



The Limulus lysate assay as a rapid and sensitive test of bacterial water quality
by Thomas Morgan Evans

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
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Montana State University
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Abstract:

The Limulus lysate assay was used to measure the endotoxin content in stream water and was found to reflect the degree of bacterial contamination as measured by coliform, enteric, gram-negative and hetero-trophic bacteria. The firm clot method was found to be a less sensitive and reproducible technique for the detection of endotoxin than the spectrophotometric modification of the Limulus lysate assay. Bound endotoxin, as determined by the spectrophotometric modification of the Limulus lysate assay, was found to be a better measure of the endotoxin associated with bacterial cells than total endotoxin.

On the basis of high positive correlations between bound endotoxin and coliform, enteric, gram-negative and heterotrophic bacteria, the measurement of bound endotoxin by the Limulus lysate assay was proposed as a rapid and sensitive test of bacterial water quality.

Because of the assumptions that are inherent in using the LLA as a measure of bacterial water quality, more research is needed before the LLA can be generally applied.

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OF BACTERIAL WATER QUALITY

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

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in

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ABSTRACT

The Limulus lysate assay was used to measure the endotoxin content in stream water and was found to reflect the degree of bacterial contamination as measured by coliform, enteric, gram-negative and heterotrophic bacteria. The firm clot method was found to be a less sensitive and reproducible technique for the detection of endotoxin than the spectrophotometric modification of the Limulus lysate assay. Bound endotoxin, as determined by the spectrophotometric modification of the Limulus lysate assay, was found to be a better measure of the endotoxin associated with bacterial cells than total endotoxin.

On the basis of high positive correlations between bound endotoxin and coliform, enteric, gram-negative and heterotrophic bacteria, the measurement of bound endotoxin by the Limulus lysate assay was proposed as a rapid and sensitive test of bacterial water quality. Because of the assumptions that are inherent in using the LLA as a measure of bacterial water quality, more research is needed before the LLA can be generally applied.

Chapter 1

INTRODUCTION

With increasing demands on water resources, bacteriological tests are becoming more important as a means of assessing the effects of multiple use on water quality. At present, bacteriological tests are the only acceptable means of assessing the sanitary quality of water supplies.

Bacteriological measurements of water quality rely predominantly on the detection and enumeration of indicator organisms. These indicator systems are based upon the detection of fecal contamination from warm-blooded animals since this is the natural link to the occurrence of pathogenic microorganisms in polluted water. The direct enumeration of pathogens is not practical because of: (1) their low numbers and (2) the expense of time and money necessary to isolate and enumerate them. Fecal coliforms, fecal streptococci and total coliforms are the usual bacterial indicators used to relate fecal contamination to the presence of pathogenic bacteria. However, bacterial indicators have, in some cases, been present in low enough numbers to reflect little fecal contamination when Salmonella typhi was present in high enough numbers to cause typhoid fever (34).

One limitation of the standard tests of water quality is the 24 to 48 hours necessary to perform the test and to obtain the results.

A rapid and simple test of bacterial water quality would have definite advantages. A test requiring only two hours to perform and which reflects the number of indicator organisms present as well as other predominant aquatic bacteria would have many applications. Such a technique would be especially useful where floods, hurricanes, earthquakes and tornadoes make time a critical factor in assessing the water quality.

Several investigators (83, 37) have suggested that the Limulus lysate assay for endotoxin may be a useful technique for rapidly determining the bacterial quality of water. This technique requires only one hour to perform and detects the lipopolysaccharide or endotoxin portion of gram-negative bacterial cell walls. The Limulus lysate assay was developed by Levin and Bang (65) while investigating the toxic effects of a marine bacterium on Limulus polyphemus, the horseshoe crab. They found that the endotoxin from this marine bacterium caused a massive coagulation of the amoebocytes in the crabs' blood (7). Further investigation showed that the protein within the amoebocytes gels in the presence of minute amounts of endotoxin (1 nanogram/ml) (59), and that the rate of gelation is proportional to the amount of endotoxin present (65). Several methods have been used to extract the protein from the amoebocytes for use in the Limulus lysate assay, the most common ones being those of Levin and Bang (65) and Jorgenson and Smith (53).

The Limulus lysate assay is performed by reacting 0.1 ml of lysate with 0.1 ml of sample, incubating at 37 C for one hour and checking the solution for the presence of a firm clot or an increase in turbidity.

Statement of Purpose

Jorgenson, et. al. (51) have found that the Limulus lysate assay can be used to determine the number of gram-negative bacteria in fluids. The amount of endotoxin in river water has also been determined by the Limulus lysate assay (22). In light of these findings, the purposes of this study are:

- (1) To assess the applicability of the Limulus lysate assay as a rapid test of bacterial water quality.
- (2) To develop the Limulus lysate assay into a sensitive, reproducible and quantitative technique for measuring the quantity of endotoxin in water.
- (3) To correlate the amount of endotoxin in water with standard measures of bacterial water quality.

Chapter 2

LITERATURE REVIEW

Bacteria as a group of microorganisms are tremendously diverse in terms of their tolerance to pH, temperature and oxygen concentrations. They can grow and survive in waters having very dilute nutrient concentrations and are able to utilize substrates which other organisms are unable to metabolize. Heterotrophic bacteria consume organic materials (many of which may be pollutants) and produce mineralized end-products. In addition, some bacteria are pathogenic and cause water-borne diseases of man. Because of these characteristics, bacteria play an important role in determining water quality.

Indicators of Water Quality

Bacteriological measurements of water quality rely predominantly on the differentiation and enumeration of indicator organisms. These indicator systems are based upon the detection of fecal contamination from warm-blooded animals, since this is the natural link to the occurrence of pathogenic microorganisms in polluted waters. Fecal coliforms, fecal streptococci and total coliforms have been used to relate fecal contamination to the presence of pathogenic bacteria in the aquatic environment (34,36,38,58, 61). These indicators do not relate directly to the other parameters of water quality such as

general bacterial densities, the occurrence of pathogens or aesthetics. Cohen (14) and Gallagher (34) have stated that any one of these organisms should not be used alone as indicators of water quality. Allen (2) proposed that Pseudomonas aeruginosa, Clostridium perfringens and Bacteroides may be suitable as other indicators of water quality. Nitrate-reducing, sulfate-reducing and fluorescent bacteria have been used as alternative indicators of water quality (84). The total viable count or standard plate count has also been used by many investigators to help assess the water quality of aquatic environments (49, 50, 85, 95).

Rapid Tests of Water Quality

Many attempts have been made to develop rapid tests of water quality. Guthrie, et. al. (39) were able to enumerate fecal strains of Escherichia coli, in 12 hours, by the combination of the membrane filter and fluorescent antibody techniques. Even though their technique compared favorably with standard methods of determining fecal coliforms, they did not apply this technique to natural aquatic environments. Abshine, et. al. (1) improved this technique so the time required to complete the assay was reduced from 12 to 3 hours. In actual field situations, this technique corresponded closely with standard methods for determining fecal coliforms. The fluorescent antibody technique has also been used to detect Lancefields' group D

streptococci (79). Strange (94) used ^{125}I - labelled homologous antibody to detect small numbers of bacteria in aqueous suspensions. Results could be obtained in 8 - 10 minutes and could detect as few as 500 bacteria. All of the above methods rely on the membrane filtration technique to either collect the antibody complex or to concentrate the bacteria.

Khanna (56, 57) developed a 4 hour technique using ^{32}P incorporated in a substrate to enumerate coliform organisms. This was accomplished without the use of the membrane filter technique by co-precipitation of the radioactive phosphorus. Strange (94) has found that the use of the membrane filter in these techniques may decrease the accuracy and sensitivity of the assay by introducing background interferences due to entrapment of interfering particles on the membrane filter. A radiometric method, based on the release of $^{14}\text{CO}_2$ from ^{14}C lactose, was used by Bachrach (6) to detect between 1 - 10 bacteria in cultures incubated for 6 hours. Using a similar technique, Levin (63) found that ^{14}C formate worked equally well to enumerate coliforms when an incubation time of 3 and 1/2 hours was used.

A rapid and sensitive method for detecting fecal and total coliforms was developed by Kenard and Valentine (55). This technique involves the detection of bacteriophage specific for coliform and fecal coliform organisms. By the addition of large numbers of

the organism in question to the water sample, the presence of a virulent bacteriophage could be detected in 6 - 8 hours. The authors found a high degree of correlation (0.95) between the number of phage and the number of fecal coliforms. This relationship held true over a wide range of fecal coliform concentrations.

In contrast to the indirect methods of using fluorescent antibody, radioactive labelled antibody, radiometric and bacteriophage techniques, several tests have been developed for the rapid and direct enumeration of indicator bacteria. Andrews and Presnell (5) used a newly formulated medium (A-1) in a 24 hour elevated temperature test to recover Escherichia coli from estuarine waters. The authors reported that this test compared favorably to the standard 72 hour MPN test in terms of recovery and the number of false positives. The usefulness of the method was further demonstrated by Andrews, et. al. (4). Francis (31) has formulated another medium for use in 7 hour elevated temperature enumeration of fecal coliforms in fresh chicken. This new technique may be applicable to water quality studies.

A new technique for the rapid, nonselective enumeration of microorganisms in water has been developed by Levin, Usdin and Slonim (62,63). This method employs the bioluminescent reaction of the firefly and is based upon two biochemical findings: adenosine triphosphate (ATP) is specifically required in the firefly bioluminescence reaction and ATP is ubiquitous to all living cells. The test involves measuring

the ATP content in a water sample using the firefly bioluminescent reaction and relating the quantity of ATP measured to the number of microorganisms responsible for this quantity of ATP. The authors report that this technique can detect as few as 100 to 300 bacterial cells in less than 1 minute. Since ATP is ubiquitous to all living cells, this technique measures all biomass whether of bacterial origin or not.

Limulus Lysate Assay

Some reference has been made to using the Limulus lysate assay for endotoxin (LLA) as a possible rapid test of water quality (37,83). This assay has been shown to be specific for the detection of gram-negative bacterial endotoxins (16,28,51,53,73,80,81,82,105), which are lipopolysaccharide moieties contained in the outer cell layer of gram-negative bacteria. Most, if not all, gram-negative bacteria possess endotoxins (19,74). Endotoxins, otherwise known as lipopolysaccharides (LPS), or pyrogens, are of medical interest because of the role they play in endotoxemia and gram-negative bacteremia. Endotoxins are pyrogenic in nature due to their ability to cause a febrile response when injected into experimental animals. The endotoxin molecule possesses three subunits (70): (1) the lipid A moiety, (2) the core region composed of ketodeoxyoctonate, 2 heptoses and 3 hexoses, and (3) side chains of repeating polysaccharides (oligopolysaccharide).

The lipid A moiety serves to link the core region and polysaccharide side chains to the cell membrane and is the site of biological activity. The core region links the polysaccharide side chains to the lipid A moiety. The repeating polysaccharide side chains are responsible for the antigenic specificity of the LPS molecule and the O antigen in the enteric bacteria. Endotoxins are usually thought to be firmly bound to the cell wall and released only upon cell lysis (74). Several investigators (20,71,103) have found free endotoxin in the culture fluid of a thymine auxotroph of Escherichia coli. This endotoxin may be due to overproduction of endotoxin and not a result of cellular lysis. The free or extracellular endotoxin is immunologically identical to the endotoxin that is bound to the cell surface (20).

Jorgenson and Smith (54) have used the Limulus lysate assay to measure free endotoxin in culture fluids. Their results indicated that free endotoxin results from increased solubilization or shedding of pre-existing cell wall material and is probably not a consequence of metabolic over production of this material, nor of cellular lysis. This conclusion was based on experiments conducted on resting or stationary phase cultures of E. coli. Appreciable amounts of free endotoxin were found in the culture filtrate when cell lysis or growth could not be detected. Even though free endotoxin may constitute 50% of the total endotoxin, the endotoxic activity of the LPS remaining

on the surface of intact cells is readily measurable by the LLA (54). Since the amount of endotoxin bound to the cell surface remains fairly constant, the quantitative measurement of bound or total endotoxin by the LLA should be a suitable method for approximating the number of gram-negative bacteria in fluids (54). Jorgenson, et. al. (51) used this principle to determine the number of gram-negative bacteria in urine by measuring the total endotoxin content with the Limulus lysate assay. By correlating the endotoxin content with bacterial counts in urine, they were able to detect as few as 1000 bacteria/ml (51).

Gram-positive bacteria, as well as ~~exotoxin~~ and extracellular products from gram-positive bacteria do not give a positive Limulus lysate test (81). However, several researchers (24, 28) have questioned the universal specificity of the lysate assay. Elin (24) has reported that a few polynucleotides and proteins (specifically, enzymes) gave a positive Limulus test. The concentrations required to elicit this response were from 10^3 to 10^7 times greater than the concentrations of endotoxin necessary to give a positive Limulus test. Wilfeuer et. al. (101) showed peptidoglycan isolated from the cell walls of gram-negative bacteria gave a positive Limulus test, however, the activity of the peptidoglycan was 1,000 to 400,000 times less than that of E. coli. Wilfeuer et. al. (101) suggested that other bacterial components should be investigated for their ability to initiate the

gelation of Limulus lysate. One investigator (28) did find that a viral RNA gave a positive Limulus test in approximately the same concentrations as endotoxin.

