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

Abstract:
The quality of water used for injuring bacteria affeccted 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 micro-graphs. 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.

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ABSTRACT

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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 refrigerated 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 $\frac{\text{TSYA count} - \text{DLA count}}{\text{TSYA count}} \times 100\%$.

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 $2\frac{1}{2}$ 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:

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

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 percent 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.

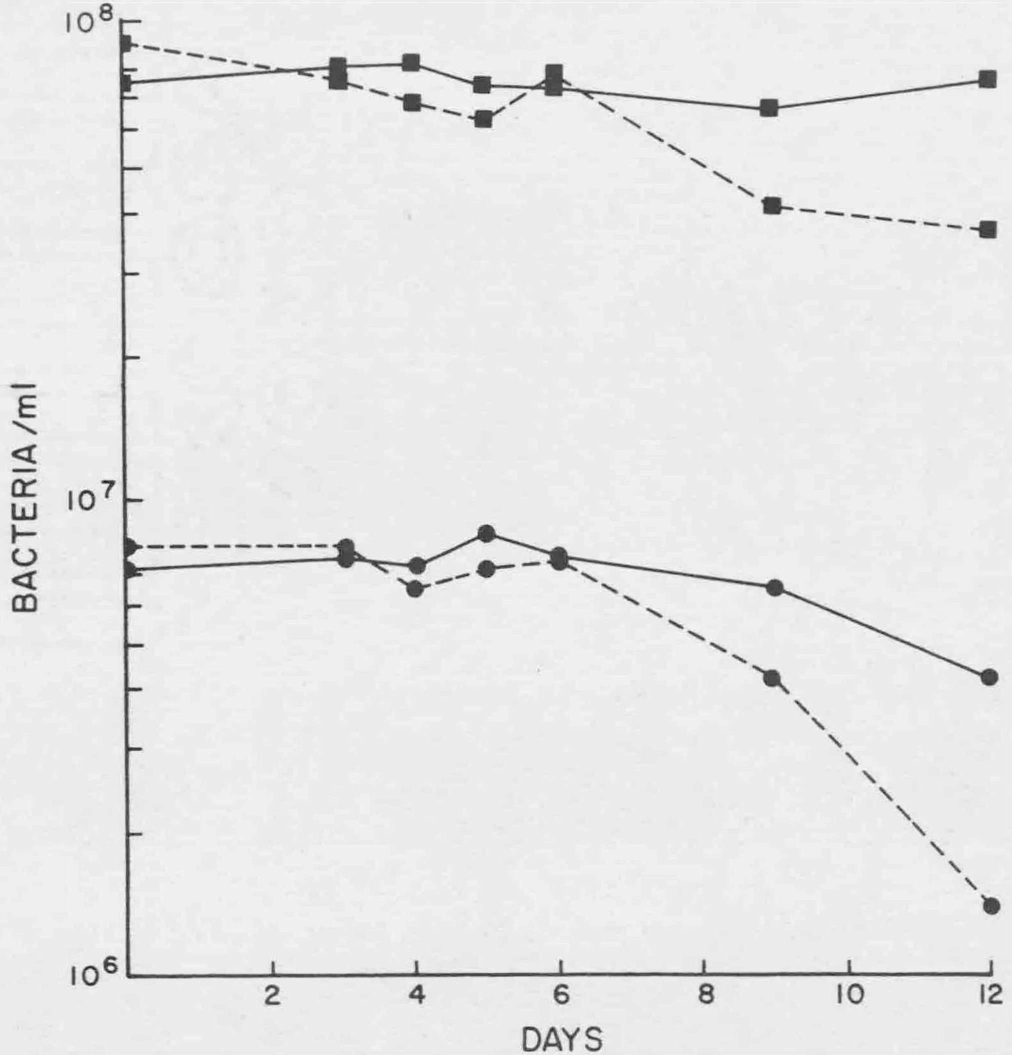
RESULTS

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.

