**MONTANA STATE UNIVERSITY**

Copper injury of Escherichia coli
by Matthew Joseph Domek

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
Low levels of copper in chlorine-free distribution water caused injury of coliform populations. Monitoring of 44 drinking water samples indicated 64% of the coliform population was injured. Physical and chemical properties were measured including the concentrations of three heavy metals (copper, cadmium, and lead). Copper concentrations ranged from 0.007 to 0.54 mg/liter. Statistical analysis of all factors was used to develop a model to predict coliform injury. The model predicted almost 90% injury using a copper concentration near the mean observed value (0.158 mg/liter) in distribution waters. Laboratory studies using copper concentrations of 0.025 and 0.050 mg/liter in a carbonate buffer under controlled conditions of temperature and pH showed over 90% injury in 6 and 2 days, respectively.

Escherichia coli injured in drinking water or by copper showed decreased oxygen utilization. Oxygraph measurements of cells injured in drinking water showed 73 to 83% decrease in oxygen utilization when cells were injured 70 to 99.5%. Copper-injured cells had less than 25% the rate of oxygen utilization of the control cells. Respirometry experiments measured rates over a longer period of time and showed similar trends.

13Carbon-Nuclear Magnetic Resonance Spectroscopy (13C-NMR) and gas chromatography were used to identify differences in metabolism between healthy and injured populations of E. coli. The rate of glucose utilization by injured cells under anaerobic conditions was 64% of the healthy cells. The rates of accumulation of lactate and ethanol were 88 and 50% of the control, respectively. 13C-NMR studies of oxygenated cultures revealed differences in the accumulation of acetate and lactate. A large pool of glutamine accumulated in healthy but not injured cells. Additional studies revealed injured cells had a decreased ability to reduce 2-(p-iodopheny1)-5-phenyl tetrazolium chloride (INT) with a variety of carbohydrate substrates. When NADH was used as a substrate, injured cells reduced greater quantities of INT than the healthy cells. Copper-injured E. coli appeared to have impaired respiration as indicated by decreased INT reduction and other respirometric indices. A comparison of metabolic end products suggested that injured cells also had considerable differences in carbon flow compared with healthy cells indicating that the injured cells were being repaired.

COPPER INJURY OF <u>ESCHERICHIA</u> <u>COLI</u>


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APPROVAL

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Matthew Joseph Domek


    This thesis has been read by each member of the thesis
committee and has been found to be satisfactory regarding
content, English usage, format, citations, bibliographic
style, and consistency, and is ready for submission to the
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# ABSTRACT

Low levels of copper in chlorine-free distribution water caused injury of coliform populations. Monitoring of 44 drinking water samples indicated 64% of the coliform population was injured. Physical and chemical properties were measured including the concentrations of three heavy metals (copper, cadmium, and lead). Copper concentrations ranged from 0.007 to 0.54 mg/liter. Statistical analysis of all factors was used to develop a model to predict coliform injury. The model predicted almost 90% injury using a copper concentration near the mean observed value (0.158 mg/liter) in distribution waters. Laboratory studies using copper concentrations of 0.025 and 0.050 mg/liter in a carbonate buffer under controlled conditions of temperature and pH showed over 90% injury in 6 and 2 days, respectively.

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## INTRODUCTION

### Isolation of Coliform Bacteria from Water

The evaluation of potable water supplies for total coliform bacteria is important in determining the sanitary qualitity of drinking water. Elevated levels of coliform bacteria indicate a contaminated source, inadequate treatment, or post-treatment deficiencies. The occurrence of injured coliform bacteria presents a significant problem in accurately estimating the number of indicator organisms in drinking water. Previous reports have documented that indicator bacteria become injured in drinking water (3) and surface water (8) and such injured bacteria are not enumerated when currently accepted media are used (37).

In general terms bacterial injury is defined as the inability of bacteria to grow under specified conditions that are satisfactory for uninjured cells (12). This may be assessed in different ways such as: measuring differences in the ability of bacteria to form colonies on minimal media versus complex media or loss of capability to grow in the presence of a selective agent (12). Sodium deoxycholate was used as a selective agent in this study since it is used in many different types of media used in

aquatic bacteriology and is known to prevent the growth of injured coliform bacteria (61).

Coliform bacteria may become injured by exposure to a variety of stressful factors in aquatic environments including: chlorine (11,13,40), heat (15,24,32), freezing (41,57), acid mine drainage (22), sunlight (25), and heavy metals (42, Domek, M.J., M.W. LeChevallier, and G.A. McFeters, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q6, p. 261). Injury of coliform bacteria by chlorine has been well-described (11,13,40) and is likely the major cause of damage to indicator organisms in drinking water. Metals can be another important cause of bacterial injury because of their common occurrence in drinking water (49,59).

Part A of this study was done to determine the role of metals in injury of _Escherichia_ _coli_ under conditions simulating municipal drinking water distribution systems. To accomplish this it was necessary to show that injured coliform bacteria could be found in drinking waters where chlorine was not present. In this part of the study, physical and chemical characteristics of water were assessed to determine the role of various factors in injury caused by distribution water. Laboratory experiments were done to test whether metals could cause injury at concentrations predicted from the physicochemical analyses of the water in which injury was observed. Strains of _E. coli_ isolated from water were used in the experiments as

representatives of the coliform bacteria. The findings
that were reported in part A supported the conclusions that
metals, in particular copper, contributed to injury and
caused significantly reduced isolations of coliform
bacteria.