The LLA is the most sensitive test for detecting endotoxin (15,24, 51, 82, 104) and can detect as little as 1 picogram of endotoxin/ml (96, 104). The LLA is at least 10 times as sensitive as the rabbit pyrogen test used by the Food and Drug Administration as the standard endotoxin test (82) and is between 10^6 and 10^7 times as sensitive as the colorimetric assay developed by Janda and Work (45, 106).

Numerous investigators have used the LLA to detect endotoxin in blood or blood fluids (11; 21, 26, 29, 30, 35, 66, 67, 68, 72, 77, 81,90), spinal fluids (75), urine (51) and tissue homogenates (102). Intravenous fluids (52, 73) and radiopharmaceuticals (23, 42, 52, 73) have also been screened for the presence of endotoxin by the LLA. The lysate assay has also been tested for its suitability as a rapid screening test of ground meats (46). Limulus lysate reacts with the endotoxin of both aerobic and anaerobic gram-negative bacteria (90). Endotoxin concentrations in well and river water have been measured by the LLA. Endotoxin concentrations ranged from 400 micrograms/ml in the Mississippi River at New Orleans, La., to 1 microgram/ml in the Cumberland River near Nashville, Tenn. (22).

The Limulus lysate assay was first described by Levin and Bang (65). This discovery was based upon the observation that the in vitro coagu-

lation of Limulus polyphemus amoebocytes is mediated by gram-negative bacterial endotoxin (7). While elucidating the mechanism responsible for the coagulation, Levin and Band (65) determined that a protein within the amoebocytes was involved in clot formation. Several methods have been designed to extract the clottable protein from the amoebocytes (65, 81, 100, 104, 105). These various methods were developed to try to improve the sensitivity of the lysate and to correct the variability in the biological activity. Sullivan and Watson (96) were able to reduce variability among different lysate preparations and improve sensitivity by chloroform extraction of an inhibitor and the addition of divalent cations.

The mechanism of gel formation in Limulus lysate was first hypothesized by Levin and Bang (65). They suggested that clot formation was due to a reaction of amoebocyte cellular protein with an endotoxin activated enzyme. This hypothesis was substantiated in a later study by Young, Levin and Prendergast (105). Sephadex column chromatography was used to determine that the lysate was composed of 3 fractions, two of which were involved in gel formation. They proposed that a heat labile, high molecular weight protein was activated by endotoxin and then gelled a second, heat stable, clottable fraction of approximately 27,000 molecular weight. Solum (88, 89) confirmed the protein nature of the fractions and this mechanism of gel formation. Yin et. al. (104) has demonstrated that the lipopolysaccharide portion of the endotoxin

molecule reacts with the lysate in the gelation reaction. These findings were corroborated by those reported by Jorgenson and Smith (98) who found that a combination of the lipid and polysaccharide moieties of the endotoxin molecule showed slightly less lysate activity than the whole endotoxin (polysaccharide + lipid + oligosaccharide), but more activity than when tested separately.

The endpoint determination of the gelation reaction in the LLA is only semi-quantitative. Most investigators (16, 51, 53, 65, 81, 104, 105) have used an increase in viscosity or a firm gel as the endpoint. Hochstein et. al. (42) has found that this method for determining the endpoint may bias the results of the LLA by reading surface tension as a firm gel. Hochstein et. al. (42) and Sullivan and Watson (96) detected the endpoint of the LLA by reading a firm gel as one that would not break when inverted 180°. This method tended to reduce investigator bias, but may still be 50% off in detecting the endpoint due to the two-fold serial dilutions used in the lysate assay. Niwa, Hiramatsu and Woguri (76) have developed a method to quantitatively measure the amount of clottable protein formed in the reaction of endotoxin and Limulus lysate. The use of this method made the LLA quantitative with a sensitivity that ranged between 1 and 10 nanograms/ml of endotoxin. The sensitivity depended on the activity of the endotoxin used to develop the standard curve. Worthington Biochemical Corporation, Freehold, New Jersey, has developed a spectrophotometric method to

quantify the LLA assay. This technique consisted of changing reaction conditions in the lysate assay so that instead of a solid gel being formed, the reactive protein precipitated yielding a turbid suspension. The absorbance of this suspension was read on a spectrophotometer, and a standard curve was developed by plotting absorbance versus endotoxin concentration. This method was sensitive to one picogram/ml of endotoxin. Watson, Woods Hole Oceanographic Institution (personal communication) has developed a similar method which was sensitive to one picogram/ml of endotoxin.

Enumeration of Gram-negative Bacteria

The Limulus lysate assay detects the endotoxin of gram-negative bacteria. If the LLA is to be useful as a rapid test of water quality, the amount of endotoxin must be correlated with the number of gram-negative bacteria. Several media have been proposed to selectively enumerate gram-negative bacteria. Holding (43, 44) used a medium consisting of 0.5% meat extract, 0.5% peptone and 1:500,000 (2 ug/ml) crystal violet to enumerate gram-negative bacteria from soil. Litsky, Mallmann and Fifield (69) showed that crystal violet in a concentration of 2 ug/ml was inhibitory to Escherichia coli. They proposed that ethyl violet would be a more suitable selective agent for gram-negative bacteria. Ethyl violet in a concentration of 1.25 ug/ml was not inhibitory to Escherichia coli, Salmonella typhi and Salmonella typhimurium

while completely inhibiting Bacillus subtilis and Streptococcus faecalis (69). Several investigators have used ethyl violet as a selective agent to isolate anaerobic gram-negative bacteria (8, 32). Nile blue was shown by El Sladek and Richards (25) to inhibit gram-positive bacteria at a concentration of 100 ug/ml without inhibiting gram-negative bacteria. Brom thymol blue, o-cresolphthalein, janus green, methylene blue, safranin o, safranin Y, methyl green and p-rosaniline have all been shown to be inhibitory to gram-positive and not gram-negative bacteria (33). However, the degree of insensitivity of gram-negative organisms to these dyes was not reported. Nitrogen containing steroids (87) and B-methylpyridino derivatives (47) show promise as selective agents from gram-negative bacteria, as well.

A detergent, Tergitol 7, was shown by Pollard (78) to inhibit many gram-positive bacteria. Chapman (13) used this selective agent and triphenyltetrazolium chloride in a culture medium to isolate and confirm Escherichia coli in 10 hours. Chapman (12) and Kulp, Mascoli and Tausharijian (60) have found that the numbers of coliform bacteria on Tergitol 7 agar were 30 to 50% greater than on either Endo or Levines' eosin methylene blue agar. Tergitol 7 agar appears to be completely non-inhibitory to most gram-negative bacteria and has been used as the coliform confirmatory medium in water analysis (60) and as a selective medium for enteric bacteria (41).

Enumeration of Heterotrophic Bacteria

The standard plate count by the method given in Standard Methods for the Examination of Water and Waste Water (3) has been shown to recover a lower percentage of the total bacterial population than the streak plate method employing casein-peptone-starch (CPS) medium (48, 92) of Stark and McCoy (93). Jones (48) and Staples and Fry (92) have shown that CPS medium gave higher counts than the medium recommended in Standard Methods. The inoculation of plates by the streak method instead of pouring tempered agar onto the inoculum accounted for most of the discrepancy between the two methods (10, 27, 48, 49, 59, 91, 107). Klein and Wu (59) have reported that the streak plate method may yield up to five times the number of bacteria as on the pour plate method. The temperature of incubation may greatly influence the count obtained by the streak and pour methods. Taylor (97) and Jones (48) have indicated that an incubation temperature of 20 C provides for the greatest yield of bacteria. Bissonnett (9) and Harrison (40) have shown that bacteria may be injured in phosphate-buffered diluent so that while the cells still remain viable, they are not able to grow as readily on selective media. The harmful effects of diluent on bacterial cells can largely be corrected by the addition of 0.1% peptone to the phosphate buffer (86).

Chapter 3

DESCRIPTION OF THE STUDY AREA

The East Gallatin River provides an ideal situation for the study of bacterial indicators of water quality. The river's tributaries start out as high, pristine mountain streams and flow along the valley floor where they drain agricultural and/or urban areas. The effluent from a primary and secondary sewage treatment plant, after chlorination, empties directly into the river. Within 25 miles, the East Gallatin River and its primary tributaries change from small pristine streams to one that is contaminated with sewage effluent. Sites selected at different locations on the East Gallatin drainage provide samples of very diverse water quality. This situation allows the determination of the relationships between the amounts of endotoxin in water and the numbers of bacteria for waters of differing bacterial quality. The sites used in this study are described in Table 1. Figure 1 indicates their location.

Table 1. Description and location of sampling sites for microbiological and endotoxin analysis

Site	Description and Location
EF1	Located on the East Fork of Hyalite Creek, approximately 3.4 miles (5.5 km) from its source, a high mountain stream.
H3	Located on Hyalite Creek, approximately 7.0 miles (11.4 km) downstream from Hyalite reservoir, a high mountain impoundment.
H4	Located on Hyalite Creek, approximately 17.0 miles (27.4 km) downstream from Hyalite reservoir.
H5	Located on Hyalite Creek, approximately 25.0 miles (40.2 km) downstream from Hyalite reservoir, after flowing through agricultural and suburban land.
M3	Located on Mystic Creek, approximately 7.0 miles (11.3 km) downstream from Mystic reservoir, a high mountain impoundment.
M4	Located on Mystic Creek, approximately 12.0 miles (19.3 km) downstream from Mystic reservoir, after flowing through agricultural land.
M5	Located on Mystic Creek, approximately 16.0 miles (25.7 km) downstream from Mystic reservoir, after flowing through the City of Bozeman.
EG4	Located on the East Gallatin River approximately 0.1 miles (0.3 km) upstream from the Bozeman Waste Water Treatment Plant outfall.
OF2	Located on the outfall of the Bozeman Waste Water Treatment Plant, a primary and secondary treatment plant with chlorinated effluent.
EG5	Located on the East Gallatin River, approximately 0.8 mile (13.3 km) downstream from the outfall of the Bozeman Waste Water Treatment Plant.

Table 1. (continued)

Site	Description and Location
EG5A	Located on the East Gallatin River, approximately 3 miles (4.8 km) downstream from the outfall of the Bozeman Waste Water Treatment Plant.

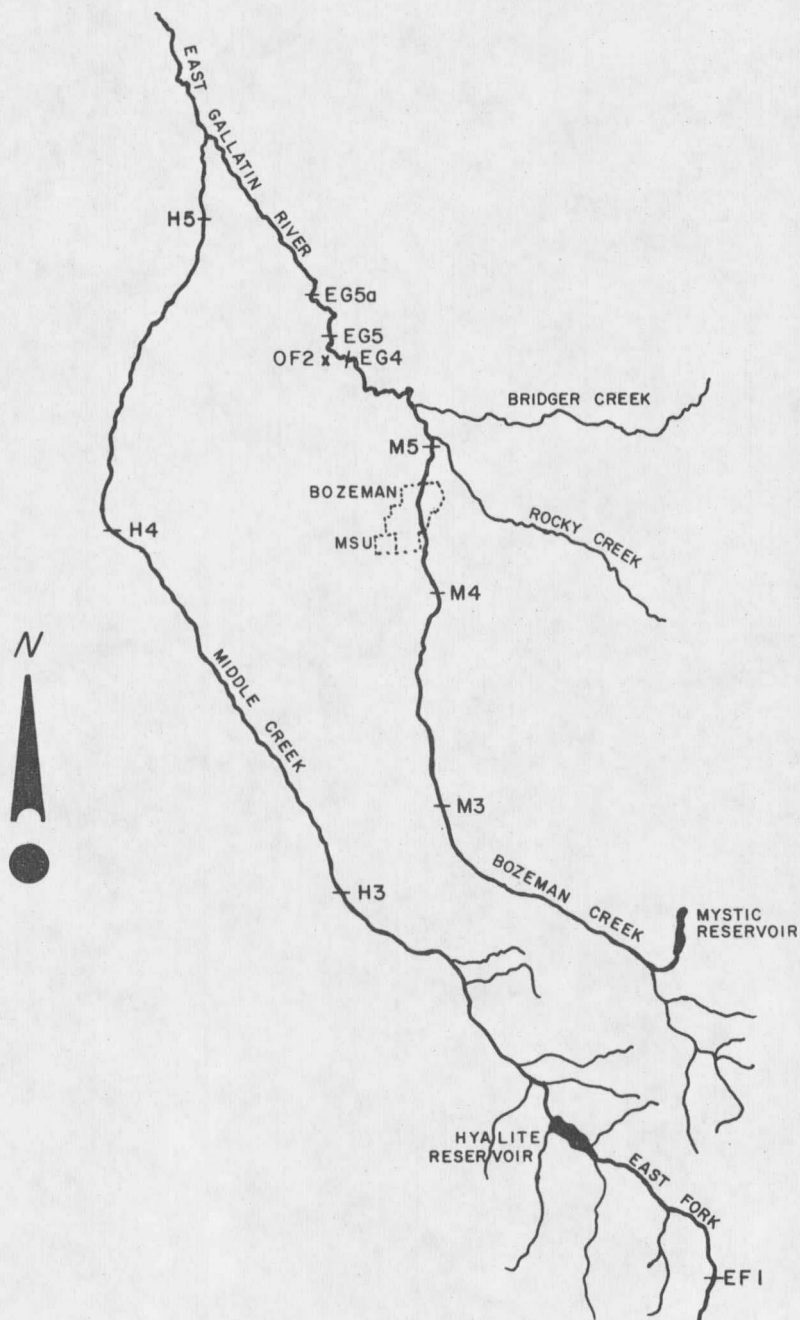


FIGURE 1. SAMPLING SITES FOR BACTERIAL AND ENDOTOXIN ANALYSIS.

