Physiological studies of chlorine injury in Escherichia coli
by Anne Kosteczko Camper

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
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Abstract:
Injury induced in E. coli cells by chlorination was studied from a physiological standpoint. The chlorination procedure used consisted of exposure of cells directly to 0.5 mg/l chlorine in pH 6.5 water for timed intervals up to sixteen minutes at 22 - 25 C. Predictable and reproducible injury was found to occur.

The injury inflicted on the E. coli cells by the chlorinated environment was reversible under certain nutrient conditions such as in overlay broth. There was an extended lag period in chlorinated cell growth not seen in control cells followed by a resumption of logarithmic growth at a rate equaling that of control rate. The nontoxic and nonselective environment provides an opportunity for injured cells to repair themselves.

The aldolase activity of cells chlorinated in vivo was equal to or slightly higher than those values obtained with control cells. This implies that aldolase is not the primary site of chlorine action as previously suggested with in vitro experiments.

Oxygen uptake experiments showed that chlorinated cells undergo a decrease in respiration. This decrease is more pronounced in rich media containing reducing agents. The cells are not immediately repaired in the presence of the reducing agents, suggesting that synthesis of new material may be of greater importance than reversal of chlorine oxidation.

Uptake of metabolites was inhibited by chlorine injury as shown with experiments using labeled glucose and algal protein hydrolysate.

Labeling before chlorination demonstrated that turnover of intracellular material continues at about the same rate after chlorination as before. Amino acids are apparently turned over very rapidly in both control and chlorine treated cells.

The results of experiments given indicate that reversible injury occurs in E. coli cells exposed to commonly used concentrations of chlorine. Further, our findings suggest that the previously accepted mechanism of chlorine damage (aldolase inactivation through sulfhydryl oxidation) is invalid and that impairment of the normal transport physiology of the cell envelope should be considered.

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Date June 17, 1977

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Approved:

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ABSTRACT

Injury induced in E. coli cells by chlorination was studied from a physiological standpoint. The chlorination procedure used consisted of exposure of cells directly to 0.5 mg/l chlorine in pH 6.5 water for timed intervals up to sixteen minutes at 22 - 25 C. Predictable and reproducible injury was found to occur.

The injury inflicted on the E. coli cells by the chlorinated environment was reversible under certain nutrient conditions such as in overlay broth. There was an extended lag period in chlorinated cell growth not seen in control cells followed by a resumption of logarithmic growth at a rate equaling that of control rate. The non-toxic and nonselective environment provides an opportunity for injured cells to repair themselves.

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The results of experiments given indicate that reversible injury occurs in E. coli cells exposed to commonly used concentrations of chlorine. Further, our findings suggest that the previously accepted mechanism of chlorine damage (aldolase inactivation through sulfhydryl oxidation) is invalid and that impairment of the normal transport physiology of the cell envelope should be considered.

Chapter 1

INTRODUCTION

Indicator organisms in receiving waters and chlorinated effluents as isolated using various media have long been used to define the efficiency of wastewater treatment plants. The accepted thought is that chlorine as used in treating wastewater effluent will cause the rapid die-off of the bacterial population. This line of thought has created a pattern for the interpretation of results and the devising of media used in these tests by restricting the view to the alternatives of the live, healthy organism or the dead one. The concept of reversible injury has been applied in other situations of environmental stress, but has been largely ignored in the explanation of results obtained in the presence of chlorine. Knowledge of the physiological mode of action of chlorine on the cell and information on the reversibility of chlorine injury could be used to define the response of the organisms to various aquatic and nutritive environments and help formulate media for isolation and detection.

The appearance of increased numbers of organisms at some distance below the discharge of chlorinated effluent has been attributed to several factors, among which are regrowth of fecal coliforms (42), regrowth of nonfecal organisms (11), regrowth of nonfecal organisms plus an unidentified point source (28,43), and the upset of the predator-

bacteria balance resulting in bacterial multiplication (25). None of these instances considers the possibility that recovery or repair of injury induced by chlorination is responsible for the reappearance of the organisms.