## Metabolism of Injured Bacteria

Differences in bacterial growth on selective and non-
selective media reveal little specific information about
the nature of the bacterial injury caused by various
stresses. However, a considerable amount of literature is
available on cellular damage in injured bacteria caused by
treatments related to food processing (12). Freeze-injured
E. coli cells release amino acids, small molecular weight
RNA, and peptides (48,58). E. coli and Salmonella
typhimurium cells that are heat-injured release lipopoly-
saccharides (24,31). Heat injury to S. typhimurium causes
RNA degradation (38,56) and alters the transport of glucose
(55). Tomlins et al. reported that ribosomal regeneration
(68), and protein (67) and lipid (69) biosynthesis are
required for the recovery of heat-injured S. typhimurium.
Despite the information available regarding bacterial
injury related to food processing little is known about
water-related injury. Zaske et al. (77) showed that the
cell envelope of E. coli is damaged by exposure to water
that is high in conductivity. During the recovery period

aquatically injured E. coli exhibits an extended lag phase (76).

The major objective of part B was to develop an understanding of the physiological processes involved when coliform bacteria were injured in aquatic environments containing metals. Physiological alterations of E. coli injured by exposure to chlorine have been reported (13,75). There is however a paucity of physiological information describing coliform bacteria that have been injured in the aquatic environment where chlorine is either not present or negligible.

## Toxic Effects of Metals on Microorganisms

There are reports about toxic effects of metals on microorganisms (for a review see Babich and Stotzky, 5). Singleton (62) observed a decrease in the diversity in a naturally occurring population of bacteria from fresh water when either mercury or copper was added to the system. A copper concentration of 0.05 mg/liter slows the growth rate of the alga, Selenastrum capricornutum, and 0.09 mg/liter of copper inhibits growth completely (6). Structural changes are observed when the alga, Ankistrodesmus braunii, and the blue green bacterium, Anabaena 7120, are grown in the presence of copper (45). Tyler reported that copper limits the rate of mineralization of nitrogen in acid forest soils (71) and decreases respiration rates of soil

microorganisms (41). A study by Albright et al. (1) showed that a copper concentration of 0.01 mg/liter doubles the rate of glucose turnover in a population of heterotrophic bacteria, and that copper and zinc toxicity is additive (2). Higher concentrations of copper are needed to cause toxic effects when the cell density or amount of organic matter in the system is increased. Lamb and Tollefson (36) showed that copper at a concentration of 5.0 mg/liter reduces by 90% the conversion of glucose to carbon dioxide in activated sludge from a sewage plant. An exposure of microorganisms in activated sludge to a concentration of 5.0 mg/liter of copper for one day causes nearly a 90% reduction in oxygen utilization (30). Malaney et al. (43) observed similar reductions in oxygen utilization when bacteria are exposed to copper concentrations in the range of 5.0 to 20.0 mg/liter.

## Approach to Studying Injured Bacteria

A variety of techniques was used in part B to assess physiological differences between the injured and non-injured populations. Respirometry experiments were done on cells injured by exposure to drinking water to investigate possible differences in energy conserving physiology between injured and non-injured _E. coli_ (Dockins, W.S., M.J. Domek, and G.A. McFeters, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, p. 179, N93). These preliminary

experiments revealed that E. coli injured in potable water
had impaired respiratory activity.  Subsequent experiments
used E. coli cells injured by copper under carefully
controlled conditions.  Injured E. coli were relatively
fragile compared to non-injured cells and did not withstand
many ordinary laboratory manipulations such as
centrifugation.  Therefore techniques involving minimal
manipulation were employed whenever possible.

An active bacterial electron transport system (ETS)
reduces 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetra-
zolium chloride (INT) to INT- formazan (53) which was
measured spectrophotometrically (48).  Injured E. coli
cells were tested for their ability to reduce this dye.

Metabolic differences between injured and healthy
organisms were obtained by using nuclear magnetic resonance
spectroscopy (NMR) to follow the fate of $^{13}$C-enriched
glucose.  This technique allowed monitoring of in vivo
metabolism under aerobic and anaerobic conditions (47).
Well-resolved spectra were obtained with 20 minute accumu-
lations of data.  This method proved to be a powerful tool
in providing information about metabolic rates and pathways
without the need for analysis by extraction of the metabo-
lites or other destructive procedures.  Also, gas chromato-
graphy was used to determine production of carbon dioxide
($CO_2$) and hydrogen ($H_2$) gases during recovery from injury.

In addition to the basic physiological contribution of this study, the information gained was used as part of a larger concurrent study conducted in the laboratory to develop a new medium (37) for the isolation and enumeration of injured coliform bateria. Ultimately it is hoped that a better understanding of aquatic injury is attained through this work and other projects (9,13,45,68,69).

## Statement of Objectives

Initial observations revealed that an E. coli suspension in a membrane diffusion chamber became injured in drinking water having less than a 0.1 mg/liter concentration of chlorine. This prompted our efforts to determine what type of physicochemical factors, other than chlorine, might cause injury in a drinking water distribution system. Additionally I sought to show that naturally occurring coliform bacteria could be isolated from distribution water thus establishing that the survival of injured coliform bacteria was not an in vitro artifact.

After determining that copper injured E. coli in drinking water, I was interested in the physiology of the injured organism. We attempted several approaches in part B. Respirometry experiments measured oxygen utilization. [13]Carbon-NMR and gas chromatography were used to study the fate of glucose. These methods were used to investigate alterations in metabolism during the recovery of E. coli

injured by copper.  INT was used to analyze electron transport activity.  The physiological data were also used to delineate the metabolic lesion(s) caused by copper.

## Materials and Methods

### Organisms

The E. coli strains used in these studies were isolated by membrane filtration from the East Gallatin River near Bozeman, Montana. The isolates were identified and maintained as previously described (77).