Chapter 4

MATERIALS AND METHODS

Sampling

Samples for bacterial analysis were collected in two liter sterile nalgene bottles from sites shown in Table 1. In the initial phase of this study, the sites collected were M3, M4, M5, H3, H4, EG4, OF2, EG5 and EG5A. Later, samples were collected at EF1, H5, M3, M4, M5, EG4, OF2, EG5 and EG5A. When sampling OF2 and EG5, 2 ml of a 10% solution of sodium thiosulfate were added to the sample bottles before autoclaving. The addition of sodium thiosulfate neutralized any residual chlorine that may have been present at these two sites. Samples for endotoxin analysis were collected in pyrogen-free screw cap culture tubes (preparation of pyrogen-free glassware is described in the section dealing with the Limulus lysate assay). Samples for endotoxin and bacterial analyses were collected simultaneously. All samples were placed on ice in a Coleman cooler, transported back to the university laboratory and held on ice until analysis. All samples were tested within six hours of collection.

Gram-negative Bacteria by the Membrane Filter Technique

In the initial phase of this research, gram-negative bacteria were enumerated on crystal violet selective medium (CV) using the

membrane filter technique. CV medium consisted of tryptic soy broth supplemented with 0.3% yeast extract, 0.5% glucose, 2 ug/ml crystal violet and 1.4% agar (unless otherwise specified, all media and media components were Difco products). The medium was autoclaved, and 10 ml portions were poured into 50 X 12 mm sterile, glass petri dishes.

All samples were thoroughly shaken before withdrawing aliquots for filtration or for dilution before filtration. Ten, 5 or 1 ml portions of the sample to be filtered were transferred to the filter funnel containing approximately 10 ml of standard phosphate buffer (3) supplemented with 0.1% peptone. One ml of the sample to be diluted was transferred to a 99 ml sterile phosphate-buffered peptone dilution blank. One, 10 or 50 ml portions from the dilution blank were then transferred to a membrane filter funnel. Two replicates of each dilution were made. After filtration, the filter funnel was rinsed four times with sterile phosphate-buffered peptone water from a sterile wash bottle. The membrane filter (Millipore Filter type HAWG 047 S0 with a pore size of 0.45 μ m) was aseptically rolled onto the CV medium. Filter and water controls were also performed. The plates were inverted and incubated for 24 h at 35 C. Plates showing between 20 and 80 colonies were counted and reported as the number of gram-negative bacteria/100 ml. Gram-negative and all other bacteria enumerated by membrane filtration were counted with the aid of a 7X view-

ing scope and incident light.

To test the selectivity of the CV medium, 20 colonies from each of 4 water sample plates were picked and transferred to sterile tryptic soy broth (TSB). Picking proceeded from one edge of the plate selecting all colonies until 20 were obtained. Following incubation for 24 h at 35 C, slides were prepared from the broth cultures and these were gram stained.

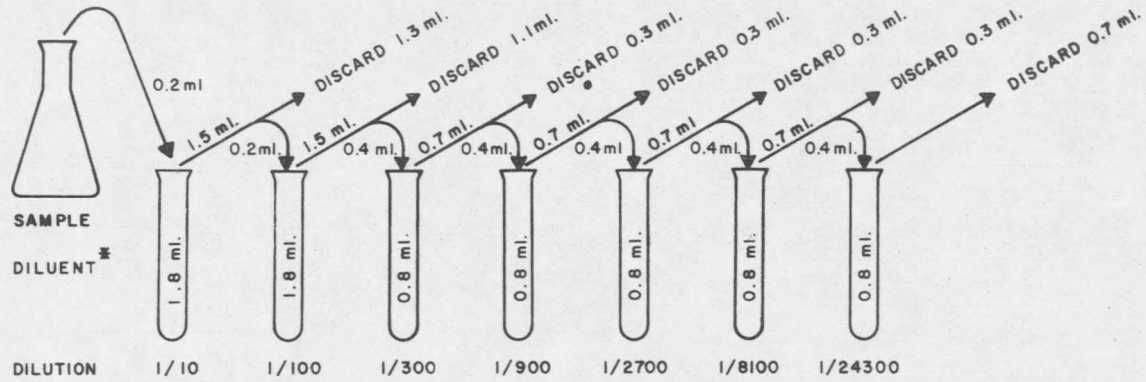
Gram-negative Bacteria by the Streak Plate Method

Preliminary results indicated that CV medium was unsatisfactory for the enumeration of gram-negative bacteria. Ethyl violet (Matheson, Coleman and Bell), Nile blue A (Matheson, Coleman and Bell) and crystal violet were compared as selective agents for gram-negative bacteria.

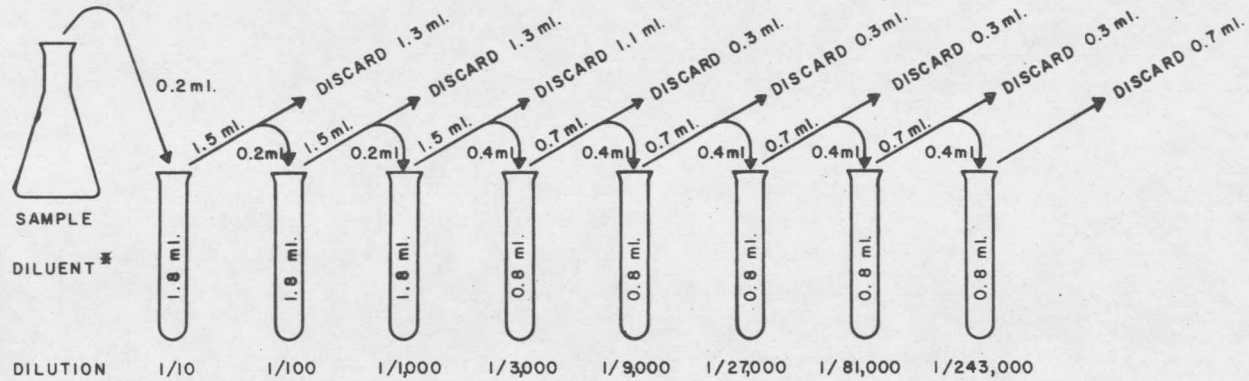
A basal medium was prepared containing differing concentrations of each dye. The basal medium was a modification of casein-peptone-starch medium (CPS) (93), consisting of g/l: 0.2 K_2HPO_4 , 0.05 $MgSO_4 \cdot 7 H_2O$, trace $FeCl_3$ (4 drops of a 0.01% solution), 0.5 peptone, 0.5 casein, 0.5 soluble starch, 1.0 glycerol and 15 agar. Jones (48) has reported that this modification does not alter the ability of CPS to enumerate heterotrophic bacteria. Twenty ml of the autoclaved medium were poured into sterile, 100 X 15 mm glass petri dishes, inverted and allowed to dry at room temperature for 24 hours. Various

aquatic bacteria were streaked onto plates containing the basal medium supplemented with one selective agent. The selective agents used and their respective concentrations included: 20 and 100 ug/ml Nile blue A, 2 and 10 ug/ml crystal violet and 1.25 and 2 ug/ml ethyl violet. The bacteria used (obtained from Montana State University's microbiological culture collection) and their gram reaction are shown in Table 2. One loopful of bacteria was transferred aseptically into 1 ml of sterile phosphate-buffered peptone, mixed and streaked onto plates divided into 6 sections. The plates were streaked by placing the inoculating loop near the center of the plate and drawing the loop in a straight line outward to the edge of the plate. All bacteria were streaked on the basal medium alone and on basal medium with added selective agents. The plates were inverted and incubated at room temperature for 48 h. The amount of growth occurring on the basal medium supplemented with one of the selective agents was scored relative to the amount of growth occurring on the basal medium alone. A value of 4+ was assigned to the amount of growth comparable to that occurring on the basal medium, 3+ indicated growth 3/4 of that occurring on basal medium, 2+ indicated growth 1/2 of that occurring on the basal medium, 1+ indicated growth 1/4 of that occurring on the basal medium and 0 indicated no growth or only the appearance of a few isolated colonies. Since not all of the gram-positive bacteria tested were inhibited by the selective agents used, an experiment was

SAMPLES OF LOW ENDOTOXIN CONCENTRATION



SAMPLES OF HIGH ENDOTOXIN CONCENTRATION



* PYROGEN-FREE 0.1% NaCl

FIGURE 2. DILUTION SCHEME FOR PREPARATION OF SAMPLE FOR LIMULUS LYSATE ASSAY.

Table 2. Bacteria used to test the selectivity of various agents for gram-negative bacteria

Bacteria	Gram reaction
<u>Escherichia coli</u> (<u>E. coli</u>)	-
<u>Bacillus megaterium</u> (<u>B. megaterium</u>)	+
<u>Enterobacter aerogenes</u> (<u>E. aerogenes</u>)	-
<u>Bacillus cereus</u> (<u>B. cereus</u>)	+
<u>Salmonella typhimurium</u> (<u>S. typhimurium</u>)	-
<u>Bacillus polymyxa</u> (<u>B. polymyxa</u>)	+
<u>Enterobacter cloaceae</u> (<u>E. cloaceae</u>)	-
<u>Streptococcus bovis</u> (<u>S. bovis</u>)	+
<u>Salmonella flexneri</u> (<u>S. flexneri</u>)	-
<u>Streptococcus faecalis</u> (<u>S. faecalis</u>)	+
<u>Alcaligenes faecalis</u> (<u>A. faecalis</u>)	-
<u>Streptococcus faecalis</u> subs. <u>zymogenes</u> (<u>S. faecalis</u> subs. <u>zymogenes</u>)	+
<u>Serratia marcescens</u> (<u>S. marcescens</u>)	-
<u>Streptomyces</u> sp.	+
<u>Klebsiella pneumoniae</u> (<u>K. pneumoniae</u>)	-
<u>Sarcina lutea</u> (<u>S. lutea</u>)	-
<u>Erwinia caratovora</u> (<u>E. caratovora</u>)	-
<u>Leuconostoc mesenteroides</u> (<u>L. mesenteroides</u>)	+

Table 2. (continued)

Bacteria	Gram reaction.
<u>Acinetobacter</u> sp.	-
<u>Pseudomonas aeruginosa</u> (<u>P. aeruginosa</u>)	-
<u>Lactobacillus brevis</u> (<u>L. brevis</u>)	+

designed to test the selective ability of ethyl violet in conjunction with penicillin. A basal medium was prepared as above and supplemented with 1.25 ug/ml ethyl violet. Potassium penicillin G (Eli Lilly and Co.) was added to this medium in varying quantities so that seven different concentrations were obtained. These concentrations included: 30, 10, 5, 2.5, 1 and 0.1 units of penicillin per ml. Twenty ml portions of the autoclaved media were poured into sterile, 100 X 15 mm glass petri dishes. After the media had cooled, the dishes were inverted and allowed to dry for 24 h at room temperature. The bacteria presented in Table 2 were streaked on the different media as previously described. Growth on the various media was scored 4+, 3+, 2+, 1+ or 0 according to the scheme already described.

As a result of the preceding experiments, aquatic gram-negative bacteria were enumerated by the streak plate method employing the previously described modified CPS medium supplemented with 1.25 ug/ml ethyl violet and 10 units/ml penicillin G. (In all future references, this medium will be designated as EVP.) Autoclaved EVP medium was poured in 20 ml portions into sterile 100 X 15 mm glass petri dishes. Upon cooling, the plates were inverted and allowed to dry for 24 h at room temperature. After drying, the plates were stored at 4 C for no longer than 48 h before use.

