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RAPID BIOASSAY MONITORING SYSTEM FOR WATER QUALITY

(RAPID DETECTION OF TOXICANTS IN POTABLE WATERS BY LASER LIGHT SCATTERING)

FINAL REPORT

PHILIP J. WYATT, Ph.D.

March 1989

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A laser-based bioassay system has been developed for the rapid detection of a broad range of toxicants in potable waters. Exponential phase cultures of two members of an isogenic set of *Bacillus subtilis* are prepared from lyophiles. Water samples to be tested are prepared by filtration, pH adjustment, and chlorine neutralization. For each *B. subtilis* strain (wild type WT 168 and mutant fh 2006-7) a 10 ml water sample is prewarmed to 39°C and inoculated with 0.15 ml of the culture. Similar control samples are prepared with deionized, filtered water. Laser light scattering measurements of the four samples are made 6 minutes and 66 minutes following inoculation. From these recorded measurements, an on-line personal computer determines the presence or absence of toxicants.

This report describes the instrumentation and protocols developed to achieve this rapid assay. The results of a detailed statistical analysis of key elements of the assay in the presence of non-toxic contaminants are presented using data from the Wyatt Technology Corporation laboratory and the Southern Research Institute. A set of 53 blind samples was subsequently processed and yielded an accuracy for detection in excess of 94%. The effects of background, non-toxic contaminants upon the various scoring algorithms are discussed. Recommendations for early field testing of a portable system are made together with a discussion of several options to expand the test for the detection of insoluble waterborne toxicants as well as those toxicants requiring metabolic activation.

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SUMMARY

A laser-based bioassay system has been developed for the rapid detection of a broad range of toxicants in potable waters. Exponential phase cultures of two members of an isogenic set of *Bacillus subtilis* are prepared from lyophiles. Water samples to be tested are prepared by filtration, pH adjustment, and chlorine neutralization. For each *B. subtilis* strain (wild type WT 168 and mutant fh 2006-7) a 10 ml water sample is prewarmed to 39°C and inoculated with 0.15 ml of the culture. Similar control samples are prepared with deionized, filtered water. Laser light scattering measurements of the four samples are made 6 minutes and 66 minutes following inoculation. From these recorded measurements, an on-line personal computer determines the presence or absence of toxicants.

This report describes the instrumentation and protocols developed to achieve this rapid assay. The results of a detailed statistical analysis of key elements of the assay in the absence of non-toxic contaminants are presented using data from the Wyatt Technology Corporation laboratory and the Southern Research Institute. A set of 53 blind samples was subsequently processed and yielded an accuracy for detection in excess of 94%. The effects of background, non-toxic contaminants upon the various scoring algorithms are discussed. Recommendations for early field testing of a portable system are made together with a discussion of several options to expand the test for the detection of insoluble waterborne toxicants as well as those toxicants requiring metabolic activation.

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FOREWORD

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PREFACE

This report is based primarily upon research performed during the period from March 1985 through July 1987, including a hiatus due to funding limitations from December 1985 through September 1986. Although the results of the 1985 studies were positive and covered a broad range of toxicants and contaminants, the basic accuracy and reproducibility of the method had not been confirmed. Indeed, the statistical studies of that period were deficient in many regards with, at the most, six replicate measurements being made at selected concentrations. A detailed statistical analysis was begun in late 1986 to confirm that, under good laboratory conditions, the toxicant detection method is reliable and reproducible. The bioassay test itself is comprised of several critical elements and procedures that had to be confirmed statistically on an individual basis.

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The measurements reported in this report were performed primarily under the direction of Dr. Richard U. Edgehill. Earlier measurement programs were directed by Dr. Richard G. Butler. Dr. I. Cecil Felkner, the developer of some of the strains used in the study and a microbial physiologist, served as a consultant to the program throughout its performance. The technical assistance of Mr. Joseph B. Estrada is gratefully acknowledged. Mr. Kendall Gray, a Ph.D. candidate at the University of Southern California, assisted in maintaining and checking the cultures periodically during the course of the statistical studies. An exceptional parallel series of measurements was performed at the Southern Research Institute under the direction of Dr. William J. Suling. The quantitative analyses were directed by Ruby H. James. Ms. Karen McAdams performed the DLS assays and Ms. Mary Emory performed the chemical stability studies. Statistical protocols were developed by Roger McDonald of Kaman TEMPO's Santa Barbara facility. The guidance, advice, and encouragement of the Contracting Officer's Technical Representative, Dr. Stephen A. Schaub, are gratefully acknowledged.

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1. INTRODUCTION

The detection of toxicants in potable waters by bacterial bioassays of various types has suffered traditionally from two major problems. First has been lack of speed, since several hours have usually been required to permit sufficient bacterial growth and a concomitant measurable response. Second has been the response of such bioassays to non-toxic contaminants often found in potable waters. These contaminants include high or low pH, surfactants and other organics, high levels of total dissolved solids, pigmentation, turbidity, chlorination, and some specific compounds such as sulfates. Laser light scattering bioassay techniques[1,2] such as developed by Wyatt Technology Corporation (WTC) staff, have been able to achieve the requirement for speed since such assays are easily completed in 60 minutes or less. The effects of contaminants, on the other hand, could result in too many false positives which would tend to make the technique impractical. Certain simple initial procedures such as filtering, pH adjustment and chlorine neutralization (the latter with sodium thiosulfate) remove many of these effects, but not others. Using two or more isogenic strains simultaneously with the laser light scattering method, such as the *B. subtilis* sets developed by I. C. Felkner and his collaborators[3-5] for plate assays, has been found to circumvent most of the contaminant-caused false positive results, provided that suitable mathematical algorithms are implemented. A wild type and mutant strain, for example, are sufficient to permit the differentiation of non-toxic contaminant effects (both strains respond *equally*) from the effects of genotoxins (each strain responds *differently*). The mutant is more greatly affected by the genotoxin as it lacks certain repair mechanisms relative to the wild type, parent strain.

Eleven representative water-borne toxicants have been studied extensively in a detailed statistical context to confirm the bioassay method and define its limitations. These include heavy metal compounds, herbicides, water soluble pesticides, organic solvents, and various metabolic poisons. The results of many of these laser bioassays are presented together with details of the method, testing protocols, and the current state of the associated instrumentation. The theoretical basis of the laser light scattering bioassay is described briefly in Sec. 2. Section 3 is concerned with instrumentation and software developed, for the most part, at WTC using both company and contract funding. Generally, the company-developed systems and software were modified and adapted under the contract to increase the efficiency or accuracy of the required testing programs. The measurement methods and materials are summarized and discussed in Sec. 4. The explicit means by which the measurements are quantified to permit the generation of dose-response curves is discussed in Sec. 5. This is followed by a description of the statistical analysis program for a non-toxic contaminant free environment in Sec. 6 and its results in Sec. 7 confirming, thereby, the basic methodology. Section 8 discusses the effects of contaminants on the assay and incorporates earlier work presented in the Interim Report of March 1986. The final section summarizes the accomplishment of the program and describes the steps remaining to obtain a practical, field-deployable system.

2. THEORY

Figure 1 is a schematic illustration of the basic laser light scattering measurement technique.[6-14] A cuvette containing a dilute suspension of exponential growth phase bacteria is illuminated along a diameter with a fine, monochromatic light beam such as that produced by a laser. The incident light is plane polarized with the electric field perpendicular to the plane of measurement. The light scattered by the bacteria is detected by an array of collimated detectors lying circumferentially about the center of the cuvette. The detectors are highly collimated so that their field of view is restricted to the very central region of the laser pencil passing through the transparent cuvette, generally a standard scintillation vial. At a bacterial concentration of 5×10^5 to 10^7 / ml, each detector collects the average scattered light from about 10^3 to 10^4 cells. For such a bacterial suspension, the recorded intensity as a function of scattering angle (0° is defined as the direction of the incident beam) may be plotted by interpolating between the discrete angles of the fixed detectors to yield the so-called differential light scattering (DLS) pattern[10] such as shown in Fig. 2 for a strain of *Bacillus subtilis*. The appearance of the pattern is similar to a diffraction pattern showing two maxima and three minima.

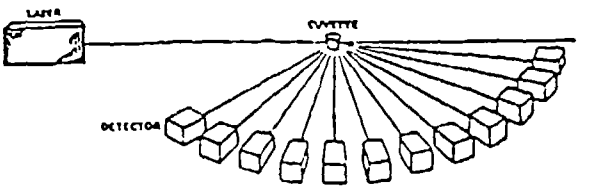

Fig. 1. Basic light scattering measurement from a suspension of bacteria illuminated by a fine laser beam.

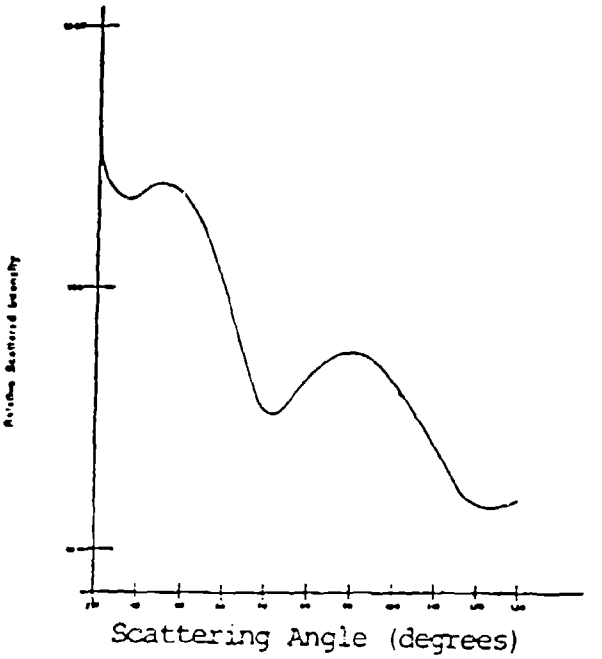

Fig. 2. Differential light scattering pattern from a suspension of exponential phase bacteria (*B. subtilis*) illuminated by a He-Ne laser at a wavelength of 632.8 nm.

The scattering of light from bacterial suspensions is rather well approximated by the Rayleigh-Gans-Debye (RGD) theory.[7,10,11-13] In this approximation, the average refractive index of the bacterial cells, n, must be very close to the refractive index of the surrounding medium (water), n_0, *i.e.*

$$| 1-(n / n_0) | << 1 \tag{1}$$

A second requirement of RGD theory is that the total phase *change* of a light ray traversing a bacterial cell is small compared to unity, *i.e.*

$$2\,ka\,| 1-(n / n_0) | << 1 \tag{2}$$

where a is the mean bacterial radius,

$$k = 2\pi n_0 / \lambda_0 \tag{3}$$

is the propagation number, and λ_0 is the incident wavelength (= 632.8nm for a He-Ne laser). Although condition (2) is rarely satisfied, the measured results remain, nevertheless, in good agreement with the RGD theory. For spherically symmetric bacteria, such as *Staphylococcus aureus*, a generalization of the exact Lorenz[16]-Mie[17] theory may be used. This theory is not subject to the limitations imposed by Eqs. (1) and (2), but requires extensive calculations, although they may be performed easily by personal computer.

Figure 3 shows the exact theoretically predicted variation of DLS patterns[7] from suspensions of homogeneous particles approximating spherical bacterial cells with a Gaussian size distribution of half width at half maximum of 23% of the mean radius, r_0. Note that with increasing size, the patterns shift to the *left*, *i.e.* smaller scattering angle. Figure 4 shows the theoretical effect of broadening the size distribution of the suspended cells; the DLS pattern washes out with increased distribution breadth.

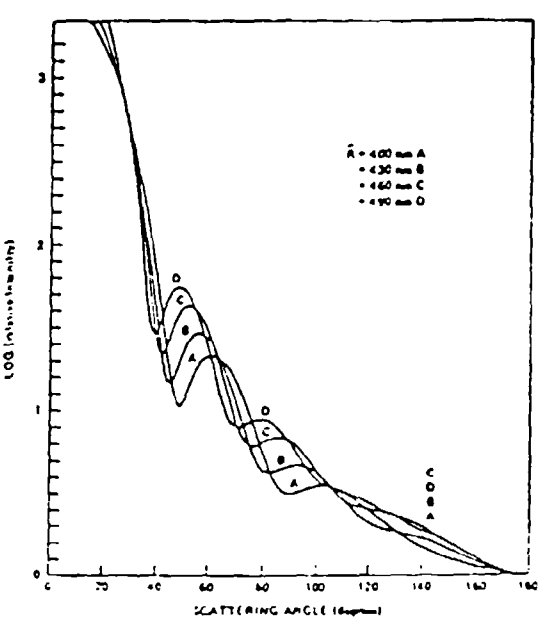

Fig. 3. Variation of DLS patterns of homogeneous spherical particles of refractive index 1.39 in water with a mean size distribution of ±13% as a function of R. Incident light is vertically polarized (λ_0 = 632.8 nm).

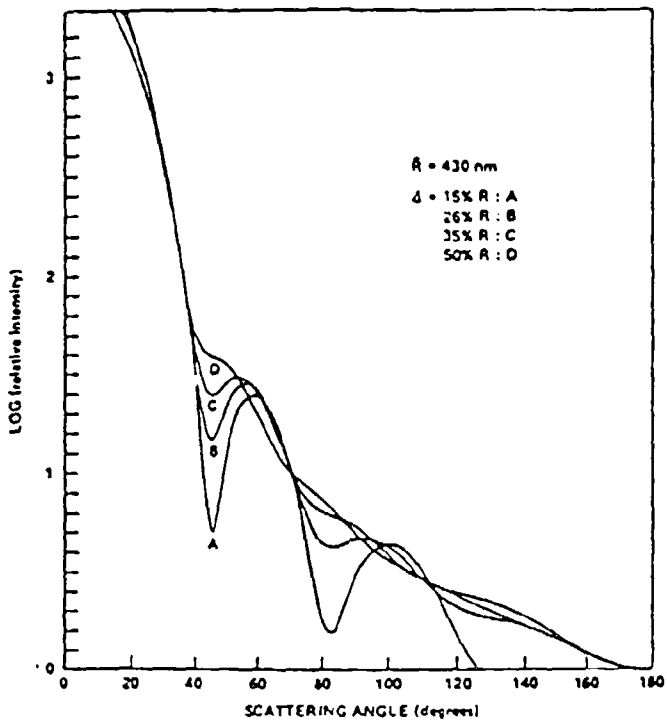

Fig. 4. The effects of changing size distributions on DLS patterns. This is the same model of particles used to generate Fig. 3.

There are two surprising results, confirmed by the RGD approximation, that measurements of the DLS patterns from exponential bacterial cells illustrate. First is the fact that the scattering patterns are sharply defined and, second, that even for rod-shaped organisms such as *B. subtilis*, the patterns remain sharp. In ideal exponential phase, cells reach a maximum volume 2 V_0 at which time they divide into two cells of equal volume V_0. Hence the ratio of the maximum volume to the minimum volume is just $2\ V_0 / V_0 = 2$, and the corresponding change in mean *radius* is just $2^{1/3}-1 \approx 23\%$, *i.e.* the distribution used to generate Fig. 3. Referring to Fig. 4, we see that such a relatively narrow distribution of cells will yield a well-defined DLS pattern.