### Preparation and Laboratory Injury of Bacteria

Cultures were grown and prepared as before (45). Injury was assessed by differential counts of colony forming units (CFU) on the non-selective TLY (Trypticase Soy Broth without dextrose, Difco, 27.5 g; Lactose, 10.0 g; Yeast Extract, Difco, 3.0 g in 1.0 liter Milli Q water, Milli Q Reagent Grade Water Systems, Millipore) agar (15 g/liter, W/V), and selective TLY-D agar (TLY with 0.1 % [W/V] sodium deoxycholate) (45). The percent of injury was calculated as follows:

$$\frac{(CFU\ on\ TLY) - (CFU\ on\ TLY\text{-}D)}{CFU\ on\ TLY} \times 100 = \%\ Injury$$

Injured populations were obtained for some experiments by placing washed cells into membrane diffusion chambers (46) immersed in water drawn from a lightly used portion of the Bozeman, MT, drinking water distribution system (45).

All glassware needed for preparation of cells injured by copper was washed with nitric acid. Copper-injured populations were obtained by placing washed cells (approximately $1.0 \times 10^5$ CFU/ml) in $CaCO_3$ buffer and adding to the buffer various amounts of copper from a stock ($CuSO_4$) solution. $CaCO_3$ buffer was prepared by adding 0.168 g $CaCO_3$ to 1 liter Milli Q water, undissolved. After autoclaving and cooling to room temperature, $CaCO_3$ was dissolved by lowering the pH by adding 1N sulfuric acid (4.0 ml/liter) and then raising it by adding 1N sodium hydroxide to pH 7.0. The buffer solution was added to 125 ml glass-stoppered bottles. The bottles were sealed with silicone stopcock grease to maintain the pH after adding the copper solution and bacterial suspension.

Since physiological experiments were run over a relatively short time interval, high cell concentrations were used. All copper-injured cells used for physiology experiments were prepared using a copper concentration of 1.875 mg/liter and a cell concentration of approximately $1 \times 10^9$ CFU.

## Physicochemical Analysis of Water

Water samples collected in sterile bottles were placed on ice, transported to the laboratory within 1 hour, and analyzed within 5 hours of collection. A complete description of sampling locations and coliform enumeration methods

has been previously published (37). Chemical and physical
analyses were done using published methods (28).

## Copper Analysis

Water samples were acidified by adding 0.1% nitric acid
(v/v) and sodium nitrate was added as an electrolyte to
final concentration of 0.1 M. An average of 3 replicates
was used to determine copper concentrations by the
differential pulse polarographic method using a model 374
Polarographic Analyzer (Princeton Applied Research,
Priceton, New Jersey) (62,63).

## Cadmium and Lead Analysis

The concentrations of these metals were analyzed in a
Woodriff Furnace Atomic Adsorption Spectrometer (33). The
average of 4 peak area measurements from replicate samples
were integrated to calculate each concentration.

## Statistical Analysis

Statistical analyses including Pearson correlation
coefficients and multiple linear regressions were done on a
Honeywell CP-6 computer equipped with a Statistical Program
for the Social Sciences (SPSS). The regression model was
developed according to published techniques (50) with the
help of statistical consultant, Georgia Ziemba. Injury was
calculated for statistical analysis as the difference

between verified coliform counts on m-T7 agar and verified coliform counts on m-Endo agar (21).

## Oxygen Uptake Analyses

Oxygen uptake measurements of E. coli injured in drinking water were done using a Gilson model KM Oxygraph equipped with a model Y.S.I. 4004 Clark-type electrode. The chamber of the instrument was maintained at 30°C. A cell suspension (1.8 ml of 5.0 X $10^8$ CFU/ml) in drinking water was added to the chamber and allowed to equilibrate for 2 minutes followed by the addition of 0.2 ml of phosphate-buffered (pH 7.0) substrate (glucose or lactose; 1%, w/v). The oxygen uptake rates were determined graphically from the slope of the lines as compared with controls without cells (13) as explained in Appendix A.

Oxygen uptake measurements of copper-injured E. coli were done similarly using a Gilson model 5/6H Oxygraph equipped with a model Y.S.I. 5331 Clark-type electrode. Substrate solutions were phosphate-buffered (pH 7.0) 10 mM (final concentration) glucose or lactose.

## Manometric Methods

Additional oxygen uptake experiments were done using a Gilson Differential Respirometer model IGP-14. The center well of each flask contained a wick with 0.5 ml of 40% KOH solution (w/v). The sidearm contained 1.0 ml of TY broth

(the same as TLY broth without the lactose) and 0.5 ml of
100 mM glucose solution. Flasks contained 1.0 ml
suspension of approximately 5.0 X $10^8$ CFU/ml E. coli, 2.0
ml Milli Q water, and 0.5 ml of mineral salts (MS) solution
(pH 7.0) containing: $K_2HPO_4$, 7.0 g; $K_1H_2PO_4$, 3.0 g; Na
citrate 2 $H_2O$, 0.1 g; $MgSO_4$ 7 $H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0
g in 500 ml Milli Q water (53). After 20 minutes of
temperature equilibration the contents of the sidearms were
tipped into each flask. Replicate flasks (3 or 4) were
shaken (100 rpm) at $30^oC$ and oxygen utilization was
measured at intervals of 10 minutes (19).