The same mode of thinking appeared during the early research leading to the selection of various media used to enumerate organisms, particularly fecal coliforms, in chlorinated effluent. Since that time the concept of reversible injury resulting from chlorination and other environmental stresses has been established (6,35,40). It has been shown that the membrane filtration procedure, such as with M-FC medium, does not enumerate chlorine injured cells that are not dead (7,27,33). The multiple-tube fermentation technique (MPN) gives higher counts on the same sample because of its initial nonselective medium, but it is cumbersome and time consuming. Improvements in the media used with the membrane filtration technique to give results of equal or higher value than the MPN results when detecting chlorine injured populations of coliforms have been accomplished (39), but without consideration as to the mechanism of chlorine on the cell. However, a new method has been developed (46) which takes into account the proposed action of chlorine as described in earlier research. The basis for these improvements seemed legitimate, but many questions were left unanswered with regard to chlorine action on the basis of previous

research.

Statement of Purpose

The understanding of the physiology of chlorine injury in indicator bacteria is of utmost importance in the interpretation of bacterial counts obtained with various media from chlorinated effluent and receiving waters. The purpose of this research is to determine if chlorine does produce injury in addition to death in a bacterial population, and whether or not that injury in reversible under conducive conditions. Specific objectives are to determine the extent of this injury, the ability of the organisms to recover, and the general physiological site of action of chlorine in producing this injury.

Chapter 2

MATERIALS AND METHODS

Test organism

The organism that was used in all of the experiments was a water isolate taken from the East Gallatin River. It produced a ++-- IMViC reaction, formed metallic green sheened colonies on Levine EMB agar (Difco, Detroit, MI), and produced gas at 44.5 C in EC medium (Difco). The organism was maintained on stock culture agar (Difco) and transferred every four weeks. At intervals of about two months an EMB plate and IMViC series were inoculated and a Gram stain done to test for cultural and genetic purity.

Media

Water utilized

The water used for the media, buffers, and chlorine demand free water was either double distilled water that had been stored in glass or reagent grade water that had been processed by a Milli-Q water system (Millipore Corp., Bedford, MA) following single distillation.

Peptone-phosphate buffer

Peptone-phosphate buffer was utilized as a diluent throughout the course of these studies. The buffer was prepared by the use of phosphate stock solution (2) with the addition of 0.1% peptone (Difco) as suggested

by Sladek, et al. (45). This buffer was also used as a wash for the membrane filter technique. Dilution blanks were stored under refrigeration until used, at which time they were put on ice.

Tris buffer

The buffer used for the oxygraph and aldolase assays was prepared in accordance with the directions given in the product flier (Sigma Chemical Company, St. Louis, MO). The buffer chosen was a 0.05 M solution of pH 7.2 at 35 C. This buffer was autoclaved and refrigerated until used.

Chlorine demand free water

Chlorine demand free water was prepared according to Standard Methods for the Examination of Water and Wastewater (2). This water was used in the laboratory chlorination experiments and as a final wash for the organisms chlorinated by that procedure.

Sodium thiosulfate solution

The sodium thiosulfate utilized to neutralize the chlorine in samples was prepared in water to give a 10% solution and filter sterilized with a 0.45 um filter (Millipore). The sterile solution was kept under refrigeration.

Reducing agent stock solutions

Reducing agents were employed in the formulation of the overlay

medium and in the production of some of the oxygraph media. Solutions of 0.25% sodium thioglycollate (Sigma Chemical Co.) and 0.25% glutathione (Sigma Chemical Co.) were prepared with water and refrigerated until used.

TSY broth

TSY broth was prepared by supplementing Trypticase soy broth (Difco) with 0.3% yeast extract (Difco) and sufficient glucose to make the final concentration 0.5%. One hundred ml portions were dispensed into flasks, autoclaved, and used to grow the organisms utilized in the experiments.