The fact that the DLS patterns from exponential phase *rod-like* bacteria, such as *B. subtilis*, averaged over their random orientations also remain sharp may be derived directly from the RGD theory[10] where it is shown that the scattering is a function of the root mean square (r.m.s.) radius. For the case of a rod of length $2b$ and diameter $2a$, the r.m.s. radius, R, is just

$$R = \sqrt{(a^2 + b^2) / 2} \tag{4}$$

Since a is essentially constant and much less than b, Eq. (4) indicates again a relatively narrow size distribution as b increases to $2b$ during exponential phase.

The presence of toxicants affects exponential phase bacterial cultures in four distinct manners:

1) Cell growth inhibition: the culture generation time is increased,
2) Cell swelling: the average cell r.m.s. radius increases;
3) Cell shrinkage: the average cell r.m.s. radius decreases; and
4) Size distribution changes: the *range* of cell sizes present increases or decreases.

In addition, toxicants can affect structural features such as wall thickness, cell surface, and organelles. These effects are incorporated into some of the above changes, although their relative contributions may be shown to be small.

All of these changes are easily observed by recording the associated DLS patterns from cells incubated in a toxicant environment in contrast to cells exposed to a control environment. Effect (1) is seen as a relative displacement *downward* of the toxicant-affected DLS pattern relative to that from the control. (The control outgrows the inhibited culture.) Effect (2) is seen as a shift to *smaller* scattering angles (to the left) of the DLS pattern from toxicant-affected cells relative to the pattern from the control culture. Effect (3), on the other hand, results in a shift to *larger* scattering angles. Effect (4) is generally observed as a *washing out* of the DLS pattern from toxicant-affected cells relative to the pattern from the control. Sometimes, however, the pattern becomes *sharper* with better pronounced extrema in the DLS patterns. Washing out corresponds to a *broadened* cell size distribution, whereas a sharpened pattern corresponds to a *narrowing* of the cell size distribution. Naturally, some toxicants can cause a combination of effects. For example, Fig. 5 shows a combination of effects (1) and (2), relative to the control. The DLS pattern (after an hour of incubation) has shifted slightly to the left and is displaced downward relative to the control

Note that the relative intensity ratio, R (θ) has been plotted in Fig. 5. This ratio is given by

$$R(\theta) = [\, I(\theta) - I_S(\theta) - I_0(\theta)] \,/\, (I_L - I_{L0}) \quad \text{where} \tag{5}$$

$I(\theta)$ = the intensity of light scattered by the suspension into the angle θ

$I_0(\theta)$ = the detector dark current measured at angle θ

$I_S(\theta)$ = the scattering of the solvent (water) into the angle θ

$I_L(\theta)$ = the laser intensity measured at the rear of the laser

$I_{L0}(\theta)$ = the laser detector dark current

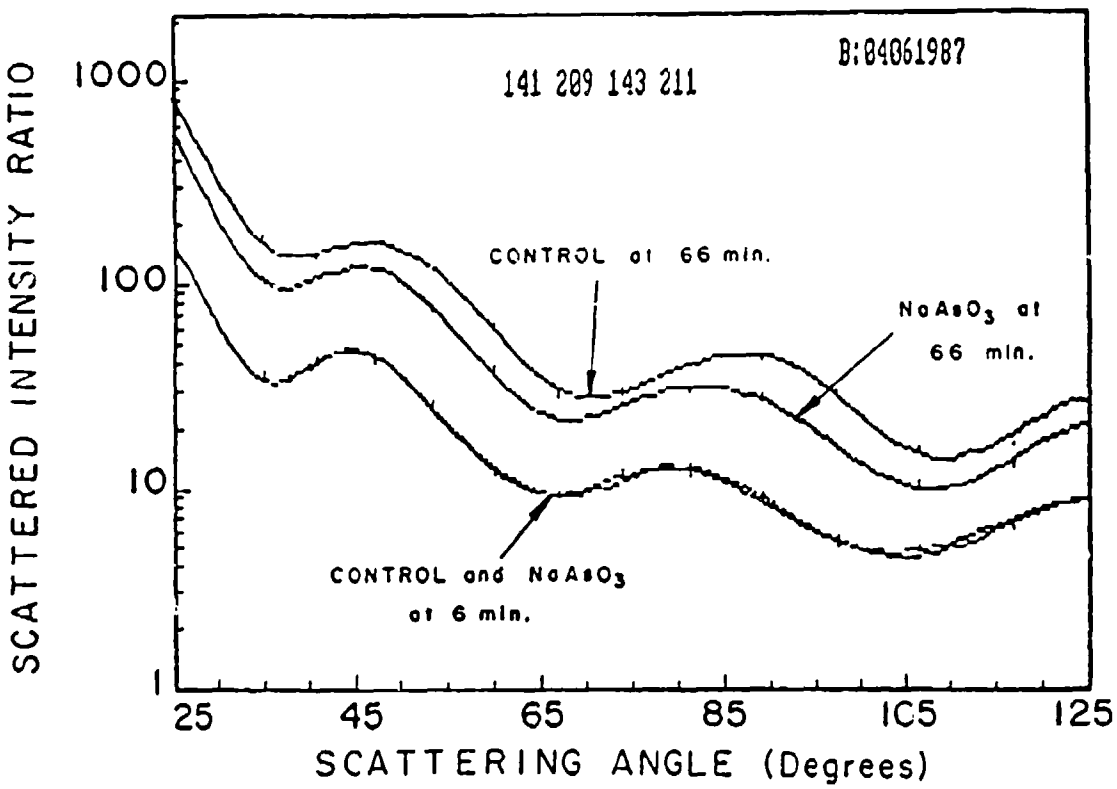

Fig. 5. Effect of $NaAsO_3$ at 60 g/ml on *B. subtilis* fh 2006-7. Topmost curve is control after 1 hr. Lowest superimposed curves are control and test sample at start of incubation.

3. INSTRUMENTATION AND SOFTWARE

Figure 6 is a schematic of the front and rear panels of the DAWN photometer. (The name DAWN was an acronym for *D*ual *A*ngle *W*eighted *N*ephelometry, an analytic means by which the multiangle measurements could be weighted and reduced to two average values at two distinct average angles. From these values, some simplified dose-response effects could be monitored.) Samples are prepared and incubated (see next section) in a customized incubator and read in the DAWN-B which transmits its readings to an analog-to-digital multiplexer (A/D MUX) in the computer or central processing unit (CPU). The computer, in turn, controls the collection and processing of the transmitted data. Figure 7 is a schematic of this system. The computer systems used most frequently with the photometer are IBM PCs or compatible with 512 K RAM, dual floppy drives, an 8087 mathematical co-processor, and a CGA or Everex graphics card for monochrome display.

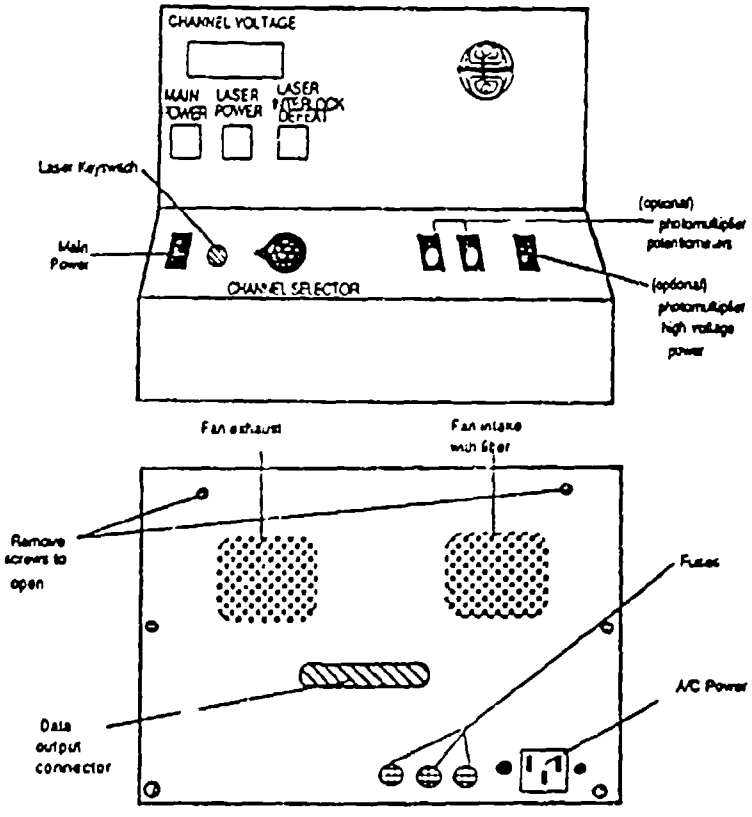

Fig. 6. Front and rear panels of the DAWN-B photometer.

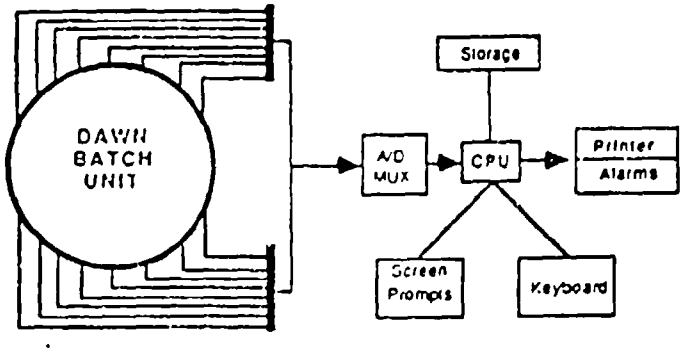

Fig. 7. Instrumentation schematics.

The incubator is comprised of a small, custom-designed, 20-cavity heating block that permits the incubation of standard cuvettes (generally scintillation vials). The custom block is heated by a Labline controller providing good temperature stability (± 0.5°C) throughout its entire structure.

Figure 8 shows the interior of a DAWN-B photometer developed in this program. The read head (uncovered in the drawing, but always covered before a measurement is made) is shown with a center stage surrounded by 15 collimated channels. At the end of each channel is a high gain hybrid transimpedence photodiode detector. Samples are placed at the center stage in standard capped scintillation vials. A helium-neon laser, oriented at 7° to the horizontal, illuminates the cuvette along a diameter with a fine beam of vertically polarized radiation. (Beam diameter about 0.4 mm, wavelength 632.8 nm, power 5-7 mW, beam divergence < 1 milliradian.) This is shown schematically in Fig. 9. The slight pitch of the beam to the horizontal reduces significantly the contributions of light scattered at the air-glass interfaces of the cuvette. The read head contains 15 collimated detectors equidistantly placed in sin θ / 2 around the cuvette to intercept radiation scattered at angles θ_i, (i = 1, ..., 15) where

$$0.2 \leq \sin \theta_i / 2 \leq 0.9 \tag{6}$$

The relative positions of the detectors around the read head are shown in Fig. 11. (See Sec. 5, p. 12.)

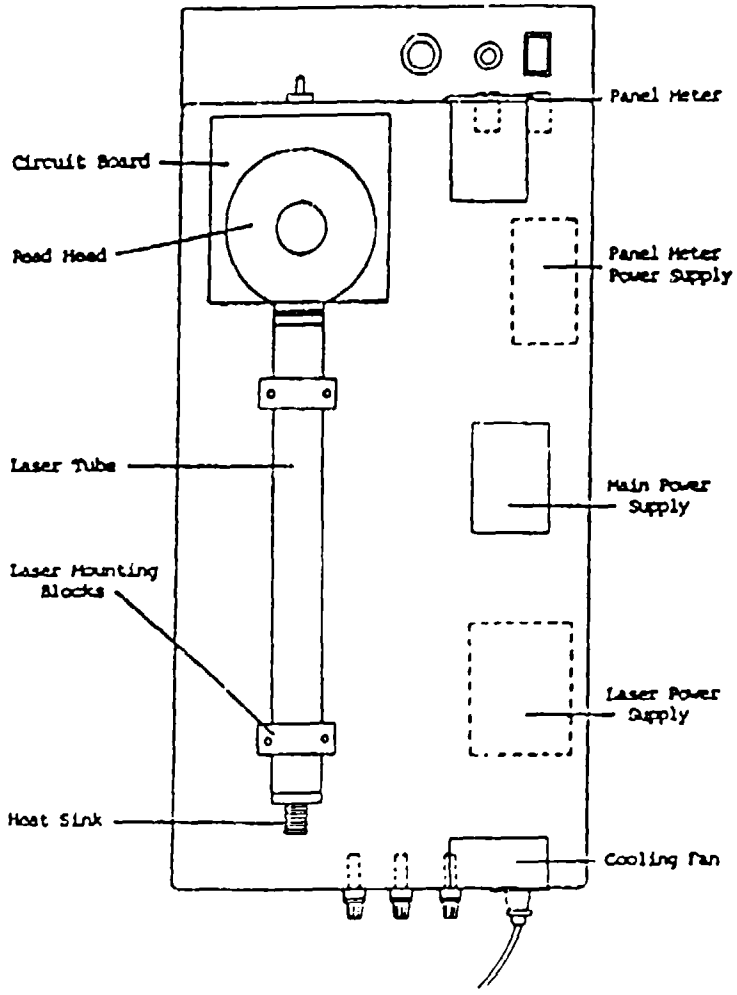

Fig. 8. Interior configuration of the DAWN-B photometer.

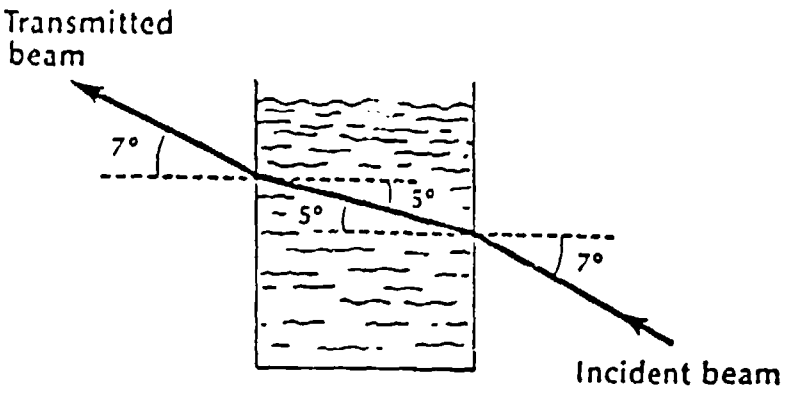

Fig. 9. Orientation of sample cuvettes with respect to incident laser beam.