## NMR Methods

A 1.8 ml suspension of E. coli cells (adjusted to
approximately 5 X $10^8$ CFU/ml) was added to a 10 mm NMR tube
containing 7.2 mg of [1-$^{13}$C] glucose (MSD isotopes) (20 mM
final concentration) or 4.7 mg of [2$^{13}$C] succinate (Stohler
Isotope Chemicals) (20 mM final concentration). Volumes of
0.1 ml MS solution and 0.1 ml TY4 broth (same as TLY broth
but 4 times the concentration) were added to 1.8 ml of a
cell suspension as buffer and additional nutrients. Oxygen
(or nitrogen for anaerobic conditions) was bubbled through
two coaxial tubes. The inner tube near the bottom of the
NMR tube bubbled at a rate of 5 ml of gas per minute
(Figure 1). The outer tube was placed just beneath the
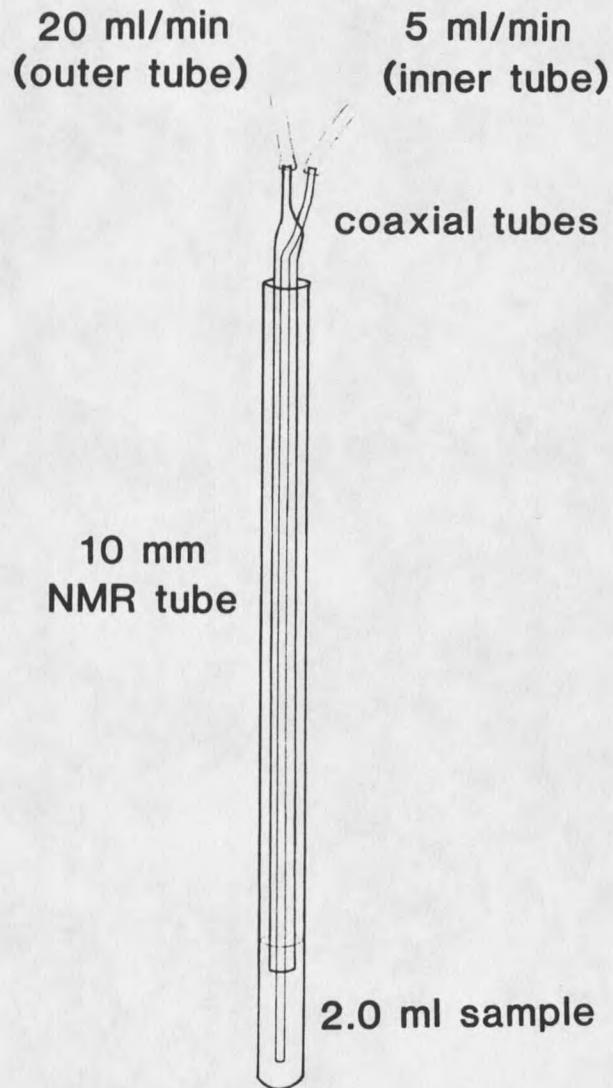surface of the 2.0 ml sample and bubbled at rate of 20 ml

20 ml/min
(outer tube)

5 ml/min
(inner tube)

coaxial tubes

10 mm
NMR tube

2.0 ml sample

Figure 1.    Apparatus for bubbling gas into a 10 mm NMR
             tube containing a suspension of E. coli cells.

of gas per minute (73). The $^{13}$C-NMR spectra of 62.83 MHz were obtained in Fourier transform mode on a Bruker WM-250 spectrometer (72). Chemical shifts of $^{13}$C-containing compounds were compared relative to tetramethylsilane used as a zero ppm standard. The alpha C-1 resonance of glucose at 92.4 ppm was used as an internal reference. A repetition time of 1.01 seconds and 45 degree pulse width were used. Data (1200 scans) were collected every 20 minutes (Appendix B).

## Gaseous End Products of Metabolism

A suspension of E. coli cells (3.6 ml, 5 X 10$^8$ CFU/ml) was added to 25 ml Erlynmeyer flasks that were fitted with No. 1.0 butyl rubber stoppers and gassed with nitrogen to purge the oxygen for anaerobic experiments. MS solution (0.2 ml) and TY4 broth (0.2 ml) were added. These flasks were incubated on a rotary shaker (100 rpm) at 35°C. Experiments were stopped at various time points by adding 1.0 ml of Zap solution (51) which consisted of 50% formaldehyde and 50% phosphoric acid (v/v).

Hydrogen was measured by removing part of the head space with a gas tight syringe (Becton, Dickinson) fitted with a Minvert valve (Supelco) and injecting the sample into a Carle model 8500 gas chromatograph (GC) having a thermal conductivity detector. The GC was equipped with a stainless steel column (2.3 meters by 3.18

mm O.D.) packed with Poroak N 80-100 mesh (Supelco). The flow rate of helium was 21 ml of gas per minute. Area unit responses were converted to umole concentrations with a Spectra Physics model 4100 integrating computer standardized against known concentrations of hydrogen (66). Afterward carbon dioxide was analyzed similarly using helium as a carrier gas at a flow rate of 21 ml per minute.

### Respiration Measurements with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride

A 0.2 ml volume of cell suspension (approximately 5 X $10^8$ CFU/ml) was added to 1.3 ml MS solution, 0.5 ml 0.2% (w/v) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution, and 1.0 ml of substrate solution (4,51). All substrates were 0.28 M and dissolved in Triton X-100 solution. The Triton X-100 solution was made by adding 1.8 ml of a 10% solution (w/v) of Triton X-100 to 98.2 ml of Milli Q water. NADH solution was made by adding 10 mg NADH to 10 ml of Triton X-100 solution. The mixtures were incubated in triplicate at $35^\circ$C on a rotary shaker (100 rpm) for 45 minutes and killed with 1.0 ml of Zap solution. The amount of INT-formazan produced was estimated spectrophotometrically at 490 nm (53) against a standard curve made with INT-formazan dissolved in 95% ethanol and diluted in MS.