All water samples were thoroughly shaken before withdrawing aliquots for plating or for dilution prior to plating. Dilutions of 10^0 , 10^{-1} ,

and 10^{-2} were made using 9 ml sterile phosphate-buffered peptone dilution blanks. While withdrawing the diluted sample, the dilution blank was mixed using a vortex mixer. Final dilutions of the sample were 10^{-1} , 10^{-2} and 10^{-3} which resulted from plating 0.1 ml of the appropriate dilution. A bent glass rod was dipped into alcohol, flamed and used to spread the inoculum evenly over the surface of the agar. Five replicates of each dilution were plated. Agar and diluent controls were also performed. The plates were inverted and incubated for 7 days at 20 C. Colonies on these plates, and all subsequent plates prepared by the spread plate technique, were counted with the aid of a New Brunswick Scientific Colony Counter and reported as the number of gram-negative bacteria/ml.

To test the selectivity of the EVP medium, 20 colonies from each of two plates from water samples were picked and their gram reaction determined in the same manner as previously described.

Enteric Bacteria Enumerated by the Membrane Filter Technique

When gram-negative bacteria were enumerated by the membrane filter technique, enteric bacteria were also enumerated by this technique. Tergitol 7 agar was the medium of choice and was prepared according to the manufacturer's instructions with one exception. One ml of a 1% solution of triphenyl tetrazolium chloride (TTC) was added to the autoclaved medium. After the addition of the TTC, 10 ml

of the autoclaved medium were poured into 50 X 12 mm sterile, glass petri dishes. The same dilutions and filtering procedure used to enumerate gram-negative bacteria by membrane filtration were used to enumerate enteric bacteria. Two replicates of each dilution were made. After filtering the sample, the membrane filter was aseptically rolled onto Tergitol 7 agar, inverted and incubated for 24 h at 35 C. The colony counts were reported as the number of enteric bacteria/100 ml.

Enteric Bacteria by the Spread Plate Technique

The same dilutions and spread plate technique used to enumerate gram-negative bacteria were used to enumerate enteric bacteria. Tergitol 7 agar was prepared according to manufacturers' instructions. The autoclaved agar was supplemented with 1 ml of a 1% solution of TTC per 100 ml of medium and 20 ml portions were poured into sterile, 100 X 15 mm, glass petri dishes. Upon cooling, the plates were allowed to dry for 24 h at room temperature. Plates were stored at 4 C for no longer than 48 hours before use. Inoculated plates were inverted and incubated at 20 C for 48 h. Colonies were counted and reported as the number of enteric bacteria/ml.

Coliforms

Total coliform bacteria were enumerated by the membrane filter

technique as described in Standard Methods for the Examination of Water and Waste Water (3). All samples were thoroughly shaken before withdrawing 250, 100, 50, 10, 5, 1 and 0.1 ml portions for filtration. The procedure used to filter the sample followed that previously described. After filtering, the membrane filters were aseptically rolled onto petri dishes containing m-Endo agar. Water and agar controls were also performed.

M-Endo-MF agar was made by preparing m-Endo broth according to manufacturers instructions and supplementing with 1.4% agar. The agar was tempered to 45 C and 10 ml portions were poured into sterile, 50 X 10 mm, glass petri dishes. M-Endo-MF agar was prepared and used the same day. After filtering, the plates were inverted and incubated for 24 h at 35 C. Two or five replicates of each quantity filtered were made. All colonies which produced a dark green metallic sheen were counted and reported as the number of total coliform bacteria/100 ml.

Heterotrophic Bacteria

Heterotrophic bacteria were enumerated by the spread plate technique employing the CPS medium of Stark and McCoy (93). This medium consisted of g/l: 0.2 K_2HPO_4 , 0.05 $MgSO_4 \cdot 7H_2O$, trace $FeCl_3$ (4 drops of a 0.01% solution), 0.5 peptone, 0.5 casein, 0.5 soluble starch, 1.0 glycerol, 1.5 agar. Twenty ml portions of the autoclaved

medium were poured into sterile, 100 X 15 mm, glass petri dishes. Upon solidification of the agar, the plates were inverted and allowed to dry for 24 h at room temperature. Plates were stored at 4 C for no longer than 48 h before use. All samples were shaken on a vortex mixer before aliquots were withdrawn for direct inoculation onto the plates or for dilution. Appropriate dilutions of the sample were made so that when 0.1 ml of the various dilutions was transferred to the plates, the final dilutions were 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} . Five replicates of each dilution were plated. Agar and water controls were also performed. Streaking and counting of the plates were by the methods previously described. The plates were inverted and incubated for 7 days at 20 C. Organisms forming colonies were recorded as the number of heterotrophic bacteria/ml.

Comparison of the Enumeration of Pure Cultures of Bacteria on Various Media

Bacteria from pure cultures of E. coli, K. pneumoniae and E. aerogenes were enumerated on the following media: Standard Methods Agar (SMA), CPS and EVP. Bacteria from stock cultures were transferred to 125 ml of sterile TSB and incubated for 24 h at 35 C. Dilutions of 10^{-5} , 10^{-6} and 10^{-7} were made from the TSB using sterile 99 ml phosphate-buffered peptone dilution blanks. Aliquots of 0.1 ml from the dilution blanks were transferred to each of the 3 media so that

the final dilutions of the TSB cultures were 10^{-6} , 10^{-7} and 10^{-8} . Five replicates of each dilution were plated on each of the 3 media. Buffer and agar controls were also performed. The plates were inverted and incubated for 96 h at 35 C. Colonies were counted and reported as the number of bacteria/ml.

Limulus Lysate Assay

4:50 4:00 8hr

Detection of Endotoxin by the Quantitative, Firm Clot Method

The Limulus lysate assay (LLA) was used to detect endotoxin in water. All glassware (pipettes, flasks, etc.) used in this assay was rendered pyrogen-free by baking at 180 C for 4 hours. Pyrogen-free distilled water (Travenol sterile water for injection) was used throughout this assay. To ensure that the distilled water was pyrogen-free, Travenol water was dispensed into pyrogen-free 250 ml, glass, erlenmeyer flasks, covered with aluminum foil, and autoclaved for 3 hours. The water in the flask was used for one day and discarded. Limulus lysate was obtained in lyophilized form (Associates of Cape Cod, Woods Hole, Mass. 02543). The lysate was reconstituted immediately before use by the addition of 5 ml of pyrogen-free water. The lysate was stored on ice until used. Any reconstituted lysate that was not used during the day was quick-frozen with a mixture of dry ice and acetone and stored at -20 C.

The LLA was performed by the method of Jorgenson and Smith (53).

Water samples collected in pyrogen-free, 16 X 150 mm, glass, screw cap culture tubes were mixed on a vortex shaker before withdrawing aliquots for the determination of endotoxin. Two-fold serial dilutions of the sample were made to range from 10^0 to 1.9×10^{-4} . An Eppendorf pipet was used to transfer 0.1 ml of the sample to tubes containing 0.1 ml of pyrogen-free water as a diluent. The Eppendorf pipet was used in all successive dilutions. The disposable tips for the Eppendorf pipet were found to be pyrogen-free. Individually wrapped, sterile, 12 X 75 mm, polystyrene tubes (Falcon) were used for the dilutions and as reaction tubes for the LLA. These tubes were also found to be pyrogen-free. The diluted samples (0.1 ml) were mixed before the addition of 0.1 ml of lysate. Controls were performed by including a tube containing 0.2 ml of lysate, and a tube containing 0.1 ml of pyrogen-free water and 0.1 ml of lysate.

Endotoxin preparations used in this research included Westphal phenol extracts of both E. coli 0111:B4 (Difco) and Klebsiella sp. ATCC # 12833 (FDA). Endotoxin stock solutions were prepared by reconstituting 1 ug of FDA endotoxin with 10 ml of pyrogen-free water so that a final concentration of 100 nanograms per ml (ng/ml) was obtained. Difco endotoxin was prepared by reconstituting 1 mg of lyophilized endotoxin with 100 ml of pyrogen-free distilled water and diluting it to a final concentrations of 100 ng/ml. Stock solutions of endotoxins were stored at 4 C for no longer than one month. Ten-

fold dilutions of the stock endotoxin were made to obtain a 1 ng/ml endotoxin solution. Two-fold serial dilutions of the 1 ng/ml endotoxin solution were made, using a 0.1 ml Eppendorf pipet, to obtain endotoxin standard solutions ranging from 1 ng/ml to 1 picogram per ml (pg/ml). The endotoxin solutions were mixed thoroughly before the addition of 0.1 ml of lysate to 0.1 ml of the solution. The sample, control and endotoxin standard tubes were incubated for 1 h at 37 C in a circulating water bath. During the incubation period, the tubes were not disturbed in any way. At the end of the incubation period, the tubes were slowly removed from the water bath and gently inverted 180°. The endpoint of the test was the highest dilution of the sample or the highest dilution of endotoxin which will clot the lysate to a degree that the clots will not break when inverted 180°. To obtain the endotoxin concentration of the sample, the sample endpoint was compared to the endotoxin standard endpoint and the value obtained was multiplied by the appropriate dilution factor. The endotoxin concentration in the sample was recorded as ng/ml.

Detection of Endotoxin by Spectrophotometric Method

In the later phases of this research, endotoxin concentration was determined by a modification of the LLA. By changing the reaction conditions of the assay, Dr. Stanley Watson of Woods Hole Oceano-

graphic Institution was able to employ a spectrophotometric method to make the LLA assay a more quantitative technique for determining endotoxin. The LLA was modified so that the reaction of lysate and endotoxin formed a turbid suspension instead of a firm clot. Dr. Watsons' method was further modified to make it more suitable for use in this research. The modifications were: (1) using pyrogen-free 0.1% NaCl as a diluent instead of the 3% NaCl as described by Dr. Watson and (2) reacting 0.5 ml of sample with 0.1 ml of lysate instead of the 1.0 ml of sample and 0.2 ml of lysate used by Dr. Watson. The method described below follows that of Dr. Watson except for these two modifications.

Samples were mixed on a vortex mixer before withdrawing aliquots for use in the lysate assay. The water samples for the determination of total endotoxin was prepared by making dilutions from 10^{-1} to 3.7×10^{-6} following the scheme shown in Figure 2. Depending upon the expected endotoxin concentrations, five dilutions were selected from this scheme to be included in the lysate assay. The amount of free endotoxin in the sample was determined from the supernatant fluid obtained by centrifuging the sample in pyrogen-free glass centrifuge tubes at 12,100 X g. A Sorvall RC-2B centrifuge and SS-34 rotor were used. The dilutions used to determine free endotoxin were the same as for total endotoxin. The amount of bound endotoxin in the sample was found by subtracting the concentration of free endo-

toxin from total endotoxin. Lyophilized lysate (Associates of Cape Cod) was reconstituted with 7.5 ml of pyrogen-free distilled water. The lysate solution was clarified by centrifuging at 6780 X g. Lysate was kept on ice during use. Unused lysate was quick frozen and stored as previously described.

The endotoxin used to standardize the LLA was E. coli 180-10 (Associates of Cape Cod). Endotoxin was obtained in lyophilized form and reconstituted with 10 ml of pyrogen-free distilled water to yield a final concentration of 100 ng/ml. The reconstituted endotoxin was stored at 4 °C for no longer than one month. This endotoxin was diluted to concentrations of 0.05 and 0.005 ng/ml by the combination of 1:10 and 1:2 dilutions. Pyrogen-free 0.3% NaCl was used as the diluent. All endotoxin dilutions were stored on ice until used. If the endotoxin dilutions were not used within 1 hour, they were discarded and new ones made. From the 0.05 and 0.005 ng/ml solutions of endotoxin, a series of endotoxin concentrations were made ranging from 1 to 30 pg/ml for use in preparing a standard curve for the LLA. The endotoxin standard solutions were prepared according to the scheme given in Table 3.

All sample dilutions and endotoxin solutions were mixed on a vortex shaker before the addition of 0.1 ml of lysate to 0.4 ml of endotoxin. The dilution scheme for endotoxin and the sample were such that the lysate could be added directly to the tubes containing

Table 3. Dilution scheme for preparation of endotoxin standards.

Stock Solutions	Desired Endotoxin Concentrations (picograms/ml)								
	1	3	5	7.5	10	15	20	25	30
	Milliliters of Stock Solution Added								
Pyrogen-free 0.1% NaCl	0.40	0.20	0	0.425	0.40	0.35	0.30	0.25	0.20
Endotoxin (0.005 ng/ml)	0.10	0.30	0.50	0	0	0	0	0	0
Endotoxin (0.05ng/ml)	0	0	0	0.075	0.10	0.15	0.20	0.25	0.30

the completed dilutions. After the addition of lysate, all samples were mixed on a vortex shaker, and incubated at 37 C in a circulating water bath for exactly 1 hour. Duplicate blanks were prepared by adding 0.1 ml of lysate to 0.5 ml of pyrogen-free 0.1% NaCl and incubating them along with each sample or endotoxin dilution series. After the incubation period, the tubes were removed from the water bath, mixed thoroughly and poured into microcuvets (Coleman) having a 1 cm light path. The absorbancy at 360 nm was immediately determined on a spectrophotometer (Varian Techtron Model 635). The spectrophotometer was zeroed with pyrogen-free 0.1% NaCl. The turbidity of the samples slowly increased with time. This necessitated the preparation of only the number of samples that could be read in a 5 minute time period. When large numbers of samples were being assayed, lysate was added at 15 minute intervals to the dilution series. By staggering the addition of lysate to the samples and reading the tubes within 5 minutes, any increase in turbidity was negligible.

