even though the bacterial particle counts remained high. Disruption of the cell envelope was also evidenced by electron micro-

graphs in which blebs are seen to protrude from the cell surface.

Oxygen uptake of injured cells placed in TSY broth had an extended lag and no oxygen uptake was found when these cells were placed in TSY broth supplemented with 0.15% sodium desoxycholate. This also indicated membrane damage as aerobic functions are part of an intact healthy cell membrane.

Although catalase added to DLA did not significantly improve enumeration on that selective medium, TSYA overlayed with DLA may allow the growth and colony formation of damaged cells and yet retain the selectivity of the medium.

Dilution blanks made with 1% nonfat dry milk or 1% peptone proved to be the best in terms of minimizing the differential bacterial counts between media.

LITERATURE CITED

1. American Public Health Association. 1976. Standard methods for the examination of water and wastewater. 14th ed., A.P.H.A., Washington, D.C.

2. Bayer, M.E. and L. Leive. 1977. Effect of ethylenediaminetetra-acetate upon the surface of Escherichia coli. J. Bacteriol., 130: 1364-1381.

3. Bissonnette, G.K. 1974. Doctor of Philosophy Thesis. Recovery characteristics of bacteria injured in the natural aquatic environment. Montana State University, Bozeman, MT.

4. Bissonnette, G.K., J.J. Jezeski, G.A. McFeters and D.G. Stuart. 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol., 29: 186-194.

5. Brewer, D.G., S.E. Martin and Z.J. Ordal. 1977. Beneficial effects of catalase or pyruvate in a Most-Probable-Number technique for the detection of Staphylococcus aureus. Appl. and Environ. Microbiol., 34: 797-800.

6. Beuchat, L.R. 1978. Injury and repair of gram-negative bacteria with special consideration of the involvement of the cytoplasmic membrane. Advances in Applied Microbiology. 23: 219-243.

7. Burman, L.G., K. Nordström, and G.D. Bloom. 1972. Murein and the outer penetration barrier of Escherichia coli K-12, Proteus mirabilis, and Pseudomonas aeruginosa. J. Bacteriol. 112: 1364-1374.

8. Chapman, A.G., L. Fall and D.E. Atkinson. 1971. Adenylate energy charge in Escherichia coli during growth and starvation. J. Bacteriol. 108: 1072-1086.

9. Camper, A.K. 1977. Masters Thesis. Physiological studies of chlorine injury in Escherichia coli. Montana State University, Bozeman, MT.

10. Clark, D.S. 1971. Studies on the surface plate method of counting bacteria. Can. J. Microbiol., 17: 943-946.

11. Clark, C.W. and Z.J. Ordal. 1969. Thermal injury and recovery of Salmonella typhimurium and its effect on enumeration procedures. Appl. Microbiol. 18: 332-336.

12. Daubner, I. 1975. Changes in the properties of E. coli under the influence of water environments. Verh. Internat. Verein. Limnol., 19: 2650-2657.

13. Dawes, E.A. and D.W. Ribbons. 1965. Studies on the endogenous metabolism of Escherichia coli. Biochem. J., 95: 332-343.

14. Dockins, W.S. and G.A. McFeters. 1978. Fecal coliform elevated-temperature test: a physiological basis. Appl. and Environ. Microbiol. 36: 341-348.

15. Dresden, M.H. and M.B. Hoagland. 1967. Polyribosomes of Escherichia coli breakdown during glucose starvation. J. Biol. Chem., 242: 1065-1068.

16. Emswiler, B.S., M.D. Pierson and S.P. Shoemaker. 1976. Sublethal heat stress of Vibrio parahaemolyticus. Appl. and Environ. Microbiol., 32: 792-798.

17. Fan, C.L. and V.W. Rodwell. 1975. Physiological consequences of starvation in Pseudomonas putida: Degradation of intracellular protein and loss of activity of the inducible enzymes of L-arginine catabolism. J. Bacteriol., 124: 1302-1311.

18. Flowers, R.S., S.E. Martin, D.G. Brewer, and Z.J. Ordal. 1977. Catalase and enumeration of stressed Staphylococcus aureus cells. Appl. and Environ. Microbiol., 33: 1112-1117.

19. Gray, R.J.H., Margaret A. Gaske and Z. John Ordal. 1974. Enumeration of thermally stressed Staphylococcus aureus MF 31. J. of Food Science, 39: 844-846.

20. Harrison, A.P. 1961. Aging and decline of bacteria in phosphate buffer. Bacteriol. Proc., p. 53.

21. Heinis, J.J., L.R. Beuchat and F.C. Boswell. 1978. Antimetabolite sensitivity and magnesium uptake by thermally stressed Vibrio parahaemolyticus. Appl. and Environ. Microbiol., 35: 1035-1040.

22. Heinis, J.J., L.A. Beuchat, and W.K. Jones. 1977. Growth of heat-injured Vibrio parahaemolyticus in media supplemented with various cations. Appl. and Environ. Microbiol., 33: 1079-1084.

