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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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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by

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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 indi-
cator systems are based upon the detection of fecal contamination
from warm-blooded animals since this is the natural link to the occur-
rance 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 expecially 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 <u>Limulus</u>

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 <u>Limulus</u>

lysate assay was developed by Levin and Bang (65) while investigating

the toxic effects of a marine bacterium on <u>Limulus</u> <u>polyphemus</u>, 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 <u>Limulus</u> 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 concentra-
tions. 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 characteris-
tics, 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
occurrance 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 occurrance 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 Psedomonas 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 inves-

tigators 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 $^{14}CO_2$ 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 usefullness 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 micro-

organisms 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). Endtotoxins, otherwise known as lipopoly-saccharides (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(oligiopolysaccharide).

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-existin 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 uring by measuring the total endotoxin content with the Limulus

lysate assay. By correlating the endotoxin content with bacterial

counts in uring, 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 <u>Limulus polyphemus</u> 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 <u>Limulus</u> lysate was first hypoth-

esized 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 endo-toxin and Limulus lysate. The use of this method made the LLA quantita-tive 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/nl of endotoxin.

Enumeration of Gram-negative Bacteria

The <u>Limulus</u> 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 <u>Escherichia coli</u>. 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 <u>Escherichia coli</u>, <u>Salmonella typhi</u> and <u>Salmonella typhimurium</u>

while completely inhibiting <u>Bacillus</u> <u>subtilis</u> and <u>Streptococcus</u>
<u>faecalis</u> (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 <u>Escherichia</u> <u>coli</u> 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.