A standard curve for the LLA was made by plotting absorbance at 360 nm against endotoxin concentration. Each absorbance value in the sample or endotoxin dilution series was corrected for the absorbancy exhibited by the blank. Total and free endotoxin concentrations in the sample were determined by averaging the values of the dilutions which fell within the range of the standard endotoxin curve. The endotoxin concentrations in the sample were reported as ng/ml of endotoxin.

Chapter 5

RESULTS

Development of a Gram-negative Selective Medium

The effects on bacterial growth of Nile blue, crystal violet and ethyl violet are summarized in Table 4. Nile blue did not totally inhibit the gram-positive bacteria at the concentrations tested while several gram-negatives (E. coli, S. flexneri and E. carotovora) were inhibited. Crystal violet completely inhibited all of the gram-positive bacteria as well as many of the gram-negative bacteria at the concentration of 2 ug/ml. Ethyl violet, at a concentration of 1.25 ug/ml, inhibited all of the gram-positive bacteria except for B. megaterium, B. polymyxa and S. faecalis sub zymogenes. None of the gram-negative bacteria tested, with the exception of Acinetobacter, which did not grow in the presence of any selective agents, were inhibited.

Since some of the Bacillus spp. and S. faecalis subspecies zymogenes grew in the presence of 1.25 ug/ml of ethyl violet, penicillin was added in various concentrations to the basal medium containing 1.25 ug/ml ethyl violet to determine the selectivity of these two compounds in combination. The effects of ethyl violet and penicillin on the growth of selected bacteria are shown in Table 5. Ethyl violet with 10 units of penicillin/ml seemed to provide the

Table 4. The effects of Nile blue, crystal violet and ethyl violet on the growth of selected bacteria

Organism	CPS ^a	Relative Growth ^c Dye ^b and Concentrations (ug/ml)					
		Nile Blue		Crystal Violet		Ethyl Violet	
		20	100	2	10	1.25	2
<u>E. coli</u>	4+	3+	3+	2+	1+	4+	4+
<u>B. megaterium</u>	4+	1+	2+	0	0	2+	1+
<u>E. aerogenes</u>	4+	4+	4+	4+	3+	4+	4+
<u>B. cereus</u>	4+	2+	2+	0	0	0	0
<u>S. typhimurium</u>	4+	4+	3+	0	0	4+	3+

a = modified casein-peptone-starch (CPS) medium without any added dye

b = dyes were added to the modified CPS medium to obtain the listed concentrations

c = the amount of growth was graded according to that occurring on modified CPS without the addition of dyes:

4+ = growth comparable to that occurring on CPS medium

3+ = approximately 3/4 of the growth occurring on CPS medium

2+ = approximately 1/2 of the growth occurring on CPS medium

1+ = approximately 1/4 of the growth occurring on CPS medium or the appearance of a few isolated colonies

0 = no growth

Table 4. (continued)

Organism	CPS ^a	Nile Blue		Relative Growth ^c Dye ^b and Concentrations (ug/ml)			
		20	100	Crystal Violet		Ethyl Violet	
				2	10	1.25	2
<u>B. polymyxa</u>	4+	1+	1+	0	0	3+	2+
<u>E. cloacae</u>	4+	4+	4+	4+	4+	4+	4+
<u>S. bovis</u>	4+	3+	3+	0	0	0	0
<u>S. flexneri</u>	4+	3+	3+	3+	0	4+	4+
<u>S. faecalis</u>	4+	4+	3+	0	0	0	0
<u>A. faecalis</u>	4+	4+	3+	0	0	4+	4+
<u>S. faecalis</u> subs. <u>zymogenes</u>	4+	4+	4+	0	0	2+	0
<u>S. marcescens</u>	4+	4+	4+	3+	1+	4+	4+
<u>Streptomyces</u> sp.	4+	0	0	0	0	0	0
<u>K. pneumoniae</u>	4+	4+	4+	4+	4+	4+	4+
<u>Sarcina lutea</u>	4+	1+	1+	0	0	0	0

Table 4. (continued)

Organism	CPS	Nile Blue		Relative Growth ^c Dye ^b and Concentration (ug/ml)			
		20	100	Crystal Violet		Ethyl Violet	
				2	10	1.25	2
<u>L. brevis</u>	4+	0	0	0	0	0	0
<u>E. caratovora</u>	4+	3+	3+	1+	0	4+	4+
<u>L. mesenteroides</u>	4+	2+	1+	0	0	0	0
<u>Acinetobacter</u> sp.	4+	0	0	0	0	0	0
<u>P. aeruginosa</u>	4+	3+	3+	4+	3+	4+	4+

Table 5. The effects of ethyl violet and penicillin on the growth of selected bacteria

Organism	Relative Growth ^c						
	CPS ^a	30	10	<u>Penicillin Concentrations^b</u> (units/ml)			
				5	2.5	1	.1
<u>E. coli</u>	4+	4+	4+	4+	4+	4+	4+
<u>B. megaterium</u>	4+	0	0	0	0	0	0
<u>E. aerogenes</u>	4+	4+	4+	4+	4+	4+	4+
<u>B. cereus</u>	4+	0	0	0	0	0	0
<u>S. typhimurium</u>	4+	4+	4+	4+	4+	4+	4+
<u>B. polymyxa</u>	4+	0	0	2+	2+	2+	2+

a = modified casein-peptone-starch medium (CPS) without any selective agents

b = modified CPS medium supplemented with 1.25 ug/ml ethyl violet and penicillin in the concentrations listed

c = growth occurring relative to that occurring on CPS without selective agents:

4+ = growth comparable to that occurring on CPS medium

3+ = approximately 3/4 the growth occurring on CPS

2+ = approximately 1/2 the growth occurring on CPS

1+ = approximately 1/4 the growth occurring on CPS or the appearance of a few isolated colonies

0 = no growth

nd = not determined

Table 5. (continued)

Organism	Relative Growth ^c						
	CPS ^a	30	10	Penicillin Concentrations ^b (units/ml)			
				5	2.5	1	.1
<u>E. cloacae</u>	4+	4+	4+	4+	4+	4+	4+
<u>S. bovis</u>	4+	0	0	nd	nd	nd	nd
<u>S. flexneri</u>	4+	0	2+	2+	2+	2+	2+
<u>S. faecalis</u>	4+	0	0	nd	nd	nd	nd
<u>A. faecalis</u>	4+	3+	3+	3+	3+	3+	3+
<u>S. faecalis</u> subs. <u>zymogenes</u>	4+	0	0	nd	nd	nd	nd
<u>S. marcescens</u>	4+	3+	4+	4+	4+	4+	4+
<u>Streptomyces</u> sp.	0	0	0	nd	nd	nd	nd
<u>S. lutea</u>	4+	0	0	nd	nd	nd	nd
<u>K. pneumoniae</u>	4+	4+	4+	nd	nd	nd	nd
<u>L. brevis</u>	0	0	0	nd	nd	nd	nd

Table 5. (continued)

Organism	CPS ^a	Relative Growth ^c					
		30	10	<u>Penicillin Concentrations^b</u> (units/ml)			
				5	2.5	1	.1
<u>E. caratovora</u>	4+	2+	2+	2+	2+	2+	2+
<u>L. mesenteroides</u>	4+	0	0	0	0	0	0
<u>Acinetobacter sp.</u>	4+	0	0	0	0	0	0
<u>P. aeruginosa</u>	4+	4+	4+	nd	nd	nd	nd

best combination for the selective enumeration of gram-negative bacteria. All gram-positive bacteria were completely inhibited. S. flexneri, A. faecalis and E. caratovora, however, were gram-negative bacteria that showed slight inhibition.

Five gram-negative bacteria were selected to compare the success of Standard Methods Agar (SMA), Casein-peptone-starch (CPS) and Ethyl Violet Penicillin (EVP) in enumerating pure cultures of bacteria. The geometric means of 5 replicate samples for the different bacteria and media are shown in Table 6. The t-values for the comparison between bacteria and media are shown in Table 7. The null hypothesis for this comparison was that the first member of the comparison was greater than the second member. In no case was SMA a better medium than EVP for the enumeration of the bacteria tested. Counts were higher on SMA than on CPS except for those of E. coli. The large difference between these two media when enumerating S. typhimurium may have been due in part to the pinpoint colonies of Salmonella on CPS. Also the differences may also reflect the reduced incubation time of 96 h instead of the 7-14 days that are recommended. This experiment was designed chiefly to compare the success of EVP and SMA in enumeration of pure cultures of gram-negative bacteria. If the counts on SMA were not greater after five days, the counts probably would not be significantly different after 7 days. EVP was a better medium for the enumeration of K. pneumoniae and S. typhimurium than CPS. CPS showed

Table 6. Numbers of bacteria enumerated on standard methods agar (SMA), casein-peptone-starch medium (CPS) and ethyl violet penicillin medium (EVP)

Bacteria	Number of Bacteria per ml ^a		
	SMA	<u>Media</u> CPS	EVP
<u>E. coli</u>	67 X 10 ⁷	73 X 10 ⁷	72 X 10 ⁷
<u>K. pneumoniae</u>	41 X 10 ⁶	33 X 10 ⁶	40 X 10 ⁶
<u>E. aerogenes</u>	65 X 10 ⁷	58 X 10 ⁷	59 X 10 ⁷
<u>S. typhimurium</u>	84 X 10 ⁷	42 X 10 ⁷	81 X 10 ⁷
<u>S. flexneri</u>	79 X 10 ⁷	90 X 10 ⁷	78 X 10 ⁷

^a = geometric mean of 5 replicates

Table 7. Comparison of the suitability of standard methods agar (SMA), casein-peptone-starch medium (CPS) and ethyl violet-penicillin medium (EVP) for enumerating selected bacteria

	SMA vs CPS	Comparison SMA vs EVP	CPS vs EVP
<u>E. coli</u>			
t-value	- .639	.641	.247
p-value ^a	> 0.25	> 0.25	> 0.25
<u>K. pneumoniae</u>			
t-value	2.17	-.644	-1.753
p-value ^a	> 0.025	> 0.25	> 0.05
<u>E. aerogenes</u>			
t-value	1.81	1.142	-.537
p-value ^a	> 0.05	> 0.10	> 0.25
<u>S. typhimurium</u>			
t-value	6.131	.498	-6.019
p-value ^a	> 0.005	> 0.25	> 0.0005
<u>S. flexneri</u>			
t-value	-1.887	-.438	2.277
p-value ^a	> 0.025	> 0.25	> 0.025

a = one-tailed test with 8 degrees of freedom

greater success in the enumeration of S. flexneri than EVP.

To test for the selectivity for gram-negative bacteria of crystal violet (CV), EVP and Tergitol 7, bacteria isolated on these media from water samples were gram-stained. The percents of gram-negative bacteria appearing on CV, EVP and Tergitol 7 are listed in Table 8. CV medium was effective in enumerating only 62% gram-negative bacteria whereas EVP and Tergitol 7 media produced 100% gram-negative bacteria.

Applicability of Limulus Lysate Assay as a Rapid Test of Water Quality

Endotoxin content and the numbers of bacteria at sites on the East Gallatin River are summarized in Table 9. Endotoxin concentrations were determined by the firm clot method of the Limulus lysate assay (LLA). Bacteria were enumerated by the membrane filter technique. Crystal violet agar was used in this phase of the research to enumerate gram-negative bacteria. Figure 3 presents an endotoxin and bacterial profile of the East Gallatin River. Generally, as the number of bacteria increased, the amount of endotoxin increased. Between sites H3 and H4, the number of coliforms, enterics, and gram-negatives increased, but the concentration of endotoxin remained constant. However, between M3 and M4, the number of coliforms decreased while the endotoxin concentration increased. This increase in endotoxin was reflected by an increase in enterics and gram-negatives. The lysate used in this

Table 8. Percent gram-negative aquatic bacteria recovered on different media

Medium	Percent gram-negative bacteria
Crystal violet ^a	62%
Ethyl violet-penicillin (EVP) ^b	100%
Tergitol 7 ^c	100%

a = bacteria enumerated by the membrane filter technique

b = bacteria enumerated by the spread plate technique

c = bacteria enumerated by the spread plate technique

Table 9. Bacteria and total endotoxin concentrations obtained at different sites on the East Gallatin River

Date and Site	Total Coliforms/100ml ^b	Enteric Bacteria/100ml ^b	Gram-negative Bacteria/100ml ^b	Total Endotoxin ^a
11/13/74				
M3	105	2,530	6,000	2.60
M4	39	5,700	5,700	0.26
M5	7,600	77,000	85,000	26.00
11/21/74				
M3	49	670	850	0.26
M4	256	2,900	1,400	26.00
M5	6,150	119,000	7,700	26.00
12/5/74				
M3	71	275	1,640	3.88
M4	42	6,800	3,640	2.60
M5	9,100	90,000	60,500	3.88
12/12/74				
M3	61	1,040	1,700	7.89
M4	54	8,100	3,900	26.00
M5	9,200	101,000	106,000	38.80
1/15/74				
EG4	3,000	16,200	29,500	7.89
EG5	4,700	55,000	50,000	78.90
OF2	25,500	390,000	124,000	260.00
1/20/75				
H3	16	570	2,550	0.26
H4	48	4,700	6,000	0.26
M5	550	96,000	38,000	33.30
2/19/75				
H3	36	380	2,550	4.16
M4	69	1,200	6,000	8.32
EG5A	1,400	13,000	18,000	16.66

a = total endotoxin concentration determined by the firm clot method and bacteria enumerated by membrane filter technique

b = arithmetic mean of two replicate samples.