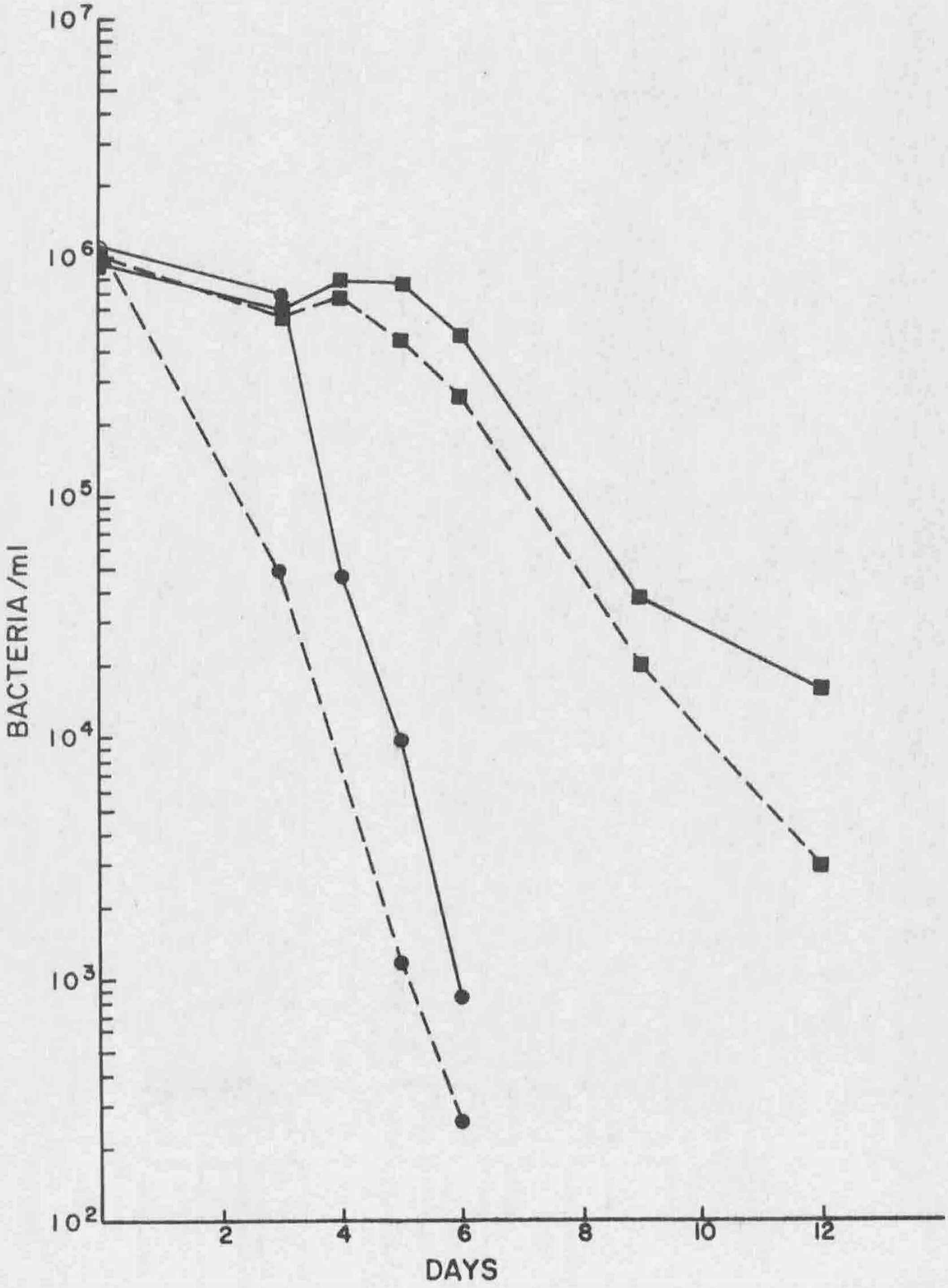
Reagent grade water with resistances of 18 and 7 Megohms-Cm were also compared as sources of debilitation. The difference between the two can be seen in Fig. 2. Water with lower resistance caused a quicker and more dramatic increase in injury. A very rapid decrease in the viable population occurred after 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 (----).



10⁷
10⁶
10⁵
10⁴
10³
10²
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 (----).