M-FC

M-FC broth (Difco) was prepared according to Standard Methods (2). The broth was prepared no more than four hours prior to use. Solid M-FC was prepared by the addition of 1.5% agar (Difco). This was dispensed in seventeen ml quantities into sterile glass petri dishes.

Overlay medium

The overlay medium was prepared according to the method described by Stuart, et. al. (46). It consisted of the overlay portion of their IM-MF medium. Five ml quantities were dispensed after autoclaving into the same type of plates used for the M-FC broth and allowed to solidify. The overlay medium was used as the index of the total viable population in the plate count procedure. The same medium without agar

was used in the recovery experiments. The overlay plates were prepared the night before use and refrigerated or the day of use.

Dulbecco's modified Eagle medium

Eagle's medium (International Scientific Ind., Inc., Cary, IL) was prepared with one-half the amount of water directed by the manufacturer, filter sterilized through a 0.22 um filter (Millipore), and then mixed with an equal volume of autoclaved water plus 1.5% agar (Difco). The medium was dispensed in five ml volumes into sterile plastic petri dishes.

Mineral salts medium

Mineral salts medium was prepared (47), filter sterilized using a 0.22 um filter (Millipore), and refrigerated.

Desoxycholate lactose agar

Desoxycholate agar (DLA) (Difco) was prepared as directed by the manufacturer and utilized in recovery experiments as a selective medium.

Preparation of glassware and utensils

All glassware was machine washed, rinsed with distilled water, and air dried. Items that were acid washed were soaked in 10% HCl for a minimum of thirty minutes, rinsed six times with tap water followed by three rinses with single distilled water and one with double

distilled water or Milli-Q reagent grade water (Millipore), and dried. Glassware sterilized by autoclaving was covered with aluminum foil and processed for fifteen minutes at fifteen pounds pressure. Pipettes and glass petri dishes were placed in metal cans or boxes and oven sterilized at 350 F for three hours.

Cell enumeration and cell density procedures

Membrane filter technique

The membrane filter technique for the enumeration of fecal coliforms as stated in Standard Methods (2) was followed. Millipore type HC filters were used throughout this procedure. The media used (M-FC, overlay, Eagle's) were dispensed in 48 x 8.5 mm tight fitting plastic plates (Millipore). Duplicate plates of two or more dilutions per sample were performed with each medium. Plates incubated at 35 C (overlay and Eagle's media) were held in a circulating air incubator while a block incubator (Millipore) and circulating water bath at 44.5 C were used for the M-FC plates. M-FC plate incubation began within ten minutes of filtering and proceeded for twenty-four hours. Colony counts were made with a binocular microscope at fifteen x magnifications.

Pour plate procedure

This procedure was performed as suggested for the standard plate count in Standard Methods (2). Overlay and DLA pour plates were incubated at 35 C. M-FC pour plates were incubated for twenty-four hours

in a 45 C circulating air incubator. Colony counts were determined with a New Brunswick colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Surface plate procedure

Depending upon the experiment, overlay, M-FC and/or DLA plates were poured and allowed to solidify. One or 0.1 ml samples were pipetted onto the surface of the agar, spread with a sterile bent glass rod, overlayed with five ml of the same medium, and incubated as described above.

Spectrophotometric determination

Cell population was determined using the Varian Techtron spectrophotometer model 635 at 420 nm.

Field chlorination

Organism preparation

The EC+ *E. coli* culture was grown for twelve hours at 35 C in TSY broth. The cells were centrifuged at 3020 x g for ten minutes and resuspended in peptone-phosphate buffer. This procedure was repeated twice with the final suspension in sterile water. Organism concentrations of approximately 5×10^5 and 6×10^9 per ml were used. The suspensions were iced and transported to the field site.