Data collection is initiated under control of the program BIOASSAY. At each of the 15 detectors, 100 measurements are made over a period of time selected by the user, generally four seconds. These data are then processed digitally to eliminate most spurious light scattering effects arising from dust, cellular aggregates, and other debris. The relative intensity of the laser output is collected similarly and is used to normalize all other recorded and processed scattered intensities. [See Eq. (5).] This reference normalization permits consistent output despite small drifts in the laser power. The digitally filtered data at each scattering angle are averaged and stored. These processed data may be used subsequently for interpolation and smoothing by a Chebyshev polynomial series. The coefficients of expansion of such a series serve as a useful quantitative basis set (number of terms ≤ 15) characteristic of the measured patterns themselves.

The recorded data are processed by means of the program BIOSKOR3 to yield a variety of important physical properties of the bacterial cultures. Most important among them are the deductions of cellular generation times (see Sec. 5, Response Algorithms) with a precision rarely found by conventional plating methods. This is due to the large number of data points involved in the comparison of two scattering patterns. Whereas plate count determinations may involve two or three replicates and a few hundred cells counted for each of two periods, the light scattering technique is based on 3200 measurements.

The program BIOASSAY is similar in most details to the standard DAWN-B data collection program marketed by the company with its DAWN-B instruments. Detailed instruction manuals for the operation of instruments and programs were delivered with the instruments to the Contracting Officer's Technical Representative. Figure 10 presents the operating menu of the BIOASSAY program. On the basis of the values entered by the user, the program collects, processes, displays, and stores scattering data. Figures 5 and 12, for example, are typical of the data displays produced by this program. Referring to Eq. (5), the dark current values $I_0(\theta_i)$, $i=1,\ldots,15$, and I_{L0} are measured by entering "y" following item 6. The solvent offset increments, $I_S(\theta_i)$ are similarly measured entering "y" following item 7. The display output may be surpressed by entering "y" at item 9. Since 100 measurements of the scattered intensities are made at each channel for each sample; a total of 1600 measurements (100 times 16 channels) are required. The *time* over which these measurements are made may be varied by changing the value for item 2. At 400 channels per second, 1600 channels would be measured over a 4 second period (1600/400). The normalization coefficients are derived by entering a "y" at item 8 and scanning the scattering pattern from an isotropic scatter such as a dilute suspension of LUDOX™. The program prompts the user during execution to perform various operations (turn off laser - item 6, place pure solvent into DAWN - item 7, etc.) consistent with these measurements. Details

of the bioassay CULTURE READY test (item 15) are discussed in Sec. 6, Test 1. The Chebyshev polynomial order (item 3) determines the order of interpolation performed on the 15 data points collected for subsequent display. The 15 detectors may be divided into any two arbitrary subsets, each of which may be assigned an additional amplification (GAIN) at the A/D board by varying item 4. The refractive index of the solvent (water), item 13, and the vacuum wavelength of the laser (632.8 nm), item 14, are readily adjusted for the other instrument configurations.

The BIOSKOR3 program is used to replot data selected by the user for any file (automatically set up by date, e.g. 04161988) and subset (run) element thereof. Details of subsequent analyses are given in Sec. 5.

```
        THE CURRENT OPERATING PARAMETERS (Ver.1.0) ARE:
 1. SET NUMBERING begins with #1 (Y) or continues (N)............. N
 2. The NUMBER of CHANNELS to be scanned PER SECOND............... 400
 3. The CHEBYSHEV polynomial ORDER (<15) to be used for the fit.... 10
 4. The GAINS for GROUPS 1 and 2 (1,2,4, or 8)................... 4  8
   GROUP#1=  1  2  3  4 GROUP#2=  5  6  7  8  9 10 11 12 13 14 15
 5. The SHIFT downward of graphs (300 units per factor of 10)...... 0
 6. Should the DARK OFFSETS be measured (Y, N, or M)?............. N
 7. Should the SOLVENT OFFSET INCREMENTS be measured (Y, N, or M)?. N
 8. Should a NORMALIZATION measurement be made (Y, N, or M)?....... N
 9. Should  graphs be SKIPPED (Y/N)?............................. N
10. Number of SAMPLES to be run (max = 5)......................... 1
11. SAVE DATA on drive C (Y/N)................................... Y
12. NUMBER of DATA VALUES (out of 100) kept...................... 10
13. SOLVENT INDEX ............................................ 1.333
14. LASER WAVELENGTH(in nanometers)........................... 632.8
15. Check CULTURE READY for bioassay (Y/N).....................N
16. ABSOLUTE CALIBRATION (Y/N)................................... N
17. EXIT PROGRAM (Y/N)........................................... N
                    MENU TIME = 17:27:55
***********In the above, Y=YES, N=NO, and M=permit a MANUAL change**********
                  Strike ANY KEY to CONTINUE
```

Fig. 10. Operating menu for the program BIOASSAY.

4. MEASUREMENT PROCEDURES

For the case of toxicant *detection* in the presence of contaminants, stock cultures are prepared for two *B. subtilis* strains from lyophile pellets (REMEL) added to small vials containing 10 ml of prewarmed (39°) BHI broth (brain heart infusion) (Difco). Alternatively, cultures were initiated from lyophiles prepared with sufficient lyophilized (BHI) broth to yield 7 ml of culture after hydration with DI (deionized) water. The strains used in this study were WT 168 (wild type) and fh2006-7 (isogenic mutant) obtained from Felkner.[3-5] Incubation at 39°C was interrupted approximately every 15-20 minutes to oxygenate the cultures by vortexing for a few seconds. Following vortexing, the vial lids were loosened to permit free access to air. After about 3 to 3-1/2 hours, each culture was tested quantitatively to establish that it was ready for use. This "culture ready" confirmation comprised the steps of adding a culture aliquot of about 0.15 ml to a prewarmed 10 ml DI control water sample. The inoculated water was incubated for six minutes and then placed in a DAWN light scattering photometer and its DLS pattern analyzed. The culture-ready subroutine confirms that the DLS pattern corresponds to that characteristic of an exponential phase culture. A culture is confirmed to be ready for assay if it has a narrow size distribution and its cell number density increases by a factor of at least 2.5 after transfer to prewarmed DI water within 60 minutes or less.

The assay of an unknown "test" water sample for the presence of a toxicant requires preparation of four separate cuvettes:

1) a DI control sample for *each* strain (WT and fh); and,
2) a test sample for *each* strain.

The pH and chlorine levels of the test samples are *generally* neutralized and the sample filtered to remove extraneous particulates before assay. For purposes of the statistical studies involving specially prepared samples from DI water, neither pH and chlorine neutralization nor filtering were required. The water sample size is fixed at 10 ml. Each sample is prewarmed to 39°C and pairs (test plus control) inoculated with 0.15 ml aliquots of the WT and fh stock cultures, respectively, yielding a final concentration of about 10^6 cells / ml.

Figure 5 of Sec. 2 shows typical changes of the light scattering properties of an exponential phase culture of *B. subtilis* (fh 2006-7). Four curves are shown: the lower two contrast the test sample to a control sample at six minutes, and the upper two contrast the test sample response to the same control sample response culture 60 minutes later (*i.e.* 66 min. after preparation). All cultures were prepared from the same exponential growth phase stock culture prepared from stock every three to four hours. Stock culture and water samples are maintained at 39°C before and following inoculation.

5. RESPONSE ALGORITHMS

The DAWN-B (batch) photometers measure the light scattered at 15 discrete angles equidistantly spaced in sin θ/2 per Eq. (5) of Sec. 2. These data are derived from 100 subsequently processed measurements as described in Sec. 3. A continuous interpolation of these data in a least squares sense is achieved by the expansion in Chebyshev polynomials[18]

$$V(\theta) = \sum_{n=0}^{N} C_n T_n(\xi) \tag{7}$$

$$\text{where} \quad \xi = (0.55 - \sin \theta/2) / 0.35, \tag{8}$$

T_n is the Chebyshev polynomial of order n, C_n are constant coefficients, and $N \leq 14$. The values

$$V(\theta) = \log_{10} [R(\theta)], \tag{9}$$

where $R(\theta)$ is the so-called Rayleigh excess ratio given by

$$R(\theta) = [I(\theta) - I_S(\theta)] / I_0 \tag{10}$$

In Eq. (10), $I(\theta)$ is the intensity of light scattered at the angle θ from the bacterial suspension, $I_S(\theta)$ is the corresponding scattering from the solution (water) absent the bacteria and including all background scattering, and I_0 is the intensity of the incident laser. [See also Eq. (5).]

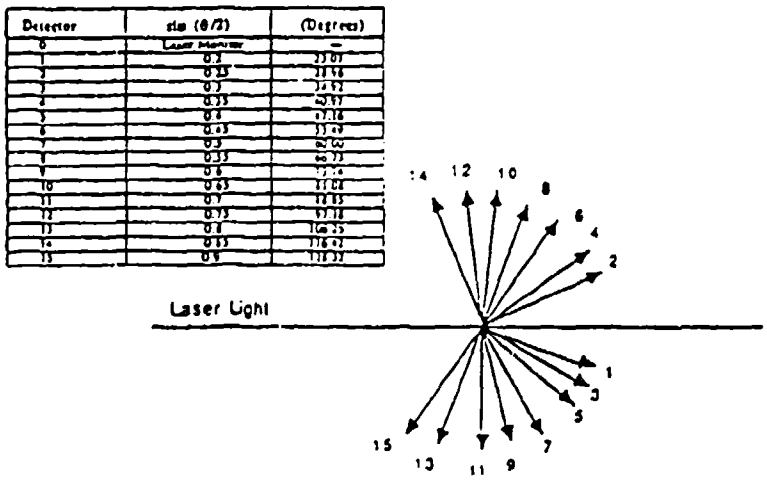

Fig. 11. Relative angular locations of the 15 detectors.

Figure 11 shows the relative angular locations of the 15 detectors viewed from above. The table in the figure lists the numerical values of these locations.

For the bacterial species used throughout this program, the number of terms, N should be set at 10. If the sample is free of debris, this polynomial order will characterize the

angular variations with great accuracy. When the samples contain high levels of debris and/or dust, the 10th order fit will effectively filter out many of these noise contributions.

It may be shown that the coefficient C_0 of Eq. (7) is approximately equal to the average value of $V(\theta)$, *i.e.*,

$$C_0 = \frac{1}{15}\sum_{i=2}^{14} V(\theta_i) + [V(\theta_{15}) + V(\theta_1)] / 30. \tag{11}$$

From this, the log weighted average value of $R(\theta)$, $\bar{R}$, may be derived

$$\bar{R} = 10^{C_0}. \tag{12}$$

Equation (12) is the fundamental connection between the light scattering properties of a bacterial suspension and its growth properties. Since the total amount of light scattered by the suspension will be directly proportional to this log weighted average intensity, which in turn is proportional to the number of cells per ml, the *ratio* of cell number n present at the time t, to the number present at time zero, n_0, is just

$$n / n_0 = \bar{R}_t / \bar{R}_0 = 10^{C_{0t} - C_{00}}. \tag{13}$$

For cells in exponential growth phase, however,

$$n = n_0 \exp(t/\tau) \tag{14}$$

where τ is the generation time. The doubling time t_2 is just 0.693 τ. The ratio n / n_0 for such an exponentially growing culture may be derived from Eq. (13) and, therefore, given the time between measurements, t, and the corresponding recorded DAWN intensities, the generation time may be calculated from Eq. (14):

$$\tau = t \log (n_0/n) = t/[(C_{0t} - C_{00}) \ln 10]. \tag{15}$$

Equation (13) has been confirmed during this program by extensive plate counts of various cultures. Although plate count variations were often of the order of 20-50%, the values derived from Eq. (13) were much more reproducible—often better than 5%.

In the event that the waters to be tested may contain non-toxic contaminants as well as toxicants, the relative response of each strain (WT and fh) must be incorporated into suitable algorithms able to discriminate between such contaminants and toxicants. More concerning this discrimination is discussed in Sec. 8. Most of the feasibility confirmation studies, however, relate specifically to the detection of toxicants absent any contaminants.

Table 1 presents a list of the most important algorithms and their definitions as used throughout these studies. For the detection of toxicants in otherwise pure water (Sec. 7), only algorithms 1, 3, 18, 19, 21 and 22 are required. Algorithms 1, 3, 18 and 19 represent various monitors of cell *growth* inhibition, while 21 and 22 are measures of mophorphological changes in the populations. In the presence of contaminants, however, algorithm 25 is often useful. Most algorithms involving curve length changes (algorithms 4-9) yield ambiguous, inconsistent results.

TABLE 1. GLOSSARY OF ALGORITHMS USED IN COMPUTER SCORING PROGRAMS

NO.	ALGORITHM	DEFINITION
1.	N/NZERO	N/NZERO represents the ratio of the number of cells at time "t" to the number of cells at time "zero." This ratio is calculated from the derived generation time τ, *i.e.*, $n = n_0 \exp(t/\tau)$. In BIOSKOR, N/NZERO is the ratio of the number of cells in the test chemical sample to the number of cells in the DI water control sample.
2.	TAU(min)	The derived generation time τ.
3.	TAU/TAUC	The ratio of the generation time of the culture in the chemical solution to that of the culture in DI water (t/τ_c). A number greater than 1.00 indicates inhibition, whereas a number less than 1.00 indicates stimulation by the test chemical.
4.	LEN6	Length of entire DLS curve at 6 min.
5.	LEN66	Length of entire DLS curve at 66 min.
6.	DLEN1	The length of the entire DLS curve at 66 min. minus the length of the entire DLS curve at 6 min.
7.	DEL(L1)	DLEN1 of the test chemical (66 - 6 min.) minus DLEN1 of the DI water control (66 - 6 min.). Same as DELWT or DELFH.
8.	DLEN2	The length of the second half of the DLS curve at 66 min. minus the length of the second half of the DLS curve at 6 min.
9.	DEL(L2)	DLEN2 of the test chemical (66 - 6 min.) minus DLEN2 of the DI water control (66 - 6 min.).
10.	DCHEB	Difference of the Chebyshev coefficients, C_1, of two samples.
11.	S6WT	100 (1 - N/NZERO) for WT at 6 min. where N/NZERO is the ratio of the number of cells in the test solution to the number in the DI water control sample.
12.	S66WT	100 (1 - N/NZERO) for WT at 66 min.
13.	S6FH	100 (1 - N/NZERO) for fh at 6 min.
14.	S66FH	100 (1 - N/NZERO) for fh at 66 min.
15.	F1	Tau of the WT strain divided by 40. Ratio of generation time obtained during experiment to an approximation of the theoretical generation time for *Bacillus subtilis*.
16.	F2	Tau of the fh strain divided by 40.

NO.	ALGORITHM	DEFINITION
17.	SKOR1	|S6WT| / F1 + |S6FH| / F2
18.	SKOR2	|S66WT| / F1 + |S66FH| / F2
19.	SKOR3	(SKOR1 + SKOR2) / 2
20.	AVGDEL	(DELWT + DELFH) / 2

For algorithms 21 through 31, scores are developed for comparing two DLS curves by reference to the eight Chebyshev coefficient $C_1, C_2, \ldots, C_8$, where the logarithm of the scattered intensity plotted as a function of the scattering angle, θ, has been expressed by

$$V(\theta) = \sum_{n=0}^{N} C_n T_n(\xi)$$

and is defined by Eq. (8).