23. Heinmets, F., W.W. Taylor, and J.J. Lehman. 1954. The use of metabolites in the restoration of the viability of heat and chemically inactivated Escherichia coli. J. of Bacteriol., 67: 5-12.

24. Hill, M.N. 1967. Action of bile salts on bacterial cell walls. Nature (London), 214: 1152-1154.

25. Hobbie, J.E., R.J. Daley and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. and Environ. Microbiol., 33: 1225-1228.

26. Hoekstra, D., J.W. VanderLaan, L.D. Leit and B. Witholt. 1976. Release of outer membrane fragments from normally growing Escherichia coli. Biochimica et Biophysica Acta., 455: 889-899.

27. Huber, T.W. and A.W. Brinkley. 1977. Growth of cell wall-defective varients of Escherichia coli: Comparison of aerobic and anaerobic induction frequencies. J. Clinical Microbiol. 6: 166-171.

28. Hurst, A. 1977. Bacterial injury: A review. Can. J. Microbiol. 23: 936-942.

29. Hurst, A. and A. Hughes. 1978. Stability of ribosomes of Staphylococcus aureus S6 sublethally heated in different buffers. J. Bacteriol., 133: 564-568.

30. Hurst, A., A. Hughes, J.L. Beare-Rogers, and D.L. Collins-Thompson. 1973. Physiological studies on the recovery of salt tolerance by Staphylococcus aureus after sublethal heating. J. Bacteriol., 116: 901-907

31. Iandolo, J.J., and Z.J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bacteriol., 91: 134-142.

32. Jones, P.C.T. and J.E. Mollison. 1948. A quantitative estimation of soil microorganisms. J. Gen. Microbiol. 2: 54-69.

33. Jones, J.G. and B.M. Simon. 1975. An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with reference to a new method for dyeing membrane filters. J. Appl. Bacteriol., 39: 317-329.

34. Jorgensen, J.H. and R.F. Smith. 1974. Measurement of bound and free endotoxin by the limulus assay (3824). Proc. of the Soc. for Experimental Biol. and Med. 146: 1024-1031.

35. Kaplan, R. and D. Apirion. 1975. Decay of ribosomal ribonucleic acid in Escherichia coli cells starved for various nutrients. J. Biol. Chem. 250: 3174-3178.

36. Kaplan, R. and D. Apirion. 1975. The fate of ribosomes in Escherichia coli starved for a carbon source. J. Biol. Chem., 250: 1854-1863.

37. Klein, D.A. and S. Wu. 1974. Stress: A factor to be considered in heterotrophic microorganism enumeration from aquatic environments. Appl. Microbiol., 27: 429-431.

38. Knox, K.W., M. Vesk and E. Work. 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of Escherichia coli. J. Bacteriol. 92: 1206-1217.

39. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

40. Martin, S.E., R.S. Flowers and Z.J. Ordal. 1976. Catalase: Its effect on microbial enumeration. Appl. and Environ. Microbiol., 32: 731-734.

41. McFeters, Gordon, A. and David G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. 24: 805-811.

42. Morgan, C. and H.S. Rosenkranz. 1970. Ultrastructure of Escherichia coli depleted of an amino acid. J. Bacteriol., 102: 584-587.

43. Mug-Opstelten, D. and B. Withholt. 1978. Preferential release of new outer membrane fragments by exponentially growing Escherichia coli. Biochimica et Biophysica Acta., 508: 287-295.

44. Nath, K. and A.L. Koch. 1970. Protein degradation in Escherichia coli. I. Measurement of rapidly and slowly decaying components. J. Biol. Chem., 245: 2889-2900.

45. Nath K. and A.L. Koch. 1971. Protein degradation in Escherichia coli. II. Strain differences in the degradation of protein and nucleic acid resulting from starvation. J. Biol. Chem., 246: 6956-6967.

46. Ng, H., J.L. Ingraham and A.G. Marr. 1962. Damage and derepression in Escherichia coli resulting from growth at low temperatures. J. Bacteriol. 84: 331-339.

47. Novitsky, J.A. and R.Y. Morita. 1976. Morphological characterization of small cells resulting from nutrient starvation of a pyschrophilic marine vibrio. Appl. and Environ. Microbiol. 32: 617-622.

48. Novitsky, J.A. and R.Y. Morita. 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. and Environ. Microbiol., 33: 635-641.

49. Obinata, M. and D. Mizuno. 1968. Intracellular localization of deoxyribonucleases in Escherichia coli. Biochim. Biophys. Acta, 155: 98-106.

50. Pierson, M.D., R.I. Tomlins and Z.J. Ordal. 1971. Biosynthesis during recovery of heat-injured Salmonella typhimurium. J. Bacteriol., 105: 1234-1236.