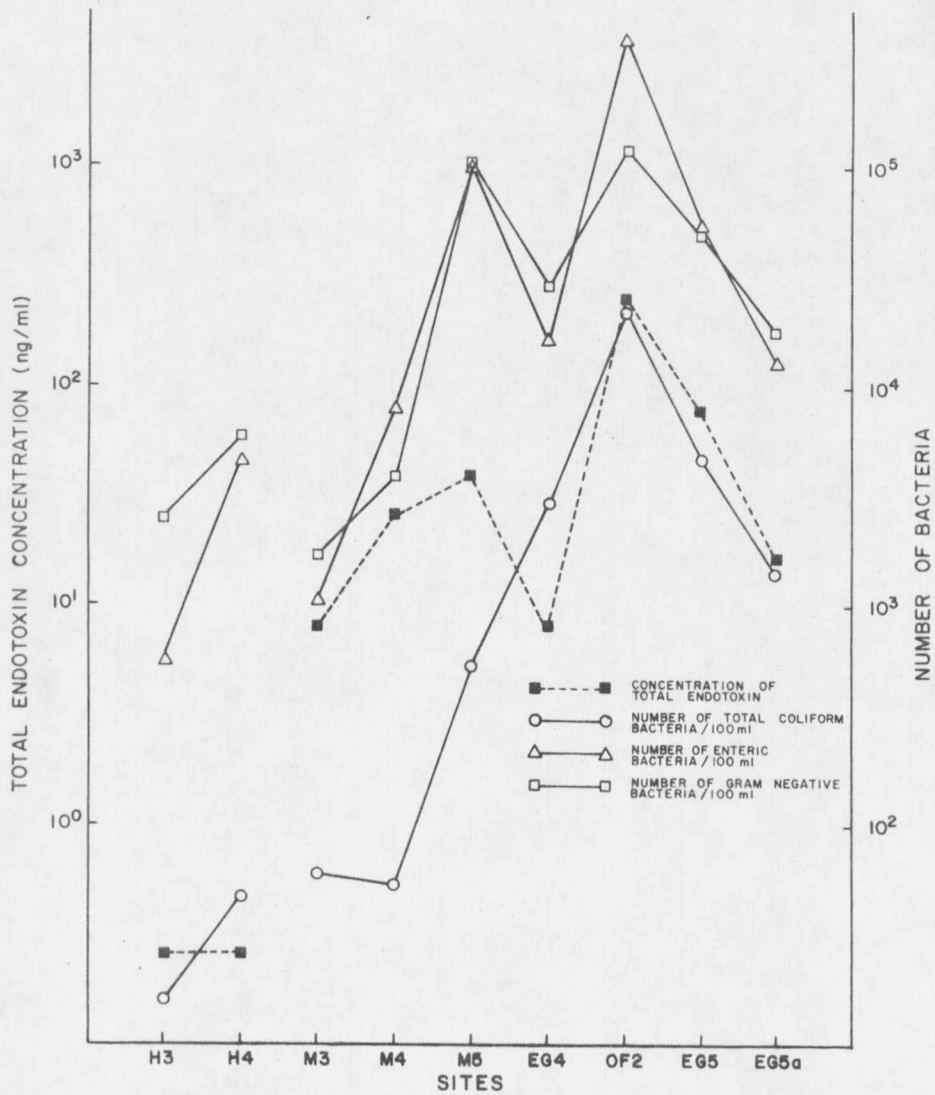


FIGURE 3. BACTERIAL AND TOTAL ENDOTOXIN PROFILE OF THE EAST GALLATIN RIVER DRAINAGE FOR SAMPLING DATES 12/12/74, 1/15/75, 1/20/75 AND 2/19/75. EACH POINT REPRESENTS THE ARITHMETIC MEAN OF TWO REPLICATE SAMPLES. BACTERIAL NUMBERS WERE DETERMINED BY MEMBRANE FILTRATION AND ENDOTOXIN BY THE FIRM CLOT METHOD OF THE LIMULUS LYSATE ASSAY.

study had a sensitivity of 0.26 ng/ml of endotoxin. Since the levels of endotoxin detected at these two sites were at the limit of sensitivity of the assay, the endotoxin content could have been different even though not reflected by the LLA. The distribution of enteric and gram-negative bacteria closely paralleled that of endotoxin. Coliform bacteria followed the same pattern with the exception of the site EG4, where the number of coliforms increased from M5 to EG4, but the endotoxin concentration decreased. Enteric and gram-negative bacteria decreased at this site, as reflected by the decrease in endotoxin.

Correlation coefficients for endotoxin content versus bacterial numbers and bacterial groups are listed in Table 10. The correlations between endotoxin and total coliforms, enterics and gram-negatives were all significant at the 1% level. Coliforms versus endotoxin was the highest correlation with 0.714. Logarithmic transformations of the data were made when it was found that the relationship between endotoxin concentration and the Log_{10} of bacterial numbers was not linear. The relationship between endotoxin concentration and enteric bacteria is shown in Figure 4. This scatter diagram shows a definite relationship between endotoxin concentration and the number of enteric bacteria, despite the large variability in the data. The high correlation coefficients between enteric bacteria and total coliforms, gram-negative bacteria and total coliforms, and gram-negative bacteria and

Table 10. Correlation coefficients for endotoxin and bacterial variables

Variables ^b	r ^a	significance level
Total Coliforms vs Total Endotoxin	.764	1%
Enteric Bacteria vs Total Endotoxin	.642	1%
Gram-negative Bacteria vs Total Endotoxin	.590	1%
Enteric Bacteria vs Total Coliforms	.878	1%
Gram-negative Bacteria vs Total Coliforms	.748	1%
Gram-negative Bacteria vs Enteric Bacteria	.870	1%

a = logarithmic transformations of endotoxin and bacterial variables

b = total endotoxin concentration determined by the firm clot method and bacteria enumerated by the membrane filter technique

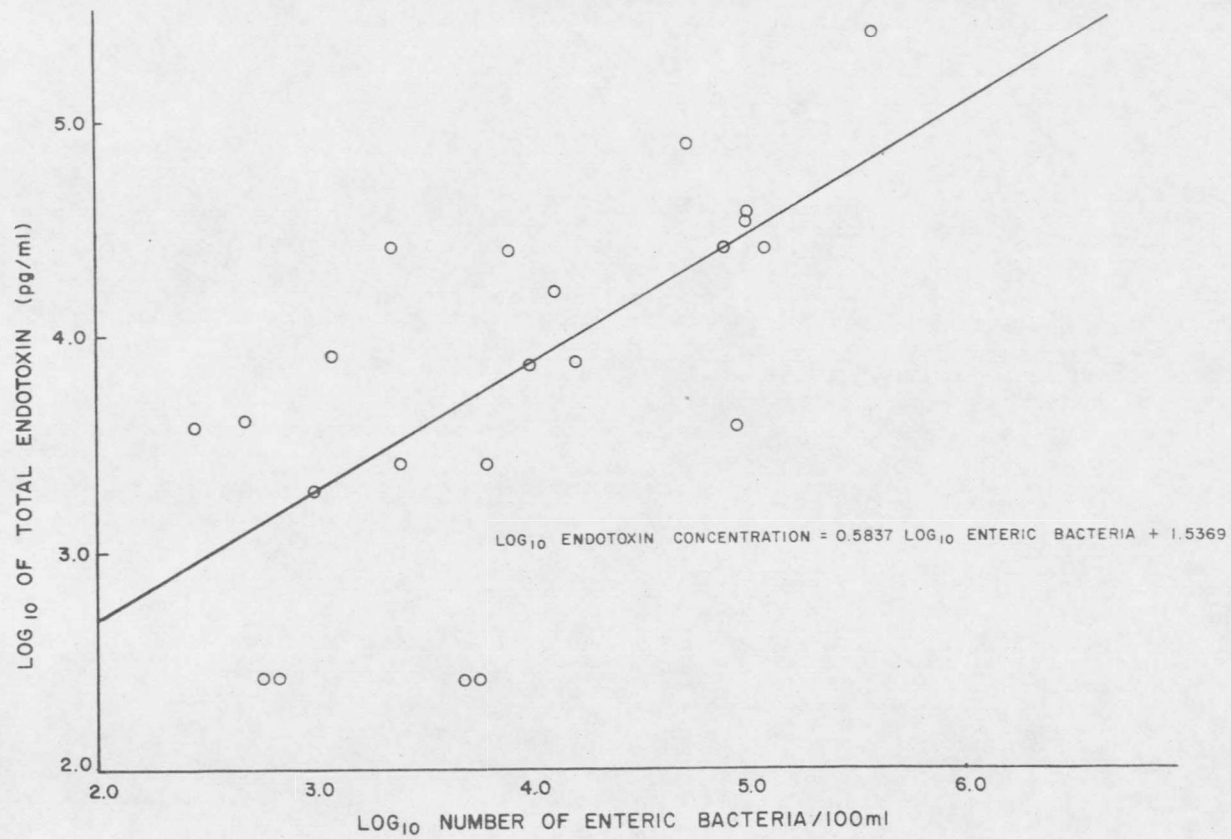


FIGURE 4. RELATIONSHIP BETWEEN TOTAL ENDOTOXIN CONCENTRATION AND ENTERIC BACTERIA ENUMERATED BY MEMBRANE FILTRATION.

enteric bacteria show the interrelationship between these bacterial groups. A scatter diagram of the relationship between enteric and coliform bacteria is presented in Figure 5.

Correlation of the Amount of Endotoxin in Water with the Number of Bacteria

After determining the applicability of the lysate assay as a rapid test of water quality, different procedures were used to detect endotoxin, gram-negative bacteria and enteric bacteria. The spectrophotometric modification of the Limulus lysate assay was used to make the assay more quantitative and reproducible. An example of a standard curve for the lysate assay using the spectrophotometric method is shown in Figure 6. As stated in the literature review, the spread plate technique is the most suitable method for enumerating the optimal numbers of bacteria. Therefore, gram-negative bacteria were enumerated on EVP medium by the spread plate technique. Enteric bacteria were enumerated on Tergitol 7 agar, also by the spread plate technique.

The concentration of bound endotoxin and the numbers of bacteria for 8 sample dates are summarized in Table 11. The number of bacteria and endotoxin content of drinking water sampled at the university laboratory is also shown in Table 11. Tap water contained an endotoxin content of 0.09 ng/ml and no coliforms or enteric bacteria. Bacterial and bound endotoxin profiles for the East Gallatin are shown in

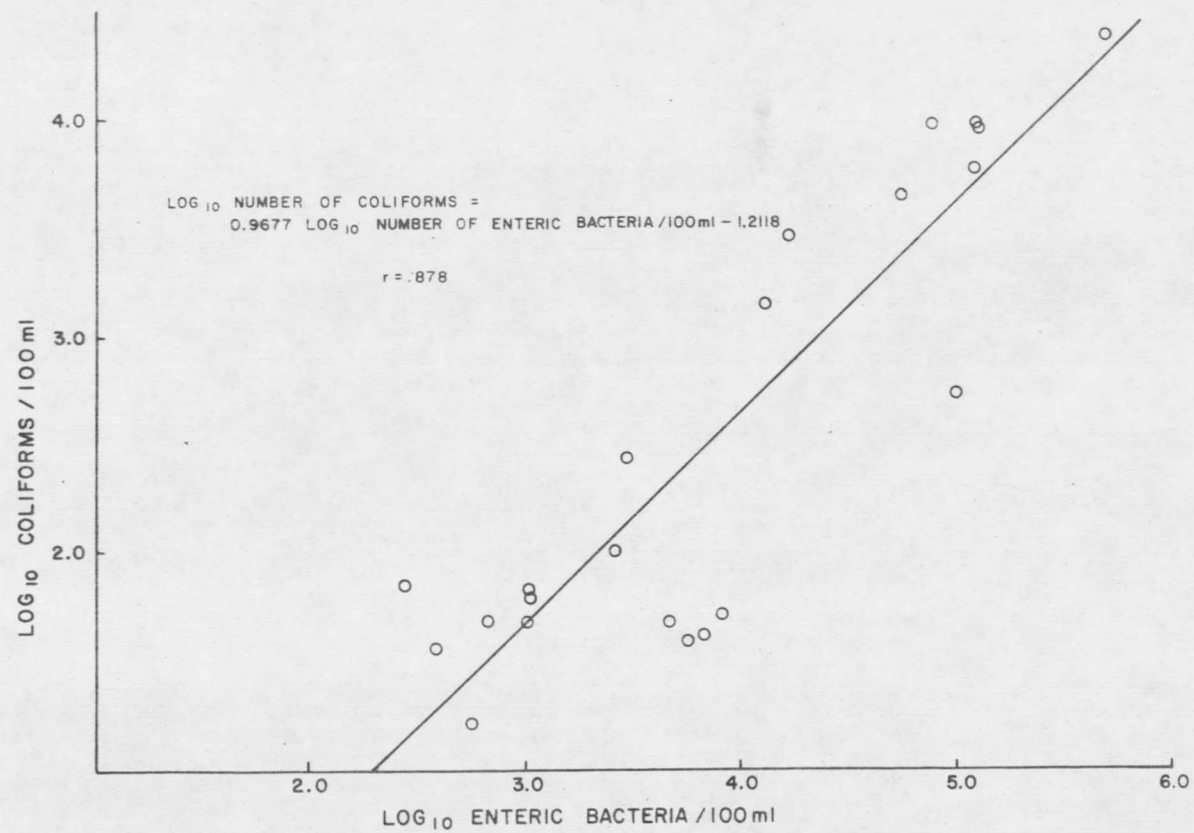


FIGURE 5. RELATIONSHIP BETWEEN ENTERIC BACTERIA AND COLIFORM BACTERIA ENUMERATED BY THE MEMBRANE FILTER TECHNIQUE.