21. WT66T.C — Compares the first 8 Chebyshev coefficients of the 66 minute curves of strain WT for the test (T) and DI control (C) by calculating

$$\text{WT66T.C} = 1000 \left\{ 1 - \frac{\sum_{n=1}^{8} C_{nT} C_{nC}}{\sqrt{\sum_{n=1}^{8} C_{nT}^2} \sqrt{\sum_{n=1}^{8} C_{nC}^2}} \right\}$$

where C_{nT} and C_{nC} are the nth Chebyshev expansion coefficients, respectively, for the test (T) and DI control (C) curves. If the curves were of identical shape, this term would equal zero.

22. FH66T.C — Same as 21, but for the strain fh.

23. WT6T.C — Same as 21, but at six (6) minutes after culture inoculation.

24. FH6T.C — Same as 23, but for the strain fh.

25. WTFH66 — A comparison (similar to 21) of the 66 minute coefficients of the WT strain vs. the 66 minute coefficients of the fh strain. This is a measure of the difference of the WT and fh strains after 66 minutes of incubation in the environment listed. If both strains were unaffected by their environment, these values would be identical to the control comparisons at 66 minutes.

26. WT6WT66 — A comparison (similar to 21) of the strain WT at 6 minutes to WT at 66 minutes. If the culture were "frozen" at its 6 minute shape, a zero value would result. At high toxic levels, these values should become smaller.

27. FH6FH66 — Same as 26, but for fh strain.

NO.	ALGORITHMS	DEFINITION
28.	WT66(C-T)	A comparison of the control (C) and test (T) Chebyshev coefficients of the WT strain at 66 minutes based on the following calculation: $$WT66(C\text{-}T) = 100 \sum_{n=1}^{8} (C_{nC} - C_{nT})^2$$ Identical curves would yield a zero value.
29.	FH66(C-T)	Same as 28, but for the fh strain.
30.	FH(66-6)	Comparison of changes of control and test differences at 6 and 66 minutes for fh strain. $$FH(66\text{-}6) = 100 \sum_{n=1}^{8} [(C_{nC} - C_{nT})_{66} - (C_{nC} - C_{nT})_6]^2$$
31.	WT-FH(6c-t)	Comparison of WT and fh changes at 6 minutes relative to their respect controls. $$WT\text{-}FH(6c\text{-}t) = 100 \sum_{n=1}^{8} \left(C_{nC}^{WT} - C_{nT}^{WT}\right)_6^2 - \left(C_{nC}^{fh} - C_{nT}^{fh}\right)_6^2$$

Other scoring algorithms are still under study.

6. STATISTICAL STUDIES

An important objective of the present study was the statistical confirmation of the methodologies. Although the light scattering techniques had yielded very encouraging and significant results over the years, a formal statistical analysis of the key instrumentation and protocol elements of the method never had been vigorously confirmed. Accordingly, a detailed statistically meaningful set of experiments was designed to confirm that under the most ideal of circumstances the laser-scattering bioassay method is reliable and reproducible. By "ideal" is meant that all samples consist of *deionized waters* filtered through 0.2 μm filters to which may be added, at various levels, pure toxicants. Certain toxicants such as nitrogen mustard and 2,4-D required buffering to a pH of about 6.2 because the salts from which the toxicants were prepared resulted in a pH significantly low enough to affect the test strains. Eventually, when field portable systems are developed, the associated water preparation reagents will include a buffering agent to insure a pH between 6.0 and 7.0 of the samples to be tested that will not affect the tester strains, *i.e.*, all solutions will be buffered.

A set of four statistical experiments was designed by Roger McDonald of the Kaman TEMPO facility in Santa Barbara to confirm the key criteria on which the laser bioassay method was based. In general, a criterion was considered confirmed if it occurred with a certainty greater than 90%, though for some tests, this figure was increased to 95%. Details of the experimental design had been reviewed earlier by the Army's statistician, Ms. Florence Broski. These statistical experiments were designed to establish the following criteria of the laser bioassay:

TEST 1. INITIAL CULTURES READY

Prior to performing the laser bioassay test, each of the two bacterial strains must have entered exponential growth phase. The determination of this "ready" status prior to test initiation is performed by preparing deionized, prewarmed water samples to which is added an aliquot of the assay culture. The suspension is then put into the DAWN unit and its light scattering data processed, recorded, and expanded in terms of its Chebyshev polynominal representation. On the basis of the values of certain expansion coefficients discussed below, the culture is determined to be "ready." This may be confirmed subsequently by continuing the sample suspension incubation for one hour. Since the doubling time of each of the two strains in the test at 39°C is of the order of 25 minutes, we *define* exponential growth as that condition whereby the cellular concentration has increased by *at least* a factor of 2.5 within the 60-minute incubation period. The relative cell number at 60 minutes to the number at the initiation of incubation is given by n / n_0 (Algorithm 1), which ratio may be calculated easily by the software. The culture ready criteria were modified somewhat during the course of these studies as media and culture initiation procedures were modified. This study of the detection of culture ready conditions was intended to confirm that a relatively untrained technician could initiate cultures from lyophile pellets and let the software confirm that he could proceed with the assay.

The culture ready criterion for *B. subtilis* strain WT was simply

$$C_5 > 0.90\,(C_4 + C_6), \text{ and} \tag{16a}$$
$$0.30 < C_0 * C_5 < 0.90 \tag{16b}$$

and for the *B. subtilis* strain fh

$$C_6 > 0.52\,(C_4 + C_5 + C_7), \text{ and} \tag{17a}$$
$$0.27 < C_0 * C_6 < 0.69 \tag{17b}$$

Criteria (16) and (17) are easily modified, with the same result. It was found early during the statistical program that cultures were invariably ready if used between 3.5 and

6.5 hours after initiation. Accordingly, these criteria were rarely needed. Indeed, good results for the bioassay could be achieved if n / n_0 were only 2.0 instead of 2.5. Most of the bacterial growth in water must be primarily endogenous since the broth concentration after inoculation is only 0.15 / 10 = 1.5%. Despite this paucity of nutrients and the almost complete lack of buffering capacity for the suspending medium, the ability of strains used in these bioassays to double within 60 minutes is impressive. Increasing the incubation time to 90 minutes generally resulted in further growth rate improvements (the first 60 minutes included at least 10-15 minutes of lag phase). Although the 90 minute incubation improved dose-response reproducibility and sensitivity, this longer incubation protocol was not pursued since test results were planned originally to be available at 60 minutes or less. Expanding the incubation period should be considered carefully for any future implementation of the method.

Note that "culture ready" criteria, Eqs. (16) and (17), are quite different from those developed during the earlier phase of this program. This change is due primarily to improved resolution of the instrument and better culture preparation and maintenance procedures. Some variations seen are also due to the significantly different quality of the incubation media (BHI broth). Early tests were made using cultures lyophilized in clean BHI broth. Later cultures—and those used for these statistical studies—were initiated from REMEL-produced lyophile pellets in BHI (see Sec. 7).

The statistical confirmation of the validity of the culture ready test was based on the following design by consultant Roger McDonald:

TEST DESIGN

Purpose: When a decision is made that a culture is ready for experimentation, there is a probability > 0, that the decision is incorrect and the culture is not yet ready for use, *i.e.*, there is a probability p that an error will be made in concluding that the culture is ready when in fact it is not. The purpose of the present tests is to estimate p in such a fashion that if $p \leq 0.05$ and the null hypothesis is accepted, the decision will be incorrect no more than 5 percent of the time.

Hypothesis: The null hypothesis is

$$H_0: \ p < 0.05$$

and the alternative hypothesis is

$$H_1: \ p > 0.05.$$

Test Criteria: For *B. subtilis* 168 WT, the culture is said to be ready when the criteria of Eqs. 16a and b are fulfilled. For *B. subtilis* fh 1006-7, the same is true when the criteria of Eqs. 17a and b are fulfilled. In both instances, the first decision that the culture is / is not ready will occur three hours after the stock culture has been initiated. If it is decided that the culture is not ready, the process will be repeated every 15 minutes until a decision is made that the culture is ready or until an hour has elapsed, whichever occurs first. If at the end of an hour, a decision has not been reached that the culture is ready, then the observation will be deleted from the test and a new observation (culture) taken. (The deleted observation will not be counted as part of the sample.)

Sample Size: Separate sequential tests will be conducted to evaluate the two bacterial strains. For each test, the type I error, α, and the type II error, β, equals 0.05 and 0.10, respectively. The minimum number of observations needed to accept H_0 equals 85 for both.

Test Procedure: Let x_k = number of failures in a sample of k trials; then,

(i) if $x_k < -3.095 + 0.072\,k$ accept H_0 (18)

(ii) if $x_k > 3.870 + 0.072\,k$ accept H_1 (19)

(iii) if neither inequality is satisfied, take another observation and proceed through steps (i), (ii), and (iii) again until either (i) or (ii) is satisfied or until $k = 100$, whichever comes first.

An observation will be said to have been a failure if (1) the observation does not satisfy the test criteria, or (2) the observation does satisfy the test criteria but the number of cells does not at least double within 60 minutes.

The power of this test has not yet been determined.

TEST 2. RESPONSE OF THE TWO ORGANISMS TO ELEVEN DIFFERENT TOXICANTS

The major part of this test concerns the determination of the response of the two *B. subtilus* strains (WT 168 and fh 2006-7) to eleven different toxicants. After the "culture ready" condition has been achieved for both cultures, the testing of a deionized water sample to which a fixed concentration of a particular toxicant has been added begins. For each such test, four cuvettes are prepared, as described earlier: two contain 10 ml of the prewarmed toxicant-laden sample, and two contain 10 ml of the prewarmed deionized water. The latter two cuvettes serve as controls. The control and toxicant cuvettes are incubated for six minutes, measured by the DAWN system under control of the program BIOASSAY and then returned to the incubator. Following 60 minutes of incubation, each sample would be read again by the DAWN and discarded. We must confirm, on the basis of selected mathematical algorithms of Sec. 5, each of which produces a response score from processing of the collected data, the following:

a) For each toxicant, is there a dose response?
b) Is the response (if it exists) near the midpoint of the dose-response range reproducible?
c) Is the dose response monotonic throughout the range tested?

Since no contaminants were to be studied in this series of experiments, only the simplest of algorithms had to be examined. Indeed, most of the toxicants produced generally inhibitory effects and could be followed easily, therefore, by the algorithms 1, 3, 18, 19, 21 or 22 (see Table 1).

The statistical verification of this test was based on the following McDonald design:

Purpose: There are three (3) parts to this test. The first is concerned with whether there is a dose response for each of the 11 toxicants in combination with the two bacterial strains, *B. subtilis* 168 WT and *B. subtilis* fh 2006-7. Each toxicant will be measured at 7 concentrations (including two controls) against two bacterial strains. This defines a sequential test. The second part is concerned with whether the midpoint of the dose response for each toxicant and bacterial strain is reproducible. (The "midpoint" is assumed to be the midpoint of the range of concentrations over which there was a response.) The dose response for a given toxicant and bacterial strain will be said to be reproducible (the same) if the means differ by no more than a multiple of 2.0. The third part is concerned with whether the dose response is monotonic (increasing or decreasing) for each measure over the test range of the toxicant. At this time a test has not been devised for quantitatively measuring whether a dose response is / is not monotonic.

Hypothesis: For part 1, let P_{ij} denote the proportion of times that there is a dose response for the ith toxicant, $i = 1, 2, \ldots, 11$, and j^{th} measure, $j = 1, 2, \ldots, 6$. Then the null and alternative hypotheses are:

$$H_0: \ P_{ij} = 0.95$$

$$H_1: \ P_{ij} \leq 0.90.$$

For part 2, let θ_{ij} denote the mean of the (midpoint) dose response for the ith toxicant, $i = 1, 2, \ldots, 11$ and j^{th} measure, $j = 1, 2, \ldots, 6$. Then under the assumption that $x_{ij} \sim N(\mu = \theta_{ij}, \sigma^2 = 1)$, the null and alternative hypotheses are:

$$H_0: \ \theta = \theta_{ij}$$

$$H_1: \ \theta > 2\theta_{ij}$$

Test Criteria: For part 1, 11 different sequential tests (one for each toxicant) will be conducted to determine whether the proportion of times there is a dose response is at least 0.95 or whether it is 0.90 or less. For each of the tests, the Type I error, α, and the Type II error, β, equal 0.05 and 0.10, respectively. For each test the minimum sample size necessary to accept H_0 is 43 observations.

For part 2, there again will be 11 different sequential tests conducted (assuming the H_0 is accepted for all of the tests in part 1). For each test, the Type I error, α, and the Type II error, β, are both equal to 0.05. In contrast to part 1, however, the H_0 could be accepted after only one observation has been taken. This is because in this case the random variable is continuous. In any event, the midpoint data results from part 1 may be used as a data base for part 2.

Test Procedure: For part 1, let x_{ijk} = number of successes in k trials of the experiment for the ith toxicant and j^{th} measure, then

(i) if $x_{ijk} \geq 3.0124 + 0.9277\,k$, accept H_0 (20)

(ii) if $x_{ijk} \leq -3.8683 + 0.9277\,k$, accept H_1 (21)

(iii) if neither inequality is satisfied, then take another observation or, if $k = 60$, terminate the test.

For part 2, let

$$\sum_{l=1}^{k} y_{ijl} = \text{sum of the 1st k observations for the } i^{th} \text{ toxicant and } j^{th} \text{ measure}$$

then

(i) if $\sum_{l=1}^{k} y_{ijl} \leq \frac{1}{\theta_0} \log\left(\frac{0.05}{0.95}\right) + \frac{3}{2}\theta_0 k$, accept H_0: $\theta = \theta_0$ (22)

(ii) if $\sum_{l=1}^{k} y_{ijl} \leq \frac{1}{\theta_0} \log\left(\frac{0.95}{0.05}\right) + \frac{3}{2}\theta_0 k$, accept H_1: $\theta = 2\theta_0$ (23)

(iii) if neither inequality is satisfied, take another observation, or if $k = 30$, terminate the test.

For both parts 1 and 2, the power of the tests has not yet been determined.

TEST 3. INSTRUMENT EFFECTS

Some of the more toxic compounds (see Sec. 7) had to be analyzed at the Southern Research Institute under a subcontract. For this purpose, a second DAWN-B (batch) instrument had been built for simultaneous use by SRI staff. If the instrumentation, software and sample preparation protocols are effective at detecting various toxicants in potable water, then all instruments should produce the same results for prepared samples. The development of field-portable systems to be used at different locations requires, as a prerequisite, that reproducibility between different instruments must be confirmed. For this test, two instruments would be run side-by-side using the same samples. In addition to measurements made at WTC on the two instruments, complete dose-response measurements for four selected toxicants were analyzed in duplicate at the two laboratories. The statistical verification of this was based on the following experimental design:

Purpose: The purpose of this test is to determine whether the differences observed in the light scattering measurements made by two different instruments are significant or not. In conducting the test, the measurements made by each instrument will be compared channel by channel across all 15 channels. For each channel, the variability between measurements within instruments will be compared with the variability of the measurements between the instruments. If there is a significant difference it will be concluded that different instruments do not read the same test results; otherwise it will be concluded that they do.