The glucose preincubation experiments were done by adding 0.2 ml cell suspension, 1.3 ml MS solution, 0.25 ml 80 mM glucose solution, and 0.25 ml of TY broth and the mixture was incubated for 45 minutes on a rotary shaker at 100 rpm. Then, 0.5 ml of INT solution and 0.5 ml of double strength Triton X-100 solution were added and the mixture was incubated for an additional 45 minutes. The cells were killed and analyzed as above.

## Results

### Part A - Injury in Relation to Water Chemistry

Preliminary experiments exposing E. coli in membrane diffusion chambers (46) to distribution water with negligible chlorine concentrations demonstrated greater than 90% injury over a 5 day period (Figure 2). This level increased to 99% with 7 days of exposure. At the same time bacterial viability (CFU on the non-selective TLY medium) within the test suspension remained high (>90%) over the entire 7 day period. These inital observations prompted further studies to determine the cause of bacterial injury seen in distribution water where levels of chlorine were either low (<0.1 mg/liter) or non-existent.

A survey was done using 44 drinking water samples collected from two drinking water systems where chlorine residuals were low in order to look for relationships between injury and various physicochemical and microbiological properties. The data for these properties were the result of a collaboration among several individuals within the laboratory. The data obtained were presented in Table 1 with my contribution being the determination of the copper, lead, and cadmium concentrations. Of particular interest were the low free and total chlorine
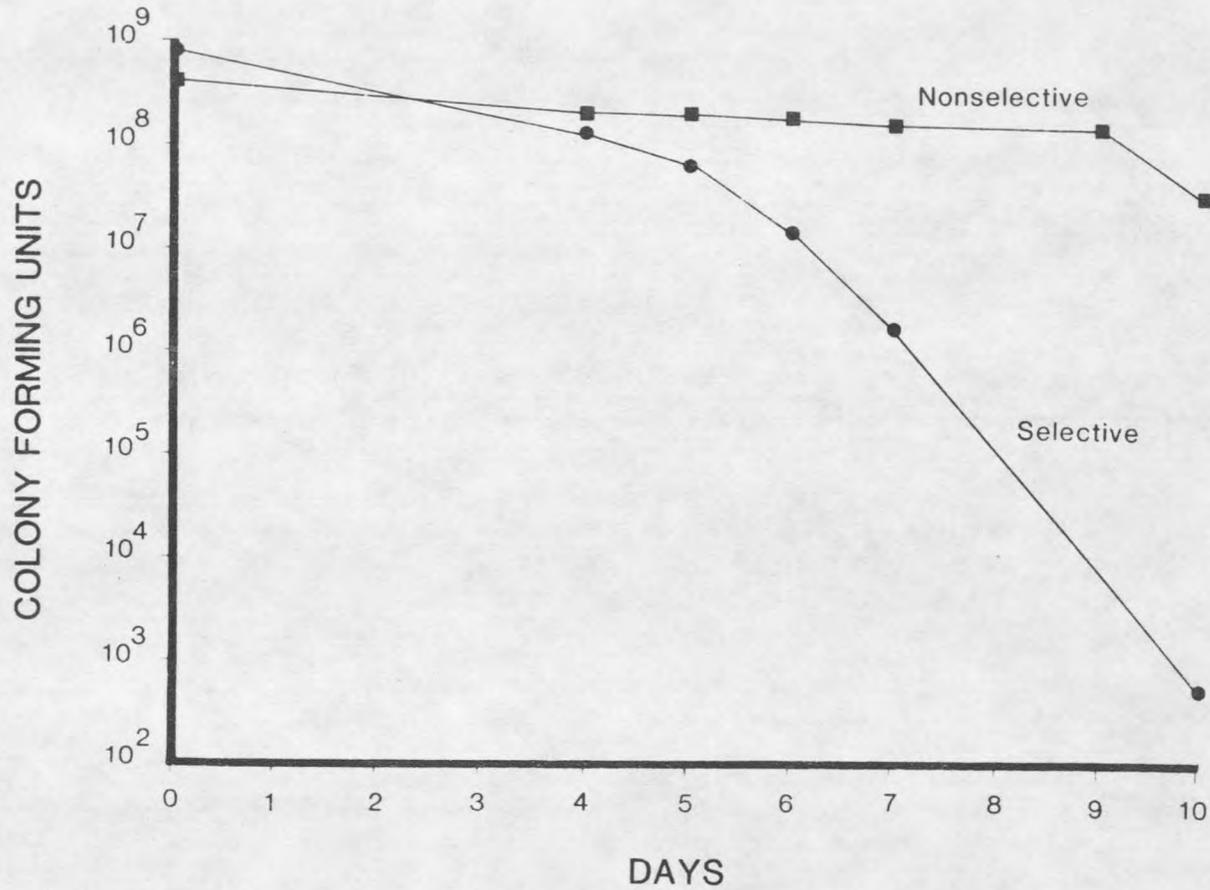
Figure 2.   Differential plate count of E. coli cells exposed to drinking
water containing less than 0.1 mg/liter of chlorine.  Cells
were plated on media that were nonselective, TLY (■), and
selective,TLY-D (●).