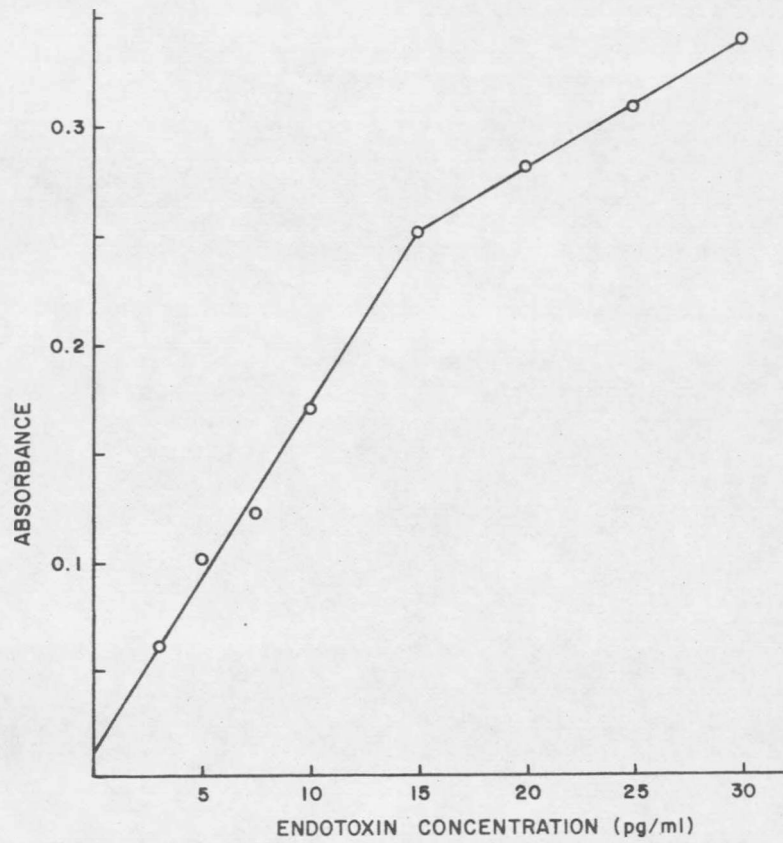


FIGURE 6. TYPICAL STANDARD CURVE FOR ENDOTOXIN ASSAY

Table 11. Bound endotoxin content and numbers of bacteria in the East Gallatin River drainage^a

Date and Site	Bound LPS ng/ml	Total Coliforms/100ml ^c	Heterotrophic Bacteria/ml ^c	Enteric Bacteria/ml ^c	Gram-negative Bacteria/ml ^c
7/3/75					
EG4	7.50	2,150	13,100	2,240	4,150
OF2	85.00	186,000	428,000	64,500	132,000
EG5	15.49	6,390	35,300	3,940	8,000
7/7/75					
M3	1.08	20	4,220	181	641
M4	2.64	99	8,490	937	3,550
M5	8.00	2,130	16,800	1,960	7,850
7/8/75					
EF1	0.70	2	1,590	133	366
H5	nd	1,410	38,500	1,950	8,810
EG5A	28.70	7,490	84,500	3,840	108,000
7/9/75					
EG4	6.80	3,360	10,500	1,880	4,990
OF2	81.00	109,000	323,000	42,900	196,000
EG5	13.50	7,260	30,600	4,146	11,000

a = endotoxin determined by a spectrophotometric application of the Limulus lysate assay, bacteria enumerated by the membrane filter and spread plate techniques

b = drinking water tap at Montana State University

c = geometric mean of 5 replicate samples

nd = not determined

Table 11. (continued)

Date and Site	Bound LPS ng/ml	Total Coliforms/100ml ^c	Heterotrophic Bacteria/ml ^c	Enteric Bacteria/ml ^c	Gram-negative Bacteria/ml ^c
7/10/75					
M3	1.65	24	3,600	153	520
M4	2.16	551	11,200	1,400	4,700
M5	11.60	152,000	67,000	4,200	11,300
7/11/75					
EG4	8.10	9,170	20,900	3,360	8,600
OF2	72.00	171,000	256,000	43,900	120,000
EG5	29.40	15,200	39,700	4,990	15,600
7/14/75					
EF1	0.84	7	720	141	420
H5	17.10	1,650	83,000	2,410	13,100
EG5A	32.40	35,800	163,000	7,780	31,800
7/15/75					
EF1	1.17	6	1,780	170	736
H5	5.28	3,030	35,300	5,800	18,500
EG5A	27.90	25,400	45,300	11,500	26,100
Tap ^b	0.09	0	46	0	3

Figures 7, 8 and 9. Bound endotoxin levels varied from 0.07 ng/ml at EF1 to 85 ng/ml at OF2. With this 1,000-fold range of bound endotoxin concentrations, the LLA is sensitive enough to adequately measure the endotoxin concentrations in river water. The same general trend can be seen as with the data in Figure 3. As the number of bacteria increased, the concentration of bound endotoxin increased. Enteric, gram-negative and heterotrophic bacteria all followed the same trend as coliform bacteria, indicators. In Figure 7, gram-negative, heterotrophic and coliform bacteria follow the same trends as endotoxin. Except for EG4, where the LPS concentration decreased and the number of enteric bacteria increased, enteric bacteria followed the same trend as endotoxin. All bacteria in Figure 8 followed the same trend as endotoxin. The endotoxin and coliform profile in Figure 9 presents drastically different results. The general trend of endotoxin and bacteria was followed at all sites with the exception of H5 and EG5A. The endotoxin concentration at these sites were measured with Limulus lysate which had been quick frozen and stored. These data indicate a reduced activity of the lysate. This was also apparent in the standard curve developed for that sampling date.

The extremes of variability in water quality between these sites are represented by the water at EF1, which is a pristine mountain stream, and that of OF2, which is the outfall of a sewage treatment plant.

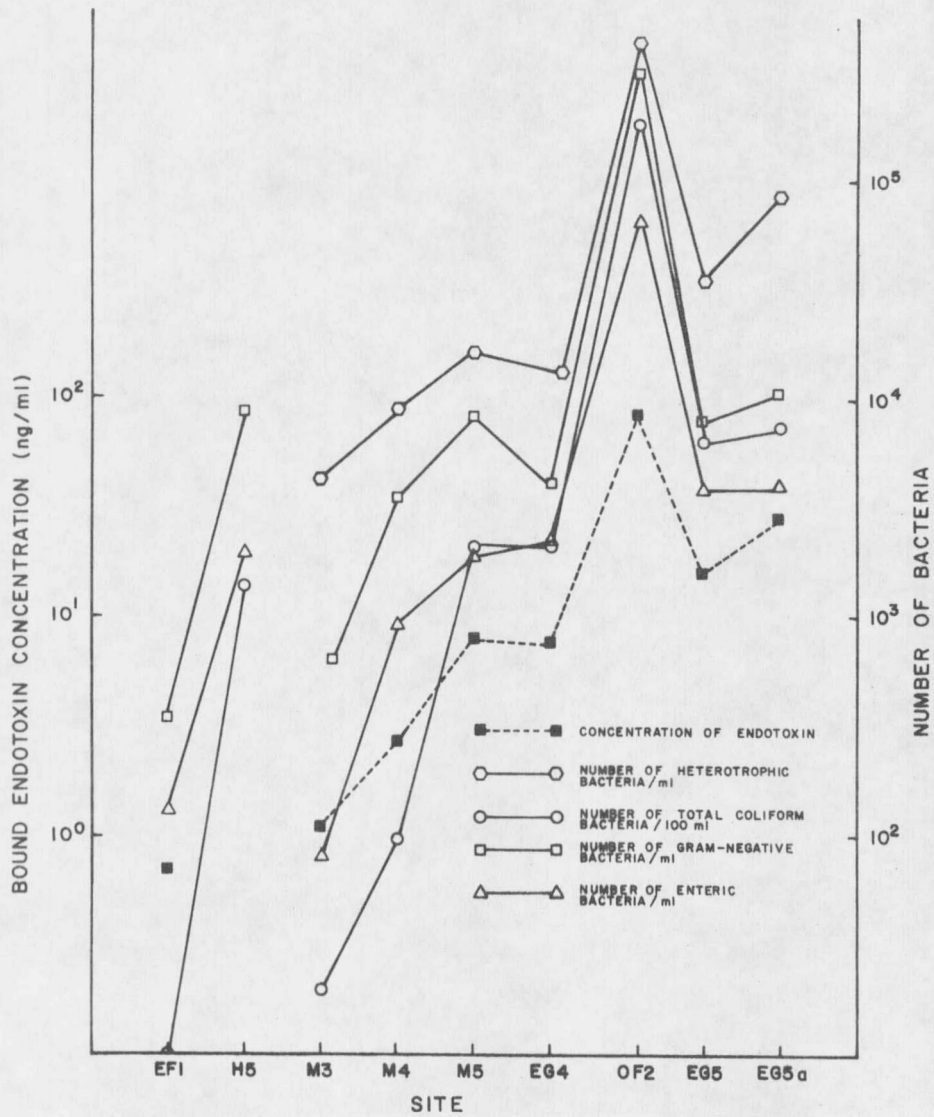


FIGURE 7. BACTERIAL AND ENDOTOXIN PROFILE OF THE EAST GALLATIN RIVER DRAINAGE FOR SAMPLING DATES 7/3/75, 7/7/75, 7/8/75. EACH POINT REPRESENTS THE GEOMETRIC MEAN OF FIVE REPLICATE SAMPLES ENUMERATED BY THE SPREAD PLATE TECHNIQUE.