Hypothesis: For the ith channel, $i = 1, 2, \ldots, 15$ and the jth instrument, $j = 1, 2$, the null hypothesis is

$$H_0: \ \mu_{i1} = \mu_{i2} \text{ for each } i.$$

The alternative hypothesis is

$$H_1: \ \mu_{i1} \neq \mu_{i2} \text{ for each } i.$$

Test Criteria: In conducting the test and analyzing the resulting data, it is assumed that the observations for the ith channel of the first instrument constitute a random sample from a normal population with mean μ_i and variance σ^2; similarly, the observations from the ith channel of the second instrument are an independent random sample from a normal density function with mean μ_2 and the same variance, σ^2. The analysis-of-variance for the ith channel is shown in Table 2, where x_{jk} denotes the kth sample observation for the jth instrument and n_1 and n_2 are the sample sizes for the two instruments. The criterion to be used for testing that the means μ_1 and μ_2 are the same is the F ratio with 1 and n-2 degrees of freedom, where

$$F = \frac{\sum_j n_j (\bar{x}_j - \bar{x})^2 / 1}{\sum_{jk} (x_{jk} - \bar{x}_j)^2 / (n - 2)} \tag{24}$$

Sample Size: For each channel, the sample sizes for the two instruments will be $n_1 = n_2 = 20$. Thus, for all 15 channels, a total of 600 observations will be taken (40 for each test). The test will be a two-tailed test at the five percent level.

Test Procedure: For the first channel, 20 light scattering measurements will be recorded by each instrument. When completed, 20 more measurements per instrument will be taken for the second channel, etc., until measurements have been taken for all 15 channels. The data will then be analyzed by channel, the mean squares estimated, and the F tests made.

TABLE 2. ANALYSIS OF VARIANCE (FOR l^{th} CHANNEL)

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE
Mean	$n\,\bar{x}^2$	1	
Inst. Effects	$\sum_j n_j(\bar{x}_j - \bar{x})^2 / 1$	1	$\sum_j \frac{n_j(\bar{x}_j - \bar{x})^2}{1}$
Deviations	$\sum_{jk} (x_{jk} - \bar{x}_j)^2$	n - 2	$\sum_{jk} \frac{(x_{jk} - \bar{x}_j)^2}{n-2}$
Total	$\sum_{jk} x^2_{jk}$	n	

TEST 4. LASER BIOASSAY VALIDATION

This final test represents a validation of the laser *bioassay* (toxicant detection system) outcome. Given a set of unknown solutions, some of which contained the toxicants, the bioassay system would be required to yield a simple "positive" or "negative" answer as to whether or not a toxicant is present. For this purpose, the set of unknowns was prepared by the COTR and his designate (Prof. Robert Jacobs of the University of California at Santa Barbara) from toxicants shown *a priori* in Test 2 to yield a response in the test organisms of this study. A midrange toxicant level was selected for the preparation of each positive sample. Negative samples contained no toxicants. After the measurements were completed and classified by the system as positive or negative, their actual identifications were made known prior to the statistical analyses. A minimum of about 50 unknowns had to be prepared.

In addition to measurements of unknowns at midrange levels, a further set of unknowns at the low end of the detectable limit was to be run. Although outside the scope of the statistical program, a successful screening of such low levels adds validity to the bioassay method.

The statistical verification of this test was based on the following experimental design:

Purpose: The purpose of the fourth and final test is to validate the test outcomes (based on a measure or combination of measures) of whether each of n test solutions is toxic or not. For each outcome, two types of errors are possible. The first is that it is concluded on the basis of the measure that the test solution is toxic when it actually is not and the second is that it is concluded that the solution is not toxic when it actually is. At a minimum, the number of test solutions will be on the order of 43 with approximately r of them being

nontoxic. Not until the test conduct is complete will it actually be known which solutions were toxic and which were not.

Hypothesis: If p denotes the probability of correctly identifying that a test solution is / is not toxic, then the null hypothesis is

$$H_0: \ p = 0.95.$$

The alternative hypothesis is

$$H_1: \ p \leq 0.90.$$

Test Criteria: The criteria used for detecting the solutions that are toxic and the ones that are not will be a measure or combination of measures proposed for adoption by WTC.

Sample Size: A sequential test will be used to determine whether the probability of correctly concluding that a test solution is or is not toxic is at least 0.95. For the test, the Type I and Type II error are 0.05 and 0.10, respectively. A minimum of 43 observations (test solutions) are required to accept H_0.

Test Procedure: Let X_i = the number of test solutions correctly identified as being toxic or nontoxic in i trials. Then:

(i) if $X_i > 3.0124 + 0.9277\,i$, accept $H_0 = 0.95$ (25)

(ii) if $X_i < -3.8683 + 0.9277\,i$, accept $H_1 \leq 0.90$ (26)

(iii) if neither inequality is satisfied, take another observation or, if k equals 60, terminate the test.

The power of the test has not been estimated.

7. EXPERIMENTAL RESULTS

A. Cultures and Reagents

The most important constituents for the performance of the laser bioassays were the cultures and their supporting growth media (brain heart infusion / bacterial broth). Before the statistical studies of Sec. 6 were begun, the procedures usually were initiated with the addition of 7 ml of DI water to the bacterial stock lyophiles. Prepared by Regional Media Laboratories, Lenexa, KS (REMEL), from cold brain heart infusion (BHI) broth rinsed exponential phase cultures, these lyophiles were produced in vials containing 7 ml of BHI broth before freeze drying. After reconstitution and incubation at 39°C, the cultures invariably required 3-1/2 to 4 hours before they reached "culture ready" conditions. This delay was due primarily to the relatively low survival rate of cells prepared by the aforementioned lyophilization procedure. Thus, it was reasoned, it required six to eight generations of cells before the scattering signal from the viable cells outweighed significantly those that were present in the reconstituted culture, but which were dead. It seemed that a simple alternative to starting with non-viable cells would be to follow the suggestion of Felkner to begin with heat shocked spores, all of which would be viable *ab initio*. Procedures to accomplish this were developed under Felkner's direction, but again led to an impasse: Cells newly germinated from spores remained in long filaments for over three hours before separating into single cells capable of producing adequate "culture ready" signatures.

REMEL then produced new lyophiles in a small tablet form (pellet), following their standard bacterial lyophization procedures that would result in nearly 100% viable cells upon reconstitution in BHI broth. Extensive microscopic examination of the reconstituted cells showed despite improved viability, considerable filament formation up to 4 hours for the mutant strain (fh) and 6 hours for the wild type (WT). It would thus appear, on the basis of the studies to date, that ideal "culture ready" conditions were not to be obtained for at least 3-1/2 to 4 hours following germination or regeneration for lyophilized vegetative cells. Rather than devote additional effort to means by which the time to "culture ready" might be reduced, two courses of action were followed.

At SRI, cultures were set up during the first week of the program (*e.g.* for arsenate assays) using the original BHI-based lyophiles. Cultures were initiated by adding 7 ml of DI sterile water and incubation to a stationary state overnight at 39°C. Stock cultures were then prepared the next morning using 0.25 ml of the overnight cultures in 5 ml of BHI broth (Difco) followed by incubation at 39°C for 2-3 hours. "Culture ready" was established then simply on the basis of growth (log weighted average intensity greater than 10). Later, when the new pellets became available, SRI began with pellet initiated cultures incubated stationary (overnight for subsequent morning assays) or for 5-6 hours in BHI broth for afternoon measurements. Stock cultures were then prepared as before using a small inoculum of these reconstituted cultures in 10 ml of BHI broth and incubation for 2-3 hours.

(REMEL had produced BHI broth in 10 ml standard vials which were not used by the SRI staff who preferred to prepare their own. At WTC, on the other hand, these prepared 10 ml BHI vials were used. The first batch prepared by REMEL was almost inhibitory to the cultures and was discarded. A replacement lot produced improved growth and it was used exclusively by WTC staff. SRI later stated that this second batch was still far below the quality of their DIFCO BHI broths. Thus, WTC cultures were invariably inferior for bioassay than those used by SRI.)

At WTC, cultures were initiated at room temperature overnight using REMEL pellets and REMEL BHI 10 ml vials. In the morning, these stationary growth phase cultures were put into incubator blocks raised to 39°C. Within two hours, the cultures were ready for assay. Their overall viability (*i.e.* as measured by the length of time that the culture could be used for assays) was inferior to those prepared by SRI, and these stock cultures could not be used as long for bioassay purposes during a test day. Alternative cultures were prepared from pellets in prewarmed (second batch) REMEL broths yielding culture ready conditions at about 4 hrs.

B. Toxicants Assayed and Chemical Stability

Eleven toxicants were examined in this study. Of the eleven, only sodium monofluoroacetate failed to yield meaningful dose-response curves. The eleven toxicants, their approximate dose-response range, the corresponding midrange level, the laboratory (WTC/SRI) where assayed, and the number of dose-response sets are listed in Table 3, below. All dose response curves were based on measurements made at 5 or more concentrations.

TABLE 3. TOXICANTS ASSAYED

TOXICANT	RANGE (mg/l)	MIDPOINT (mg/l)	LABORATORY	DOSE RESPONSE SETS
Formaldehyde	1-40	20	WTC	44
$CdSO_4$	0.002-0.7	0.1	WTC/SRI	44/36
KCN	5-100	30	WTC/SRI	43/40
$NaAsO_3$	20-200	120	WTC/SRI	48/40
Phenol	0.5-4	2.0	WTC	57
CrO_3	1-100	10	WTC	36
2,4-D	25-100	75	WTC	37
Paraoxon	28-220	100	SRI	44
Nitrogen Mustard	10-200	100	SRI	8
4NQO	0.1-1.6	0.4	SRI	37
Sodium monofluroacetate	3-100	30	SRI	4

The concentration ranges selected initially were based on COTR provided estimates of 1/100 oral LD_{50} levels for a 70 kg man consuming 5 l of water/day. This "safe" level was certainly well below levels of importance for early detection, and for the most part, was not easily detected, especially in the presence of contaminants. A more reasonable lower detection level was an order of magnitude above this safe level. A range spanning two orders of magnitude and including this latter level was ideal unless the assay strains used did not respond to such values. In this event, the range of concentrations was increased so that at the median level within the range, a reasonable dose response was clearly seen.

In general, endpoint "safe" rationales were based on animal data except for cyanide and phenol. Table 4 presents the detection levels corresponding to 1 to 2 orders of magnitude above "safe" levels.

TABLE 4. SELECTED TEST LEVELS 10- TO 100-FOLD ABOVE "SAFE"

TOXICANT	LEVEL (mg/l)	RATIONALE
Formaldehyde	4	1/10 of LD_{50} (Guinea pig) and threshold human effect
KCN	4	1/10 of LD_{50} (human)
$NaAsO_3$	7	1/10 of LD_{50} (2 mammals, ave.)
phenol	34	1/100 of LD_{50} (humans)
CrO_3	5	100 times EPA Standard
paraoxon	3	1/10 of LD_{50} (rat)
Sodium Monofluroacetate	7	1/10 of LD_{50} (rat)

Table 5 lists the source of the toxicants and their full names. Chemical stability analyses were performed by the Southern Research Institute on solutions of the chemicals prepared in deionized water at the concentrations listed in Table 6. Each solution was incubated at 39°C and analysed for chemical concentration immediately after preparation and again at 60 min. The results presented in Table 6 are reported as the observed percent change in concentration. The observed changes in concentration for all toxicants were generally too small to have any effect on the bioassay. However, a closer examination of Table 6 suggests that relatively small errors are present in the stability assay analyses themselves. Although one could anticipate the possibility of *decreased* toxicant concentration due to binding to broth constituents or some other form of deactivation, it is difficult to explain how concentrations could *increase*. SRI did not state the absolute accuracy of their assays. Accordingly, the observed concentration changes must be due to systematic errors in preparation or measurement. The errors or changes in the observed concentrations were invariably smaller than the fluctuations observed in the measurements themselves.

TABLE 5. TOXICANTS AND SOURCE

TOXICANT	SOURCE
Formaldehyde (37% in water)	Aldrich Chemical Co.
Cadmium sulfate	Sigma Chemical Co.
Paraoxon: diethyl p-nitrophenylphosphate	Sigma Chemical Co.
4NQO: 4-Nitroquinoline-N-oxide	Sigma Chemical Co.
$NaAsO_3$: Sodium arsenate	Sigma Chemical Co.
Phenol	Sigma Chemical Co.
CrO_3	Mallinckrodt, Inc.
KCN: Potassium cyanide	Mallinckrodt, Inc.
2,4-Dichlorophenoxyacetic acid	Mallinckrodt, Inc.
Nitrogen Mustard: [methyl bis (2-chloroethylamine) HCl]	National Cancer Institute
Sodium monofluroacetate	Eastman Kodak

TABLE 6. THE CHEMICAL STABILITY OF SELECTED COMPOUNDS UNDER SIMULATED BIOASSAY CONDITIONS[1]

COMPOUND	TEST DOSE (mg/l)	ANALYTICAL METHOD[2]	% CHANGE IN CONCENTRATION
Paraoxon	228.	HPLC	+0.25
	22.8		+2.0
4NQO	1.67	HPLC	+0.63
	0.10		+1.3
Sodium arsenate[3] $NaAsO_3$	12.	FAE	+4.1
	3.5		+1.9
Sodium monofluoroacetate	104.	IC	0.03
	3.12		3.0
Cadmium sulfate	0.960	IC	-
	0.192		-12.
Formaldehyde	108.	GC	-4.8
Phenol	104.	GC	-18.
Chromium trioxide	10.0	UV Spect.	0.
Potassium cyanide	Not tested		

[1]Analyses were performed on solutions of the chemicals prepared in deionized water at the concentrations listed; each solution was incubated at 39°C and analyzed for chemical concentration immediately after preparation and again at 60 min. The results are reported as the observed percent change in concentration.

[2]The analytical methods were as follows: HPLC, high performance liquid chromatography; FAE, flame atomic-emission spectroscopy; IC, ion chromatography; GC, gas chromatography; UV Spect., ultraviolet spectroscopy.

[3]The FAE measurement detected the sodium ion only; the measurement was considered to be indicative of total arsenic in solution.