Membrane diffusion chambers

Membrane diffusion chambers developed by McFeters and Stuart (32) were utilized for the field chlorination studies. Tear resistant micro-web filters (Millipore, WHWP 304 F1) were cut in circles and used. These membranes were sterilized by U.V. light for fifteen minutes per side. The chamber and dust caps were sterilized prior to assembly by autoclaving at fifteen pounds pressure for ten minutes. Chambers were assembled in the laboratory prior to transport.

Experimental location and sampling procedure

The chambers were loaded with a twenty ml sample using a sterile syringe and suspended in an eddy in the discharge ditch approximately five feet from the source of the effluent at the Bozeman Wastewater Treatment Plant. Immediately upon immersion a one ml sample was withdrawn with a sterile one ml syringe and labeled as the zero time sample. Timing began at that point and samples were withdrawn at intervals previously determined for each experiment. Before sample removal, the syringe was pumped ten times to ensure mixing of the chamber contents.

Samples taken from the chambers were deposited in a ninety-nine ml peptone-phosphate dilution blank containing one ml of a 10% sodium thiosulfate solution and shaken. If several samples were taken during the experiment, they were carried to the laboratory trailer at the

treatment plant where plating was performed. M-FC plates were incubated immediately after filtration in the portable black incubator. All plates were transported back to the laboratory at the termination of the experiment for incubation and counting.

Chlorine determinations

At the time the chamber was suspended in the effluent a sample of effluent was collected and taken to the treatment plant laboratory where chlorine concentrations were determined by the iodometric procedure described in Standard Methods (2). Samples were also taken during the experiment and at the termination in some instances.

Laboratory chlorination with membrane diffusion chambers

The procedure for the laboratory membrane diffusion chamber technique was a modification of the field chlorination studies in that the chambers were suspended in a five gallon plastic bucket. The bucket was filled with double distilled water and chlorine bleach (Chlorox) added to give a final concentration of 1.5 and 5.0 mg/l.

Laboratory chlorination

Preparation of the bleach solution

Commercial chlorine bleach (Chlorox) was purchased at least monthly during the course of the experiments and stored at refrigerator temperature as it has been demonstrated that the sodium hypochlorite

will dissipate with time. Chlorox was used as it has a 5.25% sodium hypochlorite concentration initially. A new stock solution was made each day immediately before it was to be used. For most of the chlorinations a 0.5 mg/l final concentration was desired, therefore, a 500 mg/l stock solution was prepared by diluting one ml of the bleach with ninety-nine ml of chlorine demand free water in a volumetric flask. One ml of this stock solution was then transferred to one liter of bacterial suspension in the chlorination vessel.

Preparation of the experimental chlorination vessel

A volume of 900 ml of sterile chlorine demand free water was added to a sterile, acid washed, foil covered two liter capacity DeLong flask containing a magnetic stir bar. The water was stirred constantly at a low speed. A styrofoam pad and wooden block were inserted between the flask and stirrer to reduce heat transfer from the stirrer to the water. One hundred ml of the washed E. coli culture were added to the flask and mixed thoroughly before the addition of the chlorine.

Sampling procedure

After the organisms were added to the flask and allowed to mix, the control organisms were removed and the one ml sample was transferred to a ninety-nine ml dilution blank containing iced peptone-phosphate buffer. One drop of a 10% sodium thiosulfate solution was added and the blank shaken well. One ml of the chlorine stock solution was then

added and timing commenced. At timed intervals one ml samples were removed and treated the same way as the control sample. All samples were iced until they could be filtered. Cell counts were done by the membrane filter technique and absorbances determined with these samples. When large volumes of sample were required, the aliquot was poured into a sterile flask containing 10% sodium thiosulfate solution, agitated, and iced. For a 200 ml volume, 0.5 ml of the sodium thiosulfate solution was used, and for a 400 ml sample 1.0 ml was used.