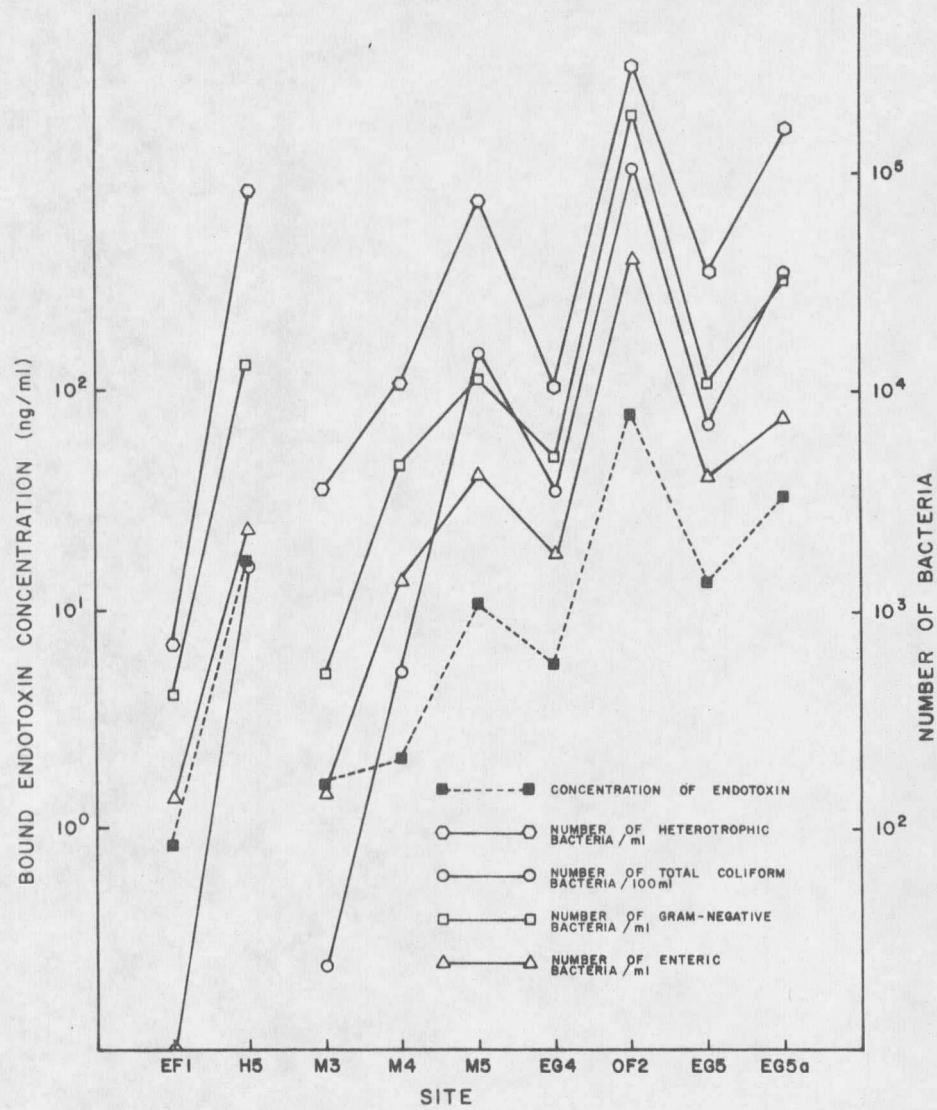


FIGURE 8. BACTERIAL AND ENDOTOXIN PROFILE OF THE EAST GALLATIN RIVER FOR SAMPLING DATES 7/9/75, 7/10/75 AND 7/14/75. EACH POINT REPRESENTS THE GEOMETRIC MEAN OF FIVE REPLICATE SAMPLES ENUMERATED BY THE SPREAD PLATE TECHNIQUE.

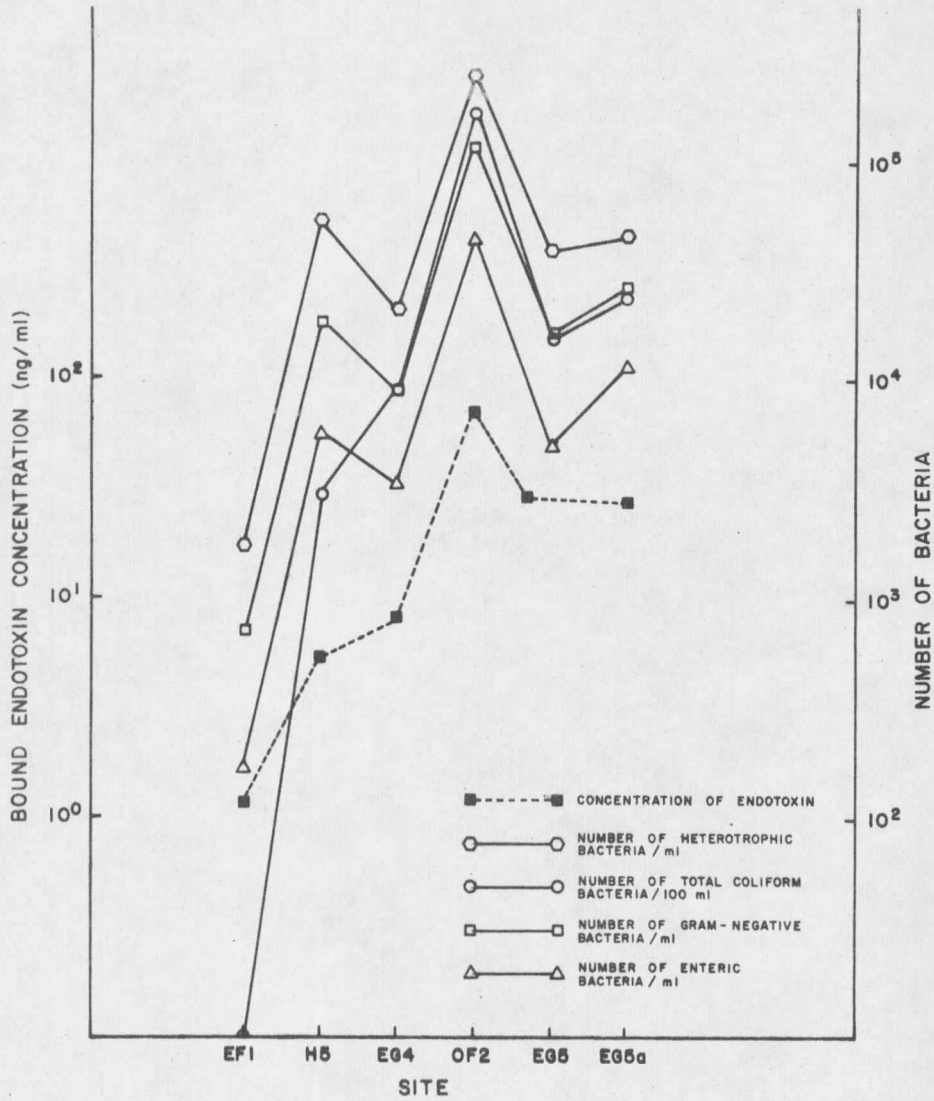


FIGURE 9. BACTERIAL AND ENDOTOXIN PROFILE OF THE EAST GALLATIN RIVER DRAINAGE FOR SAMPLING DATES 7/11/75 AND 7/15/75. EACH POINT REPRESENTS THE GEOMETRIC MEAN OF FIVE REPLICATE SAMPLES ENUMERATED BY THE SPREAD PLATE TECHNIQUE.

The relationships between bound, free and total endotoxin are summarized in Table 12. The ratio of bound/free, bound/total and free/total endotoxin are graphed in Figure 10. Total endotoxin varied 1,000-fold from 2.43 ng/ml at M3 to 1,049 ng/ml at OF2. Generally, as the total amount of endotoxin increased the numbers of bacteria also increased. The ratio of bound/free endotoxin was highest at sites H5, M5 and EG5A which were the lowest sites sampled on Hyalite, Mystic and the East Gallatin River, respectively. The ration of free/total endotoxin was highest at EF1, M3 and OF2. The correlation coefficients of endotoxin (bound and total) versus bacterial numbers are summarized in Table 13. The correlations between total endotoxin and heterotrophic, gram-negative, enteric and coliform bacteria were all greater than 0.8 and significant at the 1% level. Scatter diagrams of the relationship between endotoxin and bacterial groups are shown in Figures 11, 12, 13 and 14. Logarithmic transformations of the concentration of endotoxin in picograms/ml (pg/ml) and bacteria were computed to try to derive a linear relationship between total endotoxin and bacterial numbers. Endotoxin concentrations were expressed in pg/ml to make the computations easier. As shown in Figures 12, 13 and 14, the regression lines do not adequately fit the data. A large portion of the data points fell below the regression line. This may be a result of the three points representing data from OF2, which lie at the extreme end of the scale. These three points would

Table 12. Relationships between total, bound and free endotoxin in the East Gallatin River drainage.

Date and Site	Total LPS ng/ml	Free LPS ng/ml	Bound LPS ng/ml	<u>Bound</u> Free	<u>Ratios</u>		
					<u>Bound</u> Total	<u>Free</u> Total	
7/3/75							
EG4	19.00	11.50	7.50	0.65	0.39	0.61	
OF2	1,049.00	964.00	85.00	0.09	0.08	0.92	
EG5	53.19	37.70	15.49	0.41	0.29	0.71	
7/7/75							
M3	2.43	1.35	1.08	0.80	0.44	0.56	
M4	5.31	2.67	2.65	0.99	0.50	0.50	
M5	11.70	3.70	8.00	2.16	0.68	0.32	
7/8/75							
EF1	9.70	9.00	0.70	0.08	0.07	0.93	
H5	16.10	nd	nd	nd	nd	nd	
EG5A	40.70	12.00	28.70	2.39	0.71	0.29	
7/9/75							
EG4	22.10	15.30	6.80	0.44	0.31	0.69	
OF2	252.00	171.00	81.00	0.47	0.32	0.68	
EG5	24.30	10.80	13.50	0.32	0.56	0.44	

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a = drinking water tap at Montana State University

nd = not determined

Table 12. (continued)

Date and Site	Total LPS ng/ml	Free LPS ng/ml	Bound LPS ng/ml	<u>Bound</u> <u>Free</u>	<u>Ratios</u>	
					<u>Bound</u> <u>Total</u>	<u>Free</u> <u>Total</u>
7/10/75						
M3	4.80	3.15	1.65	0.52	0.34	0.66
M4	5.31	3.15	2.16	0.69	0.41	0.59
M5	21.00	9.45	11.55	1.22	0.55	0.45
7/11/75						
EG4	18.90	10.80	8.10	0.75	0.43	0.57
OF2	756.00	684.00	72.00	0.11	0.10	0.90
EG5	46.80	17.40	29.40	1.69	0.63	0.37
7/14/75						
EF1	2.64	1.80	0.84	0.47	0.68	0.32
H5	24.60	7.50	17.10	2.28	0.70	0.30
EG5A	48.60	16.20	32.40	2.00	0.67	0.33
7/15/75						
EF1	2.67	1.50	1.17	0.78	0.44	0.56
H5	12.69	7.41	5.28	0.71	0.42	0.58
EG5A	44.10	16.20	27.90	1.72	0.63	0.37
Tap ^a	1.19	1.10	0.09	0.08	0.92	0.08

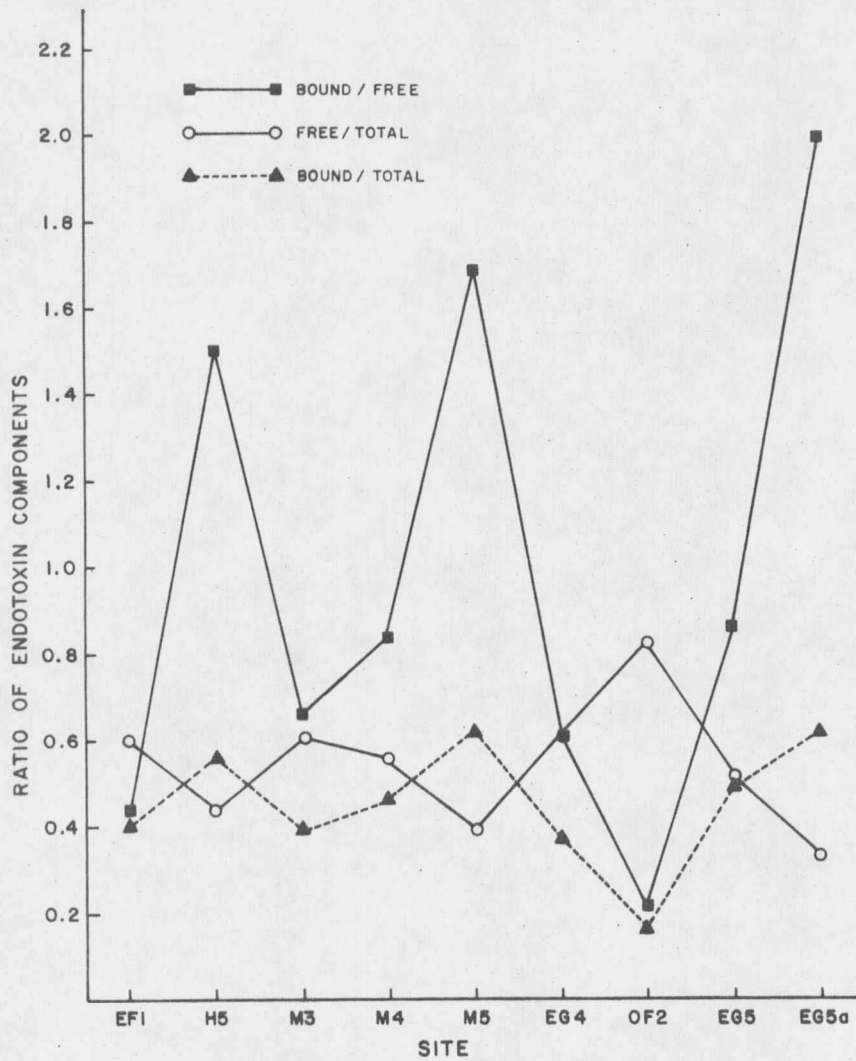


FIGURE 10. RELATIONSHIP BETWEEN BOUND, FREE, AND TOTAL ENDOTOXIN. ENDOTOXIN DETERMINED BY SPECTROPHOTOMETRIC METHOD OF THE LIMULUS LYSATE ASSAY. DATA POINTS REPRESENT AVERAGES OF ALL SAMPLING DATES FOR EACH SITE.