C. Instrument Comparison (Sec. 6, Test 3)

The two DAWN-B instruments used for this study were modified by adding a 21 gain booster amplifier to the eleventh detector (89°) on each unit. The signals from the photodiode detector could be amplified by this factor of 21 or left unamplified. With this additional gain, the scattered intensity at about 90° from a suspension of pure toluene produced a signal of about 500 mV rather than about 20 mV. With this high signal, it becomes possible to calibrate all units absolutely to reference solvents available worldwide. The standard normalization procedure, therefore, consists of the following steps:

(i) Normalize all detectors relative to channel 11 (whose normalization coefficient is set equal to 1.0) using a small particulate isotropic scatterer such as Ludox or 30 nm diameter latex spheres. (The Ludox standards provided by DuPont were found to be the most consistent.) By "normalize" we mean generate an arithmetic factor, f_i, for each detector such that the relative intensity ratio $R\theta_i$ of the ith detector [per Eq. (5) Sec. 2] when multiplied by its corresponding normalization factor f_i, produces the *same* value for each detector when an isotropic scatterer (*e.g.* Ludox) is measured, (Note that $f_{11} = 1.0$). (ii) Remove the jumper at the booster amplifier to produce an amplification of 21 at detector 11. (iii) Prepare a pure toluene standard in a clean cuvette and a pure benzene or acetone standard in another. (iv) Insert these two pure solvents sequentially, and measure using the BIOASSAY program in its calibration mode. (v) Let the program generate the absolute calibration factor based on these measurements. (vi) Insert this value into the parameter installation program INSTALLB and multiply all normalization factors by this term. (vii) Restore the booster amplifier jumper for detector 11.

INSTALLB and multiply all normalization factors by this term. (vii) Restore the booster amplifier jumper for detector 11.

This was used by SRI and WTC; yet the relative response of each unit was not identical, there being a variation of about 10%. Although this was considered satisfactory for the assays performed at both laboratories, the quantitative elements of the proposed statistical analysis had not been realized. The reason for the slight difference is that the amplifiers on each unit's number 11 detector output were slightly different. When the jumpers were replaced, it was *assumed* that each unit's number 11 detector output was reduced by the *same* factor of 21. This was not the case. Accordingly, the comparison of two instruments, following the statistical protocols described in Sec. 6, Test 3, was repeated after the experimental program had been completed.

Because of the booster amplifier differences noted above, it was decided to fine tune the calibration constants of unit #2 relative to the absolute calibration constant of unit #1. Before this fine tuning, the absolute calibration constants of the two units were 0.03 and 0.04, respectively. The strategy to achieve such fine tuning was to prepare a suspension of Ludox and compare the log weighted average intensities of both units. After both units had been allowed to warm up and after dark and solvent (water) offsets (using the same deionized and filtered water sample) had been measured, twenty measurements were made of the same Ludox sample by both systems. The averages of the twenty log weighted average intensity measurements were compared and the absolute calibration coefficient of unit #2 was adjusted to 0.0364.

We inserted a sample in instrument #1 and took twenty independent measurements. Each measurement consists of a determination of the relative intensity ratio per Eq. (5) of Sec. 2 for each of fifteen detectors. Thus, for instrument #1 we had twenty intensities for each of fifteen detectors. We then inserted the same sample in instrument #2 and repeated the procedure followed for instrument #1. We assumed the sample to be unchanged over the entire experiment.

A comparison test was then made for each detector. For example, the twenty normalized and calibrated intensities recorded for detector one of instrument #1 were compared with the twenty corresponding values recorded for detector one of instrument #2. If each normalized intensity detected on instrument #1 were identical—according to our statistical criterion—to the corresponding value on instrument #2, then the instruments would be said to be identical.

Referring to Sec. 6 for more details, the statistical test follows: Let i (= 1, 2, ..., 15) be the detector number and let j (= 1, 2) be the instrument number, then the null hypothesis is

$$H_0: \mu_{i1} = \mu_{i2} \text{ for each } i.$$

The alternative hypothesis is

$$H_1: \mu_{i1} \neq \mu_{i2} \text{ for each } i.$$

The test assumes that the observations for the ith channel of instrument #1 constitute a random sample from a normal population with mean μ_{i1} and variance σ_1^2, and similarly for instrument #2. A two-tailed F-test is used, assuming a 5 percent confidence level.

The F-ratio for each detector pair is computed using the following formula:

$$F = \frac{\sum_{2} n_j (\bar{x}_j - \bar{x})^2 / 1}{\sum_{jk} (x_{jk} - \bar{x}_j)^2 / (n - 2)}$$

n_j = sample size for instrument j
n = $n_1 + n_2$
x_{jk} = relative intensity ratio for observation k, instrument j
$\bar{x}_j$ = estimate of the mean for instrument j
$\bar{x}$ = $(x_1 + x_2) / 2$

Basically, the F-ratio is proportional to the variation between instruments divided by the variation within a given instrument. More specifically, Table 2 of Sec. 6 shows the analysis of variance for the ith channel.

The results presented below are based on the special measurements made in January 1988. The sample was a solution of Ludox in water. Ludox samples from Du Pont were centrifuged for 20 minutes at 3000 r.p.m. The supernatant was removed and diluted 1:1000 with distilled deionized water. The samples were prepared in thoroughly rinsed scintillation vials which were then sealed with Teflon tape.

Table 7 shows the data for the first 5 observations for instrument #1. (A total of 20 such measurements were made and the unlisted 15 measurements were similar to the 5 sets shown.) The numbers in the first column are the detector numbers. The numbers in the first row are the observed intensities. Thus, to read the twenty intensities for instrument 1, detector 1, we would read across the row for detector 1 in Table 7 (as well as an additional set of 15 values, not shown). The data for instrument #2 are similarly presented in Table 8. We consider the first three digits of the intensities to be significant.

TABLE 7. INSTRUMENT #1 OBSERVATIONS 1-5

	1	2	3	4	5
1	1.354549	1.353288	1.35108	1.347926	1.349503
2	1.363136	1.361372	1.353561	1.357089	1.357844
3	1.351073	1.347757	1.346541	1.344219	1.34632
4	1.3552	1.352271	1.352653	1.350105	1.351889
5	1.350755	1.348674	1.346871	1.346871	1.346871
6	1.349527	1.348804	1.348804	1.346633	1.345909
7	1.349288	1.347811	1.347154	1.34699	1.346005
8	1.352374	1.352025	1.352025	1.350103	1.350278
9	1.347383	1.347194	1.347572	1.346438	1.345682
10	1.349811	1.350033	1.349589	1.348703	1.348038
11	1.343478	1.342826	1.343043	1.342283	1.341957
12	1.346683	1.34658	1.346683	1.345657	1.345349
13	1.348428	1.347321	1.347321	1.346516	1.346718
14	1.346332	1.346152	1.34543	1.344889	1.343987
15	1.345681	1.345361	1.345361	1.34408	1.34408

TABLE 8. INSTRUMENT #2 OBSERVATIONS 1-5

	1	2	3	4	5
1	1.343692	1.338883	1.333887	1.343377	1.339332
2	1.342399	1.340228	1.339659	1.341832	1.343909
3	1.350893	1.348896	1.348696	1.351192	1.350493
4	1.352052	1.350776	1.348573	1.350892	1.351936
5	1.352534	1.351753	1.351623	1.352534	1.352795
6	1.351532	1.351203	1.350875	1.351203	1.352517
7	1.350827	1.350827	1.349395	1.350827	1.351941
8	1.351387	1.350723	1.350723	1.350944	1.35183
9	1.353179	1.350936	1.350562	1.351123	1.351871
10	1.350752	1.350129	1.350129	1.349299	1.353242
11	1.35184	1.350618	1.349803	1.350822	1.351229
12	1.351585	1.34965	1.34965	1.349408	1.351585
13	1.35189	1.350375	1.350375	1.350375	1.351241
14	1.350827	1.350652	1.350827	1.350827	1.351349
15	1.351598	1.34935	1.34935	1.349672	1.350956

Table 9 shows the mean, variance (var), standard devation (dev), and percent deviation (% dev) for the twenty intensities for each detector of instrument #1. The numbers in the first column indicate the detector number. The percent deviation for a given detector is the standard deviation as a percent of the corresponding mean. Table 10 shows the corresponding results for instrument #2.

TABLE 9. INSTRUMENT #1 MEAN VALUES

DETECTOR	MEAN 1	VAR	DEV	% DEV
1	1.347162	0.000087	0.009343	0.693596
2	1.347738	0.000086	0.009304	0.690343
3	1.342962	0.000070	0.008402	0.625650
4	1.342917	0.000066	0.008141	0.606259
5	1.341931	0.000056	0.007529	0.561128
6	1.338626	0.000061	0.007842	0.585888
7	1.341057	0.000055	0.007417	0.553103
8	1.341537	0.000077	0.008800	0.656009
9	1.339858	0.000052	0.007237	0.540197
10	1.341815	0.000062	0.007924	0.590605
11	1.336241	0.000058	0.007663	0.573475
12	1.338007	0.000073	0.008566	0.640259
13	1.340891	0.000051	0.007143	0.532712
14	1.338271	0.000065	0.008069	0.603012
15	1.336802	0.000071	0.008459	0.632790

TABLE 10. INSTRUMENT #2 MEAN VALUES

DETECTOR	MEAN 2	VAR	DEV	% DEV
1	1.333245	0.000029	0.005398	0.404883
2	1.344437	0.000005	0.002340	0.174113
3	1.346862	0.000010	0.003220	0.239138
4	1.351902	0.000008	0.002939	0.217397
5	1.351200	0.000004	0.002158	0.159751
6	1.352544	0.000004	0.002184	0.161488
7	1.348744	0.000006	0.002453	0.181883
8	1.352824	0.000007	0.002708	0.200229
9	1.351051	0.000005	0.002239	0.165747
10	1.356660	0.000021	0.004667	0.344039
11	1.350524	0.000006	0.002459	0.182130
12	1.352197	0.000007	0.002777	0.205403
13	1.352046	0.000007	0.002731	0.202021
14	1.351646	0.000005	0.002313	0.171156
15	1.350064	0.000005	0.002293	0.169854

The F-ratios are shown in Table 11. For a 95 percent confidence level, the null hypothesis is accepted if the F-ratio does not exceed 4.41. We conclude that the null hypothesis is rejected, *i.e.*, there is a significant difference between instruments. However, a cursory glance at the data indicates a remarkable level of repeatability considerably better than the limits for which we had hoped. How then could this test have failed? And of what significance is this failure?

TABLE 11. F-RATIOS FOR THE 15 NORMALIZED INTENSITIES

DETECTOR	F-RATIO
1	33.26629
2	2.367035
3	3.755991
4	21.55120
5	28.00328
6	58.45235
7	19.36148
8	30.05292
9	43.64840
10	52.10702
11	62.98956
12	49.65379
13	42.54738
14	50.76570
15	45.79584

We discussed our results with statistician Roger McDonald, and he concluded that the F-test was not appropriate for our data, since the variation of a given detector on a given instrument is relatively quite small. Thus the between-instrument variations, although acceptable to us, are significant compared to the even smaller variations on a given instrument. A more meaningful statement is that neither the percent deviation for a given detector, nor the percent difference between means for two corresponding detectors on the two instruments, is ever much in excess of one percent. Table 12 shows a comparison of the mean values for the two instruments.

TABLE 12. COMPARISON OF NORMALIZED INSTRUMENT DETECTOR MEANS

DETECTOR	MEAN 1	MEAN 2	% DIFF
1	1.347162	1.333245	1.033075
2	1.347738	1.344437	0.244895
3	1.342962	1.346862	0.289528
4	1.342917	1.351902	0.664633
5	1.341931	1.351200	0.685979
6	1.338626	1.352544	1.029034
7	1.341057	1.348744	0.559926
8	1.341537	1.352824	0.834361
9	1.339858	1.351051	0.828436
10	1.341815	1.356660	1.094249
11	1.336241	1.350524	1.057574
12	1.338007	1.352197	1.049395
13	1.340891	1.352046	0.824993
14	1.338271	1.351646	0.989526
15	1.336802	1.350064	0.982356

Since, at the beginning of the project, we had stated that a five percent difference in mean values would be acceptable in an experiment of this sort, we are satisfied with the above results. They show that the instruments, when properly calibrated and normalized, are in excellent agreement.

D. Culture Ready (Sec. 6, Test I)

The respective cultures of WT 168 and fh 2006-7 were assumed ready for assay if they were in exponential phase and if the light scattering signals from viable cells were much greater than the corresponding contributions from background debris. Exponential growth was assumed if n / n_0 in water equaled or exceeded 2.5 after one hour following transfer of 0.15 ml culture into 10 ml prewarmed DI water. On this basis, the log weighted average intensity of the suspension 6 minutes following inoculation had to exceed 10. The culture ready condition at t = 6 minutes of the water suspended inoculum that produced sharp, well-defined scattering patterns could be expressed in terms of the values of certain Chebyshev expansion coefficients of Eq. (6). For the case of the fh strain, this latter requirement was defined in Sec. 6, *viz.*

$$C_6 > 0.52\ (C_4 + C_5 + C_7),\ \text{and} \tag{17a}$$
$$0.27 < C_0 * C_6 < 0.69 \tag{17b}$$

The culture was considered "too old" when $C_0 * C_6 > 0.69$, after which n/n_0 became < 2.5. For *B. subtilis* WT 168, the conditions for exponential phase were

$$C_5 > 0.90\ (C_4 + C_6),\ \text{and} \tag{16a}$$
$$0.30 < C_0 * C_5 < 0.90 \tag{16b}$$

These and similar criteria could be established easily for each batch of REMEL-supplied lyophiles and broth medium. Such criteria were consistent for the entire batch during the three-month period following receipt. Since the SRI protocol did not use REMEL broth, these criteria were inapplicable in their studies. Indeed, the SRI cultures as prepared by their staff were always in a culture ready (CR) state (see Sec. 7A). No attempt was made by WTC to develop culture ready criteria for the SRI protocol. The purpose of the CR test was to confirm that associated with a set of prepared lyophiles and associated medium, a quantitative measure of the condition could be derived. The SRI cultures were laboratory cultures, produced by trained laboratory personnel and, therefore, always perfect for the assay. This test was intended to confirm that the software could guide an untrained technician in the preparation of the test. The statistical significance of the CR test was formalized after completion of the program.

The statistical studies performed during the course of the contract did not follow the test design proposed. Accordingly, they had to be repeated during the preparation of this final summary report. It should be noted that the tester strain WT 168 was generally ready for use by 3-1/2 to 4 hours after culture initiation. The slower growing fh 2006-7 mutant strain, on the other hand, invariably required 4-1/2 to 5 hours before cultures were ready for assay.

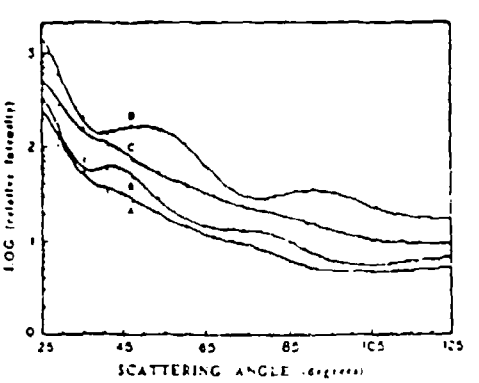

Fig. 12. Comparison of light scattering patterns from "too young" cultures and "ready" cultures. A=too young fh; B=ready fh; C = too young WT; D=ready WT.