Preparation of the culture for chlorination

Cell preparation was the same as that for the field membrane diffusion chamber experiments except that final suspension was in a volume of chlorine demand free water equal to that of the growth medium (100 ml) to give a final concentration of approximately 7×10^9 organisms per ml. If a cell count of approximately 7×10^5 was used, the appropriate dilution was made with chlorine demand free water. When a large amount of control organisms were required two flasks of bacteria were grown and prepared as above. One 100 ml portion was added to the chlorination vessel and a 1:10 dilution made of the remaining organisms to give a desired quantity of control suspension and then iced.

Recovery experiments

Experiments were performed to determine if the chlorine injured organisms were capable of recovering if exposed to a suitable environ-

ment. Recovery experiments involved the use of organisms that had been exposed to chlorinated effluent for 2½ hours at the treatment plant or those that had been chlorinated by the laboratory procedure for eight minutes.

In the first group of experiments, treatment plant chlorinated organisms were transferred to the laboratory as previously described. The control organisms were retained in the refrigerator during the chlorination time. Four ml of the control and chlorinated bacteria were placed in separate flasks containing 36 ml of overlay broth. The zero time samples were removed and the flasks incubated at 35 C. Samples were removed hourly for six hours and plated by the surface plate method on DLA and overlay media. Incubation was at 35 C.

For the second group of experiments the procedure was changed and M-FC surface plates replaced the DLA plates.

A similar experiment was done where M-FC and overlay pour plates were used and incubated at 35 C as before. Another experiment was performed in which the M-FC plates were incubated at 45 C.

The final group of experiments utilizing field chlorinated organisms involved the performance of the membrane filter technique. M-FC broth plates were incubated at 44.5 C and overlay plates at 35 C.

The studies for which the organisms were chlorinated in the laboratory involved the use of the membrane filter technique as the cell count determination procedure.

Preparation of the cells for the oxygraph and aldolase assays

The 1:10 dilutions of the control and experimental cells removed from the chlorination vessel were centrifuged at 3020 x g for ten minutes at 1 - 3 C in sterile tubes. The supernatant was discarded to rid the cells of thiosulfate or cellular debris. This method insured that only intact cells were used for the oxygraph and aldolase assays. The pellet was then suspended in a volume of 0.05 M tris buffer one-fourth that of the original volume dispensed into the tubes. In most cases a volume of 200 ml was resuspended in a volume of 50 ml. The concentrated cells were then placed in fifty ml glass stoppered, acid washed flasks and immediately iced. Oxygraph experiments were done immediately after the organisms were concentrated. The concentrated cell suspension was then refrigerated over night to be disrupted by sonication the next morning for the aldolase assay.

Aldolase assay and protein determination

Sonication of the cells

It was determined through experimentation that the sonication time needed for 98% disruption of the cells used was fifteen minutes with the Bronwill Biosonic sonicator set at 90% of maximal intensity. This amount of sonication was accomplished in bursts of three minutes each with cooling periods allowed between sonications. A three minute burst resulted in a temperature rise of approximately 16 C if a volume of

forty ml was sonicated. To prevent enzyme denaturation the cell suspension was allowed to cool in an ice bath until it had reached 1 - 3 C before it was again treated by sonication. This sonicate was diluted 1:10 with tris buffer for the aldolase and protein determinations.

Protein determination

The protein determination of Lowry, et al. (30) was done with the crude extract. A standard curve utilizing bovine serum albumin was performed in conjunction with each protein determination. Absorbance readings were taken at 625 nm in the Varian spectrophotometer.

Aldolase assay

The aldolase assay used was a modification of the hydrazine assay as described by Jagannathan (24). Reaction progress was followed in the Varian spectrophotometer at 240 nm. The automatic chart was set at 10 mV and a speed of 3½ cm/min. The reaction was monitored for 3½ minutes. Temperatures of 35 or 45 C were maintained around the reaction cuvette by means of a Heto ultrathermostat model 623 EUL circulating heater. The hydrazine sulfate solution used was allowed to reach room temperature prior to use in an attempt to minimize bubble formation on the cuvette walls. The fructose 1,6-diphosphate solution and sonicated cells were kept on ice. Triplicate readings were taken for each sample.