51. Pine, M.J. 1973. Regulation of intracellular proteolysis in Escherichia coli. J. Bacteriol., 115: 107-116.

52. Postgate, J.R., J.E. Crumpton and J.R. Hunter. 1961. The measurement of bacterial viabilities by slide culture. J. Gen. Microbiol., 24: 15-24.

53. Ray, B., J.J. Jezeski, and F.F. Busta. 1971. Repair of injury in freeze-dried Salmonella anatum. Appl. Microbiol, 22: 401-407.

54. Ray, B., and M.L. Speck. 1972. Metabolic process during the repair of freeze-injury in Escherichia coli. Appl. Microbiol., 24: 585-590.

55. Ray, B. and M.L. Speck. 1973. Descrepancies in the enumeration of E. coli. Appl. Microbiol., 25: 494-498.

56. Ribi, E., R.L. Anacker, R. Brown, W.T. Haskins, B. Malmgren, K.C. Milner, and J.A. Rudbach. 1966. Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium deoxycholate. J. Bacteriol. 92: 1493-1509.

57. Scherer, C.G. and C.W. Boylen. 1977. Macromolecular synthesis and degradation in Arthrobacter during periods of nutrient deprivation. J. Bacteriol., 132: 584-589.

58. Scheusner, D.L., F.F. Busta and M.L. Speck. 1971. Injury of bacteria by sanitizers. Appl. Microbiol. 21: 41-45.

59. Scheusner, D.L., F.F. Busta and M.L. Speck. 1971. Inhibition of injured Escherichia coli by several selective agents. Appl. Microbiol., 21: 46-49.

60. Schillinger, J.E. and D.G. Stuart. 1976. Coliform recovery and distribution changes in the East Gallatin River. Abstracts of the Annual Meeting of the American Society for Microbiology.

61. Sinskey, T.J. and G.J. Silverman. 1970. Characterization of injury incurred by Escherichia coli upon freeze-drying. J. of Bacteriol. 101: 429-437.

62. Sorrells, K.M., M.L. Speck, and J.A. Warren. 1970. Pathogenicity of Salmonella gallinarum after metabolic injury by freezing. Appl. Microbiol., 19: 39-43.

63. Stevenson, H.L. 1978. A case for bacterial dormancy in aquatic systems. Microbial Ecology, 4: 127-133.

64. Strange, R.E. 1968. Bacterial "glycogen" and survival. Nature, 220: 606-607.

65. Strange, R.E., F.A. Dark and A.G. Ness. 1961. The survival of stationary phase Aerobacter aerogenes stored in aqueous suspension. J. Gen. Microbiol., 25: 61-76.

66. Strange, R.E., H.E. Wade and F.A. Dark. 1963. Effect of starvation on adenosine triphosphate concentration in Aerobacter aerogenes. Nature, 199: 55-57.

67. Sykes, G. 1963. The phenomenon of bacterial survival. J. Appl. Bacteriol. 26: 287-294.

68. Tamaki, S. and M. Matsuhashi. 1973. Increase in sensitivity to antibiotics and lysozyme on deletion of lipopolysaccharides in Escherichia coli strains. J. Bacteriol., 114: 453-454.

69. Tomlins, R.I., and Z.J. Ordal. 1971. Requirements of Salmonella typhimurium for recovery from thermal injury. J. Bacteriol., 105: 512-518.

70. VanSoestbergen, A.A. and C.H. Lee. 1969. Pour plates or streak plates? Appl. Microbiol., 18: 1092-1093.

71. Walker-Simmons, M. and D.E. Atkinson. 1977. Functional capacities and the adenylate energy charge in E. coli under conditions of nutritional stress. J. Bacteriol., 130: 676-683.

72. Warseck, M., B. Ray, and M.L. Speck. 1973. Repair and enumeration of injured coliforms in frozen foods. Appl. Microbiol. 26: 919-924.

73. Watson, S.W., T.J. Novitsky, H.L. Quinby, and F.W. Valois. 1977. Determination of bacterial number and biomass in the marine environment. Appl. and Environ. Microbiol. 33: 940-946.

74. Weiler, William A. and S.E. Hartsell. 1969. Diluent composition and the recovery of Escherichia coli. Appl. Microbiol. 18: 956-957.

75. Woldringh, C.L. 1970. Lysis of the cell membrane of Escherichia coli K12 by ionic detergents. Biochim. Biophys Acta., 224: 288-290.

76. Wu, S. and D.A. Klein. 1976. Starvation effects on Escherichia coli and aquatic bacterial responses to nutrient addition and secondary warming stresses. Appl. and Environ. Microbiol., 31: 216-220.

77. Yem, Daniel and Henry C. Wu. 1978. Physiological characterization of Escherichia coli mutant altered in the structure of murein lipoprotein. J. Bacteriol. 133: 1419-1426.

78. Yoshpe-Purer, Yona and Y. Henis. 1976. Factors affecting catalase level and sensitivity to hydrogen peroxide in Escherichia coli. Appl. and Environ. Microbiol. 32: 465-469.

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