Table 1. Physicochemical and microbiological properties of
drinking water samples taken from several
localities in southwestern Montana.

| Microbiological, Physical or Chemical parameter | # of samples | unit of measure | Mean | Minimum | Maximum |
|---|---|---|---|---|---|
| Standard plate count bacteria | 41 | CFU's/ml | 110.8 | 1.0 | 1270.0 |
| Temperature | 44 | °C | 10.5 | 4.0 | 26.0 |
| Turbidity | 44 | nephlometer turbidity units | 0.20 | 0.01 | 4.5 |
| Conductivity | 44 | μ mho | 403.1 | 32.0 | 650.0 |
| pH | 44 | pH units | 7.7 | 7.2 | 8.4 |
| Free chlorine | 44 | mg/L | 0.15 | 0.00 | 1.50 |
| Total chlorine | 44 | mg/L | 0.18 | 0.00 | 1.60 |
| Phosphate | 44 | mg/L | .257 | 0.00 | 2.20 |
| Nitrite | 44 | mg/L | .002 | 0.00 | 0.10 |
| Nitrate | 44 | mg/L | 1.21 | 0.05 | 1.76 |
| Alkalinity[a] | 43 | mg/L | 208.9 | 171.0 | 231.0 |
| Hardness[a] | 43 | mg/L | 222.7 | 88.0 | 278.0 |
| Total organic carbon | 37 | mg/l | 18.4 | 0.0 | 66.8 |
| Copper | 36 | mg/L | 0.117 | 0.007 | 0.540 |
| Lead | 40 | mg/L | 0.008 | 0.001 | 0.088 |
| Cadmium | 40 | mg/L | 0.001 | 0.000 | 0.011 |

[a] Measured as Ca CO$_3$ in mg/L.

residuals (mean 0.15 and 0.18 mg/liter respectively), and the high copper levels (median 0.117 mg/liter). Data from this collaborative survey were analyzed for correlations between properties measured and injury observed in waterborne bacteria within those samples. Significant ($p < 0.05$) correlations are found between bacterial injury in drinking water and temperature, pH, copper, total organic carbon (TOC) as well as the sum of all metals tested (Table 2). None of the other properties listed in Table 1 show a significant simple correlation with injury in drinking water.

Injury is probably a result of complex interactions between the bacteria and the various physicochemical properties of drinking water. Simple correlations give some although limited insight into these problems. A statistical model was developed, using multiple linear regression to make a more comprehensive approach to determine the cause of bacterial injury in drinking water. All of the properties listed in Table 1 were used in building the model. Injury, however, was found to be influenced primarily by copper, temperature, pH, and alkalinity (Table 3). Among these four factors, copper is the most significant variable while the other properties probably influenced the availability and toxicity of copper ions in water. The coefficient of multiple determination, $R_2$, expressed as a percent, indicates that 50.8% of the

Table 2. Significant[a] correlation coefficients for injury in drinking water.

| Parameter | Injury[b] | | |
| --- | --- | --- | --- |
| | Correlation coefficient (r) | No. of samples | Significance (p) |
| Temperature | 0.257 | 44 | .044 |
| pH | 0.479 | 44 | <.001 |
| Copper | 0.312 | 36 | .032 |
| Total organic carbon | 0.301 | 37 | .035 |
| Metals[c] | 0.537 | 44 | <.001 |

[a] Only values significant at the 95 percent level (p $\leq$ 0.05) are presented.

[b] Injury is calculated as the difference between verified coliform counts on m-T7 agar minus the verified coliform count on m-Endo agar.

[c] The metals parameter represents the sum of copper, lead and cadmium values observed.

Table 3.  Statistical model derived to predict coliform injury.

| Model | Coefficient of Multiple Determination ($R^2$) | Number of Observations (N) | Total Squared Error (C) | F Values |
|---|---|---|---|---|
| Injury[a]= -261.7 | 0.508 | 30 | 5.7 | - |
| +150.0 (Copper) | | | | 4.82 |
| + 2.04 (Temperature) | | | | 3.48 |
| + 21.69 (pH) | | | | 2.02 |
| + 0.314 (Alkalinity) | | | | 1.19 |

a.  Injury is calculated as the difference between verified coliform counts on m-T7 minus the verified coliform count on m-Endo agar.

variation in injury was explained by the variables in the model. The total squared error for injury is 5.7. Other factors were also shown by statistical analysis to affect coliform injury. These factors included TOC, species of coliform, as well as free and total chlorine, but these properties either rarely occurred or were infrequently measured. Therefore, they could not be developed into an adequate model. Other factors that could not be measured, such as duration of aquatic exposure and the physiological state of the organism (7,14) as it entered the aquatic environment were also thought to be important.

The survey results were verified by laboratory experiments testing the capacity of copper, lead, and cadmium to cause measurable injury in a strain of E. coli isolated from water. Based on preliminary chemical analysis of water, metal levels for our first experiments were: copper, 0.25 mg/liter; cadmium, 0.017 mg/liter; and lead, 0.07 mg/liter. Initial experiments showed coliform injury occurred at these metal levels in reagent grade water, a phosphate-buffered system (pH 7.0), and in a carbonate-buffered system (pH 7.0). The carbonate-buffered system was preferred since this buffer is the dominant buffering system of most distribution waters (54) and metals were able to react without interference from phosphate or magnesium. The potential of these metals to cause injury was tested individually, and it was determined that copper

alone caused as much injury as a mixture of all the metals examined.

The progressive injury of E. coli caused by copper (0.05 mg/liter and 0.025 mg/liter) in a carbonate buffer is shown in Figure 3. Greater than 90% injury occurred in this system within 2 days ($4^{o}$C) at 0.05 mg/liter copper. After 5 days exposure to 0.05 mg/liter copper, cells demonstrated greater than 99.5% injury. Injured cells started to die with longer contact times at this copper concentration resulting in the slight apparent decrease in injury seen on day 7. At lower copper concentrations (0.025 mg/liter), injury progressed more slowly taking 6 days to reach the 90% level (Figure 3).