Oxygraph assay

Oxygraph assays were performed with a Gilson Oxygraph model KM equipped with a YSI model 4004 Clark oxygen probe. Constant temperature was maintained by a Heto ultrathermostat model 623 at 35 C. The substrates used were kept at room temperature. One ml of the concentrated cells which had been kept on ice was pipetted into the reaction chamber and allowed to equilibrate for one minute and one ml of substrate was then added and allowed to equilibrate for thirty seconds. The chart was then started and the course of oxygen uptake followed for three minutes. This was duplicated for each organism with each medium.

Media used in the oxygraph experiments included mineral salts medium, mineral salts medium plus 2% glucose, mineral salts medium plus 2% glucose plus 0.025% glutathione and 0.025% sodium thioglycollate, mineral salts medium plus 2% glucose and 0.025% glutathione and sodium thioglycollate plus 0.01% yeast extract, and TSY.

ATP determinations

Cells used for the oxygraph experiments were extracted by a modification (48) of the Bancroft, et al. procedure (3). ATP determinations were made in a DuPont Luminescense Biometer.

Labeled glucose and algal progein hydrolysate experiments

Preparation of the radioactive material

D-glucose [^{14}C (U)] with a specific activity of approximately 5.0 mCi/mmole (New England Nuclear, Boston, MA) was ordered in 0.05 mCi amounts. The glucose was dissolved in 10.2 ml double distilled water and two ml volumes were dispensed into vials. The vials were stoppered and frozen. Just prior to use one vial was thawed, two one ml quantities removed with one ml syringes and the syringes refrigerated if there was a slight delay before dispensing. A one ml quantity was used for each flask of organisms used in the experiments.

Algal protein hydrolysate [^{14}C (UL)] with a specific activity of 1.0 mCi/mmole (International Chemical and Nuclear Co., Irvine, CA) was ordered in 0.05 mCi amounts. Preparation and utilization was identical to the afore mentioned procedure.

Liquid scintillation procedure

Air dried filters were placed in a 105 C oven for twenty minutes to ensure complete dryness. The filters were rolled and placed in poly-Q scintillation vials (Beckman Instruments Co., Irvine, CA), made transparent with four ml of toluene, and nine ml of Aquasol liquid scintillation cocktail was added (New England Nuclear). The vials were shaken, wiped clean of fingerprints, and placed in a Beckman L.S.C. 100 counter set at 5% error, one hundred minutes termination time, and single automatic cycle. The counter was programmed to perform labeled carbon counts and external standards on each vial.

Pre-chlorination experimental design, long term labeling

Two flasks, each containing 100 ml TSY broth, were inoculated with the experimental organism and incubated for ten hours at 35C. One ml of labeled glucose was added to each flask and incubation was continued for one hour. One flask of organisms was prepared and chlorinated as previously described in the laboratory chlorination section. The other flask of organisms was prepared as described for the control organisms. One hundred ml of the chlorinated cells and 1:10 diluted control cells were centrifuged at 3020 x g for ten minutes and resuspended in one hundred ml of TSY at 35 C. Ten ml zero time samples were removed, filtered through a type HC Millipore filter, rinsed with chilled buffer, and allowed to air dry. These were used to determine radioactivity in the scintillation counter. At the same time, one ml samples were taken from the flasks and added to chilled ninety-nine ml peptone-phosphate buffer blanks. Appropriate dilutions were made and the membrane filter technique employed to determine cell numbers on M-FC and overlay media. Turbidities at 420 nm were also determined. The flasks were incubated at 35 C in a water bath during the experiment. Samples were taken at fifteen minute intervals during the first hour and at twenty minute intervals during the second hour.