Figure 12 shows an example of "too young" and "ready" cultures of the fh and WT strains. Note the washed out appearance of the "too young" curves, especially at larger angles, indicative of debris and (for fh) multicellular aggregates. Because of its long "lag phase," the mutant strain does not appear to grow as well as the wild type (WT 168). This is probably due to the continuing production of waste toxins eventually decreasing the period of time within which the culture was ready. As discussed later, consultant I.C. Felkner believes that more rapid culture initiation procedures starting from spores will be readily achievable. He also feels that aggregate dispersion may be accelerated by slight media changes.

A total of 86 culture ready assays were perfomed for the WT strain with no failure and 86 for the fh strain with two failures. These measurements were performed over a period of two weeks. Table 13 presents some typical results of culture ready studies. The strain ID is followed by the 6 and 66 minute assay times with their corresponding file set numbers. Then come the calculated values of n/n_0 (normalized to 60 min) and tau (the generation time in min).

TABLE 13. SCORE RESULTS FOR SELECTED ASSAYS SATISFYING THE INITIAL CULTURE-READY CRITERIA OF EQS. (16) OR (17).

ID	#	TIME	#	TIME	n/n_0	tau
WT-1	1	14.06	26	15.06	3.7	45
WT-2	2	14.08	27	15.07	3.7	46
WT-3	3	14.09	28	15.08	4.6	39
WT-4	4	14.09	29	15.09	4.4	41
WT-5	5	14.10	30	15.10	4.4	41
WT-6	6	14.11	31	15.11	4.5	40
WT-7	7	14.18	38	15.18	4.0	43
WT-8	8	14.19	39	15.19	3.8	45
WT-9	9	14.20	40	15.20	4.4	40
fh-1	11	15.50	41	16.51	2.6	62
fh-2	12	15.51	42	16.52	2.7	61
fh-3	13	15.52	43	16.53	2.7	60
fh-4	14	15.59	50	17.00	2.7	59
fh-5	15	16.00	52	17.02	2.8	59
fh-6	16	16.01	53	17.03	2.6	63
fh-7	17	16.02	54	17.04	2.8	59
fh-8	18	16.03	55	17.05	3.1	53
fh-9	19	16.04	56	17.06	2.8	58

Referring to the McDonald statistical design of Sec. 6, Test 1, we must apply Eqs. (18) or (19)

For WT 168

$$0 < -3.095 + 0.072 * 86 = 3.097\text{: accept } H_0\text{, reject } H_1$$

For fh 2006-7

$$2 < -3.095 + 0.072 * 86 = 3.097\text{: accept } H_0\text{, reject } H_1$$

(Note that there was a misprint in the originally submitted test design: The term -3.095 was inadvertently stated as -6.095.)

The original test design provided only that the cultures should double in the 60-minute incubation period. Our criterion of 2.5 is more strict and corresponds to a more rapidly growing culture. We believe, further, that once a stock culture aliquot has been transferred to a water sample, the cultures tend toward a quasi-synchronous division for a period from 40-120 minutes. The analysis of Table 13, for example, shows that there is often considerable variation of n / n_0 for cultures prepared within minutes of one another at the 60 minute measurement. If cultures were dividing in a quasi-synchronous manner near 66 minutes for their second division after inoculation, then these rather broad fluctuations would be explained.

E. Response of Two Organisms to Eleven Toxicants (Sec. 6, Test 2)

Of the eleven toxicants tested, only sodium monofluroacetate did not yield a dose-response curve. (See Table 3 of Sec. 7B.) Differences in choice of scoring algorithms at WTC and SRI were discussed in Sec. 7A. The much greater quality of the BHI broth used by SRI is reflected in their tighter results. Nevertheless, results from both laboratories, listed below in Tables 14 (WTC) and 15 (SRI), confirmed the statistical significance and reproducibility of the dose response for each trial reported. (Details of the statistical analyses follow.) Although the generation of a minimum of 43 complete dose-response curves was required to confirm the test to a certainty of 95%, some concentrations of some toxicants were not tested this many times due to time limitations as well as inadvertantly selected toxicant levels between the key levels chosen for the assay confirmations. All data generated at WTC and SRI are presented with their associated standard deviations. SRI data, plotted using n/n_0 and TAU/TAUC scoring algorithms, also include estimates of the toxicant level required to reduce n/n_0 to 1/2. (The so-called ED_{50}. See footnote, Table 15.)

TABLE 14. WTC DOSE-RESPONSE RESULTS FOR SELECTED TOXICANTS
MEAN VALUES ±SD

DOSE (mg/l)	# of TRIALS	SKOR2	SKOR3
		PHENOL	
0.0 control	57	0	0
0.5	29	32 ± 17	26 ± 10
1.0	57	91 ± 20	53 ± 10
1.5	29	183 ± 32	102 ± 18
2.0	57	307 ± 54	163 ± 28
2.5	47	581 ± 97	300 ± 48
3.0	28	693 ± 83	360 ± 42
3.5	23	749 ± 84	403 ± 43
4.0	10	645 ± 53	370 ± 25
		FORMALDEHYDE	
0 control	44	0	0
1	44	25 ± 16	20 ± 10
5	44	113 ± 19	64 ± 10
10	44	215 ± 30	117 ± 15
20	44	344 ± 60	182 ± 27
40	44	567 ± 89	298 ± 43

TABLE 14 (CONTINUED)

DOSE (mg/l)	# of TRIALS	SKOR2	SKOR3
		CrO_3	
0.0 control	36	0	0
1	36	48 ± 21	42 ± 11
5	36	127 ± 38	72 ± 16
10	36	181 ± 41	99 ± 21
30[FP]	5	331 ± 36	193 ± 18
50[NP]	36	234 ± 41	146 ± 15
100[LP]	31	76 ± 42	122 ± 18

FP = faint pigmentation; NP = noticeable pigmentation; LP = large pigmentation

		2,4-D	
0.0 contol	37	0	0
25	37	22 ± 7	2 ± 34
50	37	151 ± 40	85 ± 21
		POTASSIUM CYANIDE	
0.0 control	43	0	0
5	43	61 ± 17	40 ± 13
10	43	149 ± 24	85 ± 20
30	43	432 ± 74	231 ± 47
60	43	672 ± 137	353 ± 73
100	43	898 ± 242	472 ± 132
		SODIUM ARSENATE	
0.0 control	48	0	0
20	48	19 ± 12	17 ± 9
60	48	74 ± 34	45 ± 19
90	48	151 ± 67	85 ± 34
120	48	245 ± 99	131 ± 50
200	48	496 ± 221	260 ± 124
		CADMIUM SULFATE	
0.0 control	44	0	0
0.001	44	33 ± 21	23 ± 11
0.005	44	113 ± 34	46 ± 18
0.01	44	119 ± 53	67 ± 27
0.04	44	233 ± 77	124 ± 39
0.7	44	564 ± 133	290 ± 68

TABLE 15. THE MEAN ±SD FOR N/N_0 AND TAU/TAUC AT DIFFERENT TEST CHEMICAL CONCENTRATIONS

(Southern Research Institute Data)

Test Chemical	Dose (mg/l)	No. of Trials	N/N_0 for Strain WT168	N/N_0 for Strain fh2006-7	TAU/TAUC for Strain WT168	TAU/TAUC for Strain fh2006-7	ED_{50} (mg/l) w.Strain[1] WT168	ED_{50} (mg/l) w.Strain[1] fh2006-7
Sodium arsenate	0	40	3.93 ± 0.38	3.48 ± 0.32	1.00	1.00	73	69
	20	40	3.77 ± 0.49	3.29 ± 0.29	1.05 ± 0.13	1.05 ± 0.085		
	60	40	2.78 ± 0.34	2.44 ± 0.34	1.34 ± 0.26	1.44 ± 0.25		
	90	40	2.11 ± 0.32	1.81 ± 0.24	1.94 ± 0.47	2.22 ± 0.56		
	120	40	1.69 ± 0.23	1.48 ± 0.18	2.78 ± 0.80	3.72 ± 1.43		
Cadmium sulfate	0	36	3.97 ± 0.62	3.03 ± 0.55	1.00	1.00	0.0085	0.0035
	0.002	36	3.28 ± 0.57	2.21 ± 0.64	1.21 ± 0.27	1.36 ± 0.33		
	0.006	36	2.74 ± 0.54	1.86 ± 0.18	1.44 ± 0.38	1.90 ± 0.43		
	0.018	36	2.02 ± 0.50	1.35 ± 0.28	2.47 ± 1.57	3.63 ± 1.64		
	0.036	36	1.77 ± 0.34	1.24 ± 0.33	2.86 ± 1.31	5.29 ± 5.03		
4NQO	0	37	3.57 ± 0.43	3.13 ± 0.41	1.00	1.00	0.25	0.07
	0.1	20	2.80 ± 0.47	1.64 ± 0.15	1.29 ± 0.19	2.38 ± 0.53		
	0.2	13	2.33 ± 0.49	1.68 ± 0.16	1.59 ± 0.47	2.23 ± 0.51		
	0.4	37	2.03 ± 0.48	1.76 ± 0.16	1.87 ± 0.41	2.05 ± 0.33		
	0.8	37	1.64 ± 022	1.46 ± 0.23	2.79 ± 0.81	2.96 ± 0.62		
	1.6	37	1.28 ± 0.14	1.24 ± 0.09	6.34 ± 3.34	6.31 ± 3.1		
Potassium cyanide	0	40	3.60 ± 0.28	3.00 ± 0.41	1.00	1.00	12	11
	4	40	2.90 ± 0.47	2.60 ± 0.26	1.25 ± 0.23	1.15 ± 0.13		
	10	40	2.35 ± 0.24	2.10 ± 0.18	1.52 ± 0.18	1.52 ± 0.30		
	20	40	1.85 ± 0.24	1.64 ± 0.18	2.21 ± 0.48	2.16 ± 0.30		
	40	40	1.41 ± 0.20	1.32 ± 0.10	5.74 ± 11.0	4.48 ± 3.0		
Paraoxon	0	44	3.70 ± 0.30	3.10 ± 0.32	1.00	1.00	155	130
	28	44	3.30 ± 0.59	2.80 ± 0.29	1.13 ± 0.19	1.12 ± 0.10		
	55	44	3.10 ± 0.53	2.60 ± 0.48	1.19 ± 0.19	1.18 ± 0.14		
	110	44	2.70 ± 0.52	2.20 ± 0.25	1.39 ± 0.26	1.43 ± 0.21		
	220	44	2.00 ± 0.39	1.60 ± 0.19	1.96 ± 0.55	2.51 ± 0.86		
Sodium monofluoroacetate	0	4	3.30 ± 0.22	2.95 ± 0.40	1.00	1.00	>100	>100
	3	4	3.30 ± 0.83	3.10 ± 0.17	1.04 ± 0.17	0.97 ± 0.10		
	10	4	3.40 ± 0.85	3.10 ± 0.25	1.03 ± 0.30	0.96 ± 0.06		
	30	4	3.20 ± 0.40	3.00 ± 0.19	1.04 ± 0.14	0.99 ± 0.10		
	100	4	3.10 ± 0.30	2.90 ± 0.18	1.05 ± 0.11	1.02 ± 0.10		
Nitrogen mustard	0	8	3.50 ± 0.44	3.00 ± 0.26	1.00	1.00	92	50
	10	8	3.90 ± 0.65	2.70 ± 0.32	0.93 ± 0.16	1.12 ± 0.10		
	30	8	2.90 ± 0.62	2.20 ± 0.18	1.24 ± 0.27	1.41 ± 0.10		
	100	8	2.20 ± 0.27	1.70 ± 0.17	1.60 ± 0.22	2.11 ± 0.36		
	200	8	1.20 ± 0.11	1.10 ± 0.10	8.57 ± 6.50	20.9 ± 13.1		

[1] ED_{50}: The concentration of test chemical expected to yield 50% of the N/No value of the control.

Table 16 contrasts toxicants measured at both laboratories in terms of the SKOR2 and SKOR3 algorithms. The SRI raw data was rescored for these three toxicants so that the comparison could be made. The effects of the better growth medium used by SRI are clearly evident in these data. Nevertheless, the associated standard deviations of individual measurements overlap. Figures 13 through 18 present the corresponding plotted dose-response curves.

TABLE 16. COMPARISON OF SRI AND WTC RESULTS FOR CADMIUM SULFATE, POTASSIUM CYANIDE, AND SODIUM ARSENATE

DOSE (mg/l)	# of TRIALS	SOURCE	SKOR2	SKOR3
		CADMIUM SULFATE		
0.001	44	WTC	33 ± 21	23 ± 11
0.002	35	SRI	113 ± 47	74 ± 41
0.005	44	WTC	79 ± 34	46 ± 18
0.006	35	SRI	254 ± 99	147 ± 72
0.01	44	WTC	119 ± 53	67 ± 27
0.018	35	SRI	411 ± 143	227 ± 8
0.036	35	SRI	441 ± 120	241 ± 80
0.04	44	WTC	233 ± 77	124 ± 39
0.7	44	WTC	564 ± 133	290 ± 68
		POTASSIUM CYANIDE		
5	31	SRI	61 ± 9	40 ± 16
	43	WTC	61 ± 17	40 ± 13
10	31	SRI	142 ± 24	82 ± 16
	43	WTC	149 ± 27	85 ± 20
30	31	SRI	247 ± 35	135 ± 3
30	43	WTC	432 ± 74	231 ± 47
40	31	SRI	367 ± 49	194 ± 30
60	43	WTC	672 ± 137	353 ± 73
100	43	WTC	898 ± 242	472 ± 32
		SODIUM ARSENATE		
20	43	SRI	35 ± 36	29 ± 23
	48	WTC	19 ± 12	17 ± 9
60	43	SRI	122 ± 55	72 ± 32
	48	WTC	74 ± 34	45 ± 19
90	43	SRI	237 ± 91	132 ± 52
	48	WTC	151 ± 67	85 ± 34
120	43	SRI	345 ± 74	184 ± 38
	48	WTC	245 ± 99	131 ± 50
200	48	WTC	496 ± 221	259 ± 124

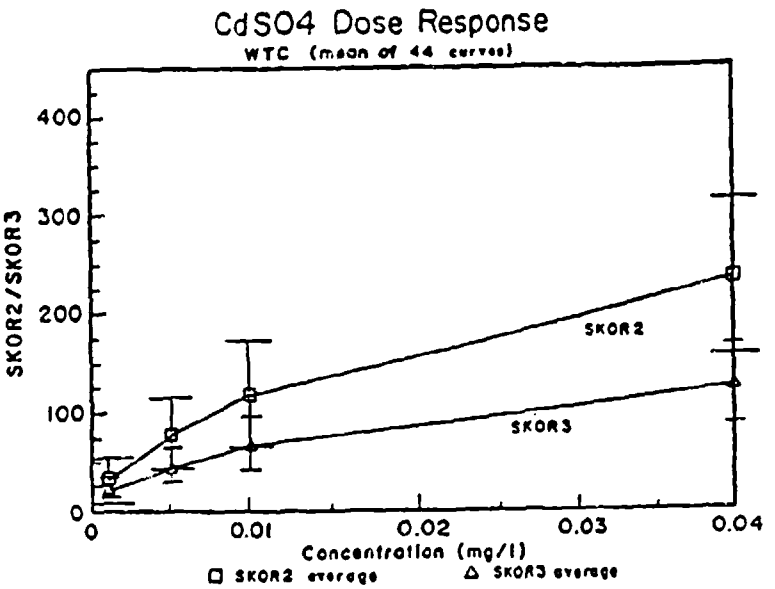

Fig. 13. Cadmium sulfate dose-response curves using scoring algorithms SKOR2 and SKOR3 for WTC data. Standard deviations as indicated.