This experiment was repeated with 6 other strains of E. coli isolated from water. Table 4 shows the typical pattern of progressive injury and loss of viability upon exposure to copper concentrations of 0.025 mg/liter and 0.050 mg/liter with one of the seven isolates. In comparisons among isolates, 6 out of 7 isolates showed less than 90% loss in viability in 5 days upon exposure to 0.025 mg/liter of copper, whereas 4 out of 7 isolates had greater than 90% loss in viability at 0.050 mg of copper per liter. At 0.025 mg/liter of copper 6 out of 7 isolates reached 95% injury in 3 days. Increasing the concentration to 0.050 mg/liter increased the degree and rate of injury observed. Only one isolate had greater than 63% injury in 5 days in
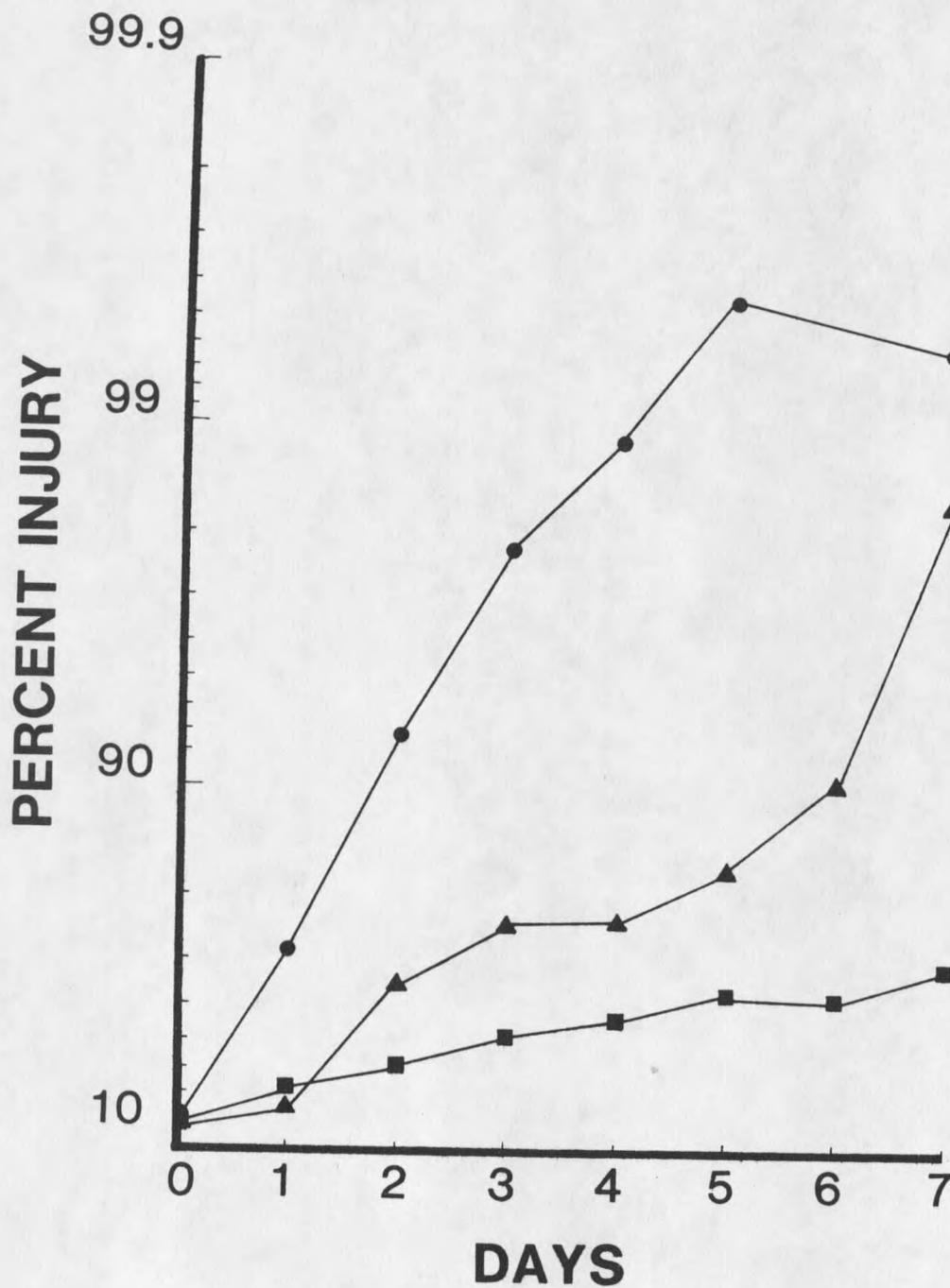
Figure 3. Injury of E. coli cells exposed to copper in carbonate buffer. E. coli (1.1 x 10$^5$ CFU/ml) was exposed to 0.050 mg of Cu/liter (●), 0.025 mg of Cu/liter (▲), and carbonate buffer (pH 7.0) (■).