Pre-chlorination experimental design, pulse labeling

The cell preparation was identical to the above with the exception that the exposure time to the labeled glucose was ten minutes instead of one hour. Ten ml samples were removed for radioactive analysis and absorbance readings at 0, 15, 30, 45, 60, 80, 100, and 120 minutes. Plate counts were performed with samples taken at 0, 30, 60, and 80 minutes by the membrane filter technique. The pre-chlorination algal protein hydrolysate experiments were identical to this.

Post-chlorination experimental design

In this group of experiments the organisms were grown in TSY as before, half were chlorinated by the laboratory method for eight minutes, the control cells diluted 1:10, 400 ml of control and chlorinated cells spun down at 3020 x g for ten minutes, resuspended in one hundred ml 35 C TSY broth plus one ml prepared labeled glucose or algal protein hydrolysate, and allowed to incubate at 35 C for ten minutes. The supernatant was discarded and the cells washed twice with chilled peptone-phosphate buffer. After the final centrifugation they were resuspended in 35 C TSY broth, zero time samples removed as previously described, placed in a 35 C water bath, and timing commenced. Ten ml samples were removed at five minute intervals for radioactive and absorbance analysis.

Chapter 3

RESULTS

Field Chlorination

The objectives of the project necessitated the development of a dependable and reproducible method by which organisms could be chlorinated. The first chlorination and injury experiments were performed in the chlorinated effluent at the Bozeman Wastewater Treatment Plant. Bacterial suspensions were removed from membrane diffusion chambers which had been immersed in chlorinated effluent and plated on selective and nonselective media to determine the extent of bacterial injury. The data in Figure 1 represent the desired result. The results showed the population exhibiting a difference in counts on selective (M-FCO and nonselective (overlay) media beginning after one hour exposure to the chlorinated effluent, increasing to a maximal difference at 2½ hours, and then going into a death stage in which the counts on the two media were not different and dropped rapidly. A log or more separation in counts on selective and nonselective media at a predictable time was required for the performance of later experiments. Thirteen experiments with an initial cell population averaging 4.6×10^5 per ml and fourteen of an initial population of 5.6×10^9 per ml were performed. Figure 2 presents the data obtained on four dates. The numerical data for both the M-FC and overlay counts for these experiments are given in Table 1. The initial counts (Figure 2) were comparable, but no

Table 1. Initial chlorine levels and plate count data obtained on M-FC (selective) and overlay (nonselective) media at timed exposure to chlorinated effluent on four dates using membrane diffusion chambers.

Date	Initial Chlorine mg/l	Medium	Plate count 0 hr	1 hr	1½ hr	2 hr	2½ hr	3 hr	3½ hr	4 hr
8-19-75	.65	M-FC	$3.4x10^4$	$3.0x10^4$	$3.1x10^4$	$1.3x10^4$	$4.5x10^3$	$7.3x10^3$	$1.0x10^0$	
		Overlay	$4.1x10^4$	$4.8x10^4$	$5.2x10^4$	$4.5x10^4$	$5.9x10^4$	$2.4x10^3$	$3.9x10^2$	$5.0x10^1$
9-9-75	.97	M-FC	$5.8x10^4$	$4.4x10^4$	$3.8x10^4$	$2.2x10^4$	$2.0x10^4$	$1.6x10^4$	$2.0x10^4$	$3.2x10^3$
		Overlay	$5.6x10^4$	$4.5x10^4$	$4.5x10^4$	$4.5x10^4$	$5.0x10^4$	$2.1x10^4$	$2.4x10^4$	$4.3x10^3$
9-18-75	.70	M-FC	$4.6x10^4$	$1.1x10^4$	$4.9x10^4$	$3.1x10^4$	$2.5x10^3$	$2.4x10^3$	$1.0x10^2$	$1.0x10^0$
		Overlay	$5.2x10^4$	$2.4x10^4$	$2.3x10^4$	$2.0x10^4$	$1.8x10^4$	$1.2x10^4$	$1.0x10^2$	$1.0x10^0$
9-23-75	.75	M-FC	$4.8x10^4$	$2.0x10^4$	$1.1x10^4$	$4.3x10^3$	$4.3x10^3$	$1.8x10^3$	$1.0x10^0$	
		Overlay	$5.2x10^4$	$3.1x10^4$	$2.4x10^4$	$2.2x10^4$	$1.9x10^4$	$7.3x10^3$	$1.0x10^0$	

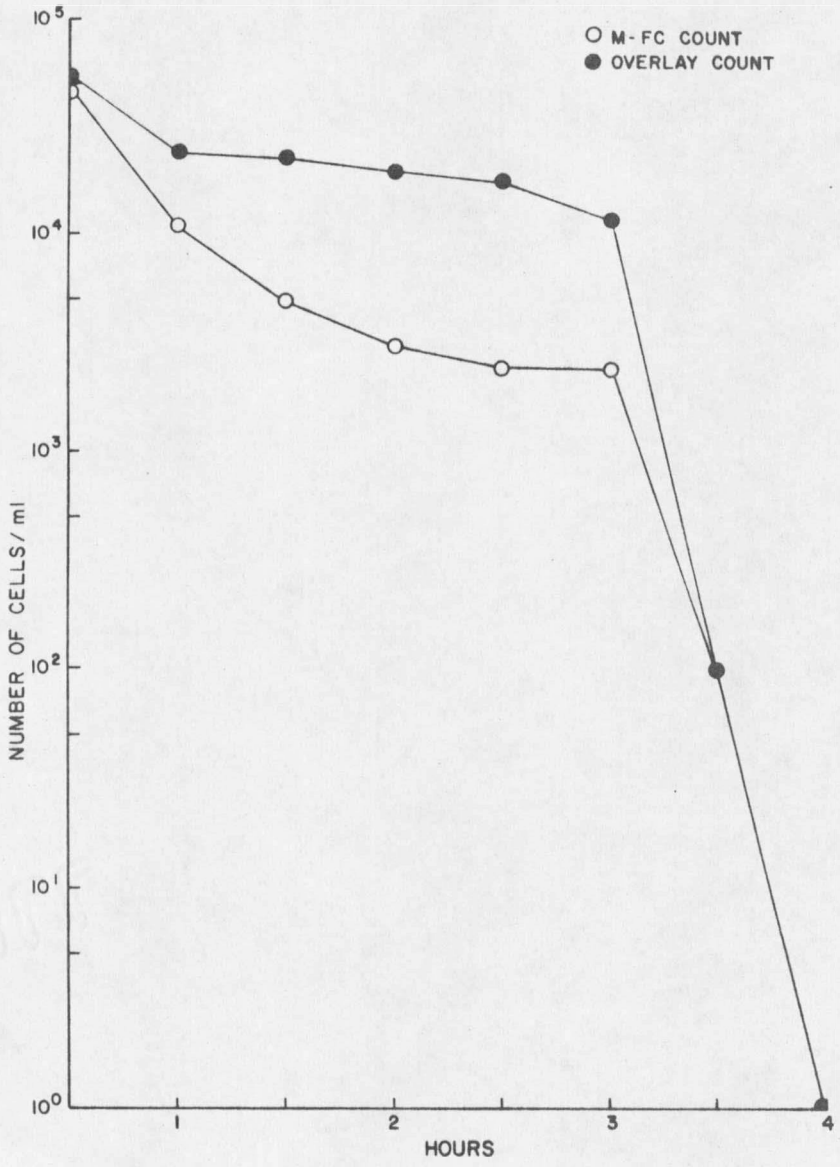

Figure 1. Progression of injury and die-off of E. coli suspended in chlorinated effluent in a membrane diffusion chamber using M-FC and overlay media.