Table 4. Effect of copper on viability and injury of _E. coli_, isolate No. 10.

| | Control[a] | | 0.025 mg/liter of copper[b] | | 0.050 mg/liter of copper | |
| | Percent | | Percent | | Percent | |
| Days | Loss in viability[c] | Injury[d] | Loss in viability | Injury | Loss in viability | Injury |
|---|---|---|---|---|---|---|
| 0 | 0 | 2.07 | 0 | 9.20 | 0 | 13.78 |
| 1 | 0.56 | n.d. | 12.27 | 90.48 | 27.04 | n.d. |
| 2 | -10.56 | 49.66 | 23.93 | 93.44 | 77.55 | 99.89 |
| 3 | 8.47 | 47.33 | 41.72 | 95.70 | n.d. | n.d. |
| 4 | 3.95 | n.d. | 54.42 | 98.15 | 96.22 | 99.78 |
| 5 | 6.78 | 57.09 | 54.79 | 98.89 | 98.67 | 99.64 |

a. _E. coli_ cells (1 x $10^5$ CFU/ml) suspended in a carbonate buffer.

b. _E. coli_ cells (1 x $10^5$ CFU/ml) suspended in a carbonate buffer with a copper concentration of 0.025 mg/liter.

c. Loss in viability calculated as: $\frac{day\ 0 - day\ X}{day\ 0}$ x 100 = %.

d. Injury calculated as: $\frac{TLY\ CFU - TLY\text{-}D\ CFU}{TLY\ CFU}$ x 100 = %.

the control populations without copper and none had greater
than 28% loss in viability.

Raising the temperature above 4°C accelerated the rate
of copper injury.  This conclusion is based on a limited
number of experiments.  The time necessary to cause 90%
injury at 0.05 mg/liter copper was only 1 day at 22°C.  In
additon, higher metal concentrations caused injury at a
faster rate (90% in less than 1 day at 4°C), but large
decreases in cell viability were also observed.

## Part B - Physiology Studies

E. coli cells placed in membrane diffusion chambers and
exposed to distribution water from a quiescent portion
(i.e., no chlorine detected) of the Bozeman drinking water
distribution network were examined to determine the
physiological implications of aquatic injury.  Initial
experiments revealed a trend of decreased oxygen use with
increased injury.  Oxygen utilization (during metabolism of
lactose) measured on a Gilson Oxygraph over a short time
period (approximately 5 minutes) decreased 73 to 83% when
cells were injured 70 to 99.5%, respectively (Figure 4).
When cells were grown on glucose, oxygen utilization
decreased 47% for 70% injured cells (Figure 4).  Similar
results were obtained with long term (2 hours) respirometry
experiments (Figure 5).  Bacteria incubated in an enriched
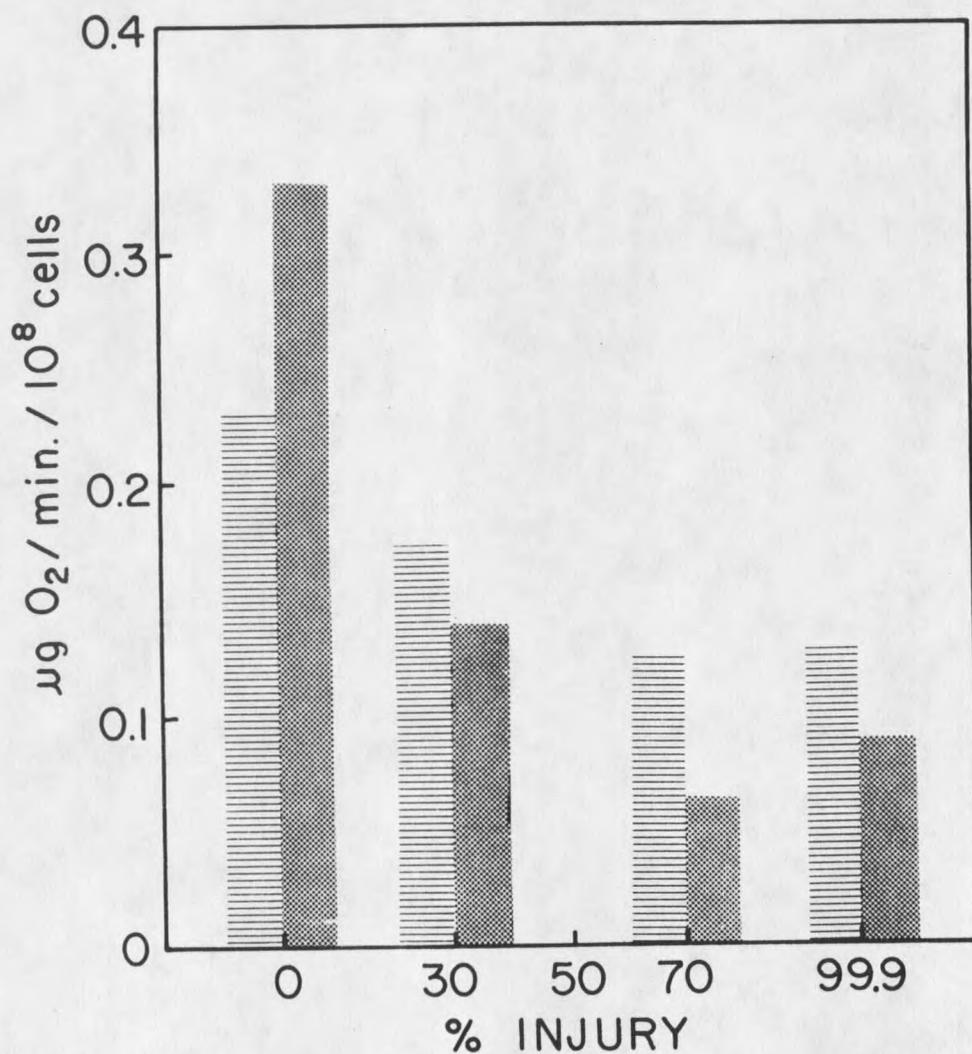medium containing either glucose or lactose required

Figure 4.    Short term uptake of oxygen by E. coli cells
            injured in drinking water.  Substrates used
            were 1.0% glucose (bars) and 1.0% lactose
            (stippled).