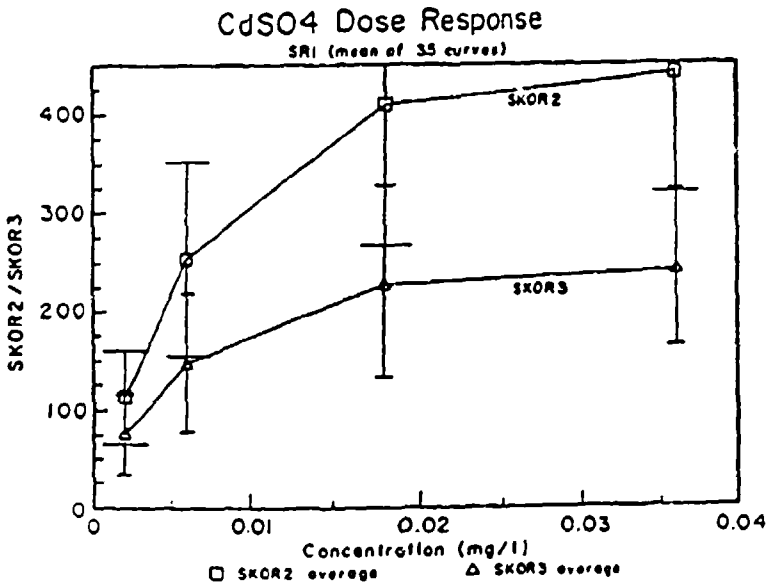

Fig. 14. Cadmium sulfate dose-response curves using scoring algorithms SKOR2 and SKOR3 for SRI data. Standard deviations as indicated.

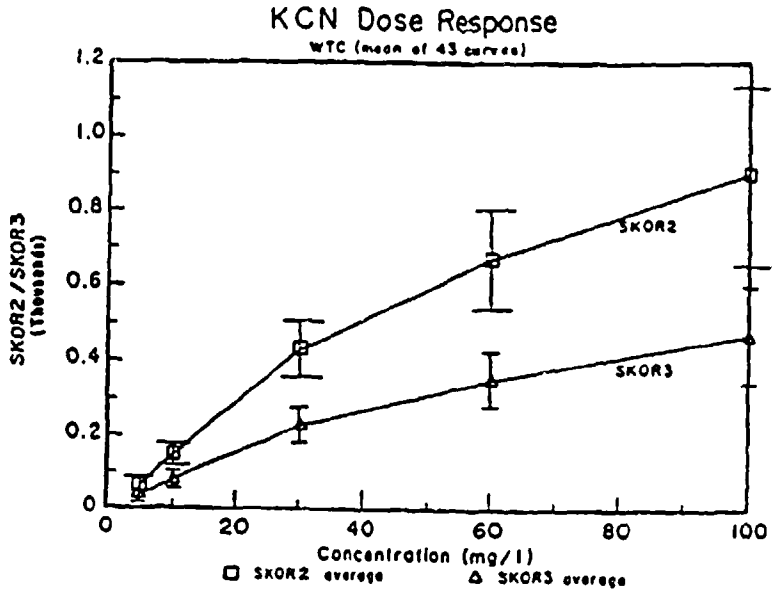

Fig. 15. Potassium cyanide dose-response curves using scoring algorithms SKOR2 and SKOR3 for WTC data. Standard deviations as indicated.

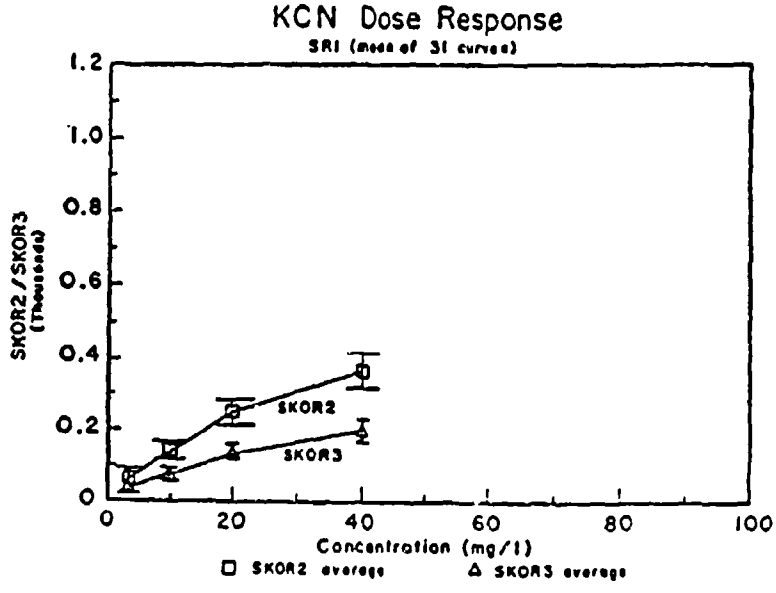

Fig. 16. Potassium cyanide dose-response curves using scoring algorithms SKOR2 and SKOR3 for SRI data. Standard deviations as indicated.

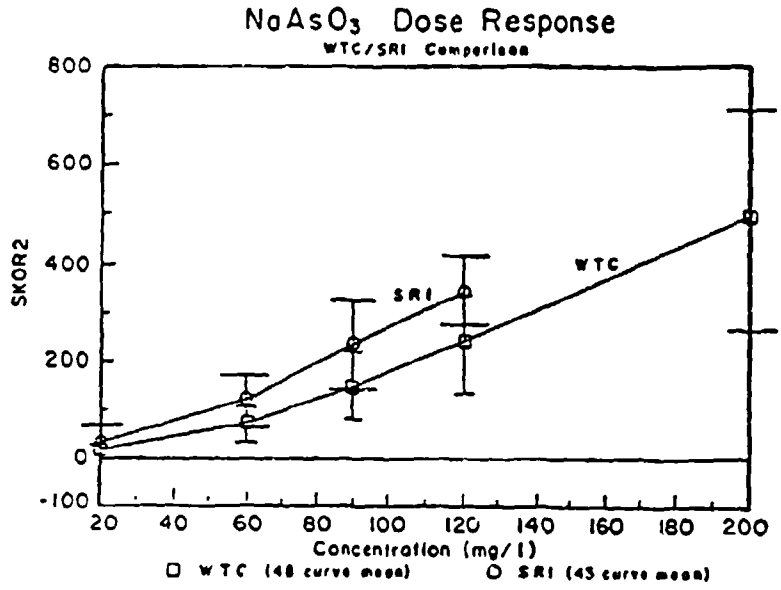

Fig. 17. Sodium arsenate dose response curves comparing scoring algorithms SKOR2 for WTC and SRI data.

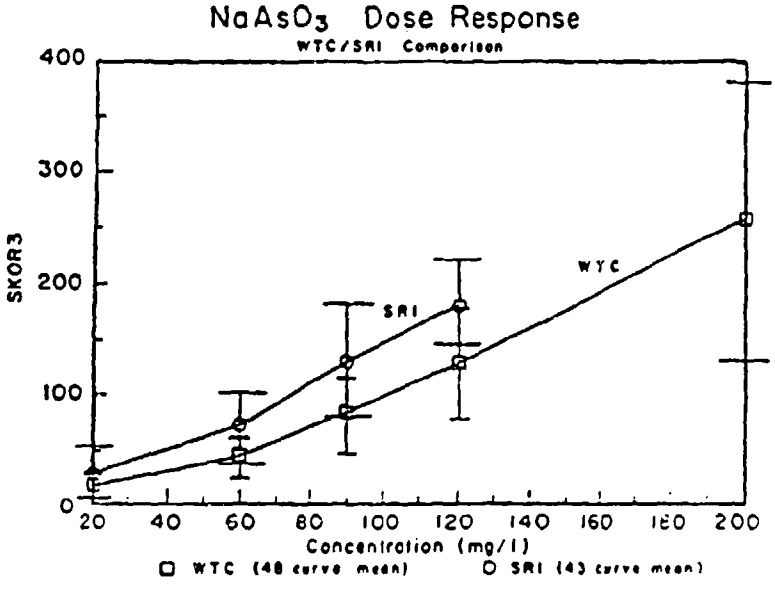

Fig. 18. Sodium arsenate dose response curves comparing scoring algorithms SKOR3 for WTC and SRI data.

The SRI weekly / bi-weekly reports / letters summarized their dose-response results. Although, as described earlier, their culture preparation protocols were different than those used at WTC, their results were consistent with our own. The SRI statistical test results are summarized in Table 17.

TABLE 17. SRI DOSE-RESPONSE STATISTICS

TOXICANT	RANGE (ug/ml)	TRIALS / FAILURES	RESULT
$NaAsO_3$	20 - 120	44 / 0	Accept H_0
$CdSO_4$	0.002 - 0.036	44 / 0	Accept H_0
4NQO	0.2 - 1.6	44 / 0	Accept H_0
KCN	5.0 - 40	44 / 0	Accept H_0
Paraoxon	28 - 220	44 / 0 WT	Accept H_0
		48 / 5	Ambiguous
Nitrogen mustard	10 - 200	8 / 0	Insufficient data
Na-Monofluroacetate	3 - 100	44 / 44	Accept H_1

The statistical results for WTC are summarized in Table 18.

TABLE 18. WTC DOSE-RESPONSE STATISTICS

TOXICANT	RANGE (mg/ml)	TRIALS / FAILURES	RESULT
Formaldehyde	1 - 40	44 / 0	Accept H_0
$CdSO_4$	0.001 - 0.7	44 / 0	Accept H_0
$NaAsO_3$	20 - 200	43 / 0	Accept H_0
Phenol	0.5 - 4	57 / 0	Accept H_0
CrO_3	1 - 100	57 / 0	Insufficient data
2,4-D	25 - 50	37 / 0	Insufficient data
KCN	5 - 100	43 / 0	Accept H_0

The McDonald specification required 5 toxicant levels plus two controls (one of which was to serve as the zero concentration value). Our measurements only provided for *one* control. The SRI data were based on a similar set since no standard deviations were attributed to any zero concentrations results. Nevertheless, all results confirmed overwhelmingly that a dose response existed at both laboratories for all toxicants except sodium monofluoroacetate. Note that the insufficient data or ambiguous listings on Tables 17 and 18 suggest (on the basis of the limited data available) that a dose response is the usual case.

Referring to Tables 14, 15 and 16 and comparing the reported standard deviations with the various responses reported at the midrange measured for each toxicant (except for sodium monofluoracetate) confirms the null hypothesis that these midrange responses are reproducible (at each laboratory performing the tests) far more accurately than the "factor of two" criterion (See Sec. 6).

F. Laser Bioassay Verification (Sec. 6, Test 4)

Unknown samples at midrange level were prepared and measured over a period of several days. High toxicity compounds were prepared for these trials by Prof. Robert Jacobs at the University of California at Santa Barbara (4NQO; paraoxon; 2,4-D; mustagen). Other toxicants were prepared by the COTR, Dr. Stephen A. Schaub at the WTC laboratories. Water or buffered blanks, as required, were also added to the unknowns tested. Dr. Jacobs also prepared an additional 21 samples at low toxicant levels. The compounds and levels used for these trials are summarized in Table 19.

TABLE 19. UNKNOWN SAMPLES (mg/l) FOR BLIND STUDIES

COMPOUND	MID RANGE (M)	LOW RANGE (L)	# of TRIALS
KCN	30 mg/l	20. mg/l	6M 3L
Sodium Arsenate	100 mg/l	60. mg/l	5M 3L
Cadmium Sulfate	0.1 mg/l	0.02 mg/l	3M 3L
CrO_3	10 mg/l	2.5 mg/l	3M
Formaldehyde	20 mg/l		6M
Phenol	1500 mg/l	1200. mg/l	6M 3L
Paraoxon	100 mg/l	20. mg/l	3M 3L
4NQO	0.5 mg/l	0.1 mg/l	3M 3L
2,4-D	75 mg/l		3M
Mustagen	100 mg/l		3M
Blanks			12M 3L

The results for the mid-range levels yielded 50 out of 53 samples correctly identified for a 94.3% accuracy. There were 1 false negative and 2 false positive results. At the low range, 19 out of 21 were correctly identified yielding 90.5% accuracy. There was one false positive and one false negative result. Details are presented in Table 20.

The letter "L" following the *Test ID* indicates low-range tests. The other Test ID suffixes correspond to different sets. Sets 1 and 2 were prepared by Dr. Schaub, while set 3 was prepared by Professor Jacobs at the University of California, Santa Barbara. The column *Result* lists the positive (P) or negative (N) sample assessment based on the light scattering measurement. *Composition* refers to the sample as prepared. These were made available after the tests.

TABLE 20. RESULTS OF BLIND STUDIES

TEST ID	RESULT	COMPOSITION	TEST ID	RESULT	COMPOSITION
5-1	P	Cyanide	1-2	P	Arsenic
6-1	P	Cadmium	2-2	P	Formaldehyde
7-1	P	Cyanide	3-2	N	Water-Blank
8-1	N	Arsenic	4-2	P	Arsenic
9-1	N	Water-Blank	5-2	P	Cyanide
10-1	P	Cadmium	6-2	P	Phenol
11-1	P	Phenol	7-2	P	Arsenic
12-1	P	Formaldehyde	8-2	N	Water-Blank
13-1	P	Chromium	9-2	P	Formaldehyde
14-1	P	Cadmium	10-2	P	Phenol
15-1	N	Water-Blank	11-2	P	Cyanide
16-1	P	Formaldehyde	12-2	N	Water-Blank
17-1	P	Chromium	13-2	P	Formaldehyde
18-1	P	Phenol	14-2	P	Arsenic
19-1	N	Water-Blank	15-2	P	Cyanide
20-1	P	Chromium	16-2	P	Phenol
21-1	P	Phenol			
23-1	P	Formaldehyde			

TABLE 20. (CONTINUED)

TEST ID	RESULT	COMPOSITION	TEST ID	RESULT	COMPOSITION
1-3	P	Paraoxon	1L	P	Arsenic
2-3	N	Water-Blank	2L	P	Cyanide
3-3	P	4-NQO	3L	P	Phenol
4-3	P	Paraoxon	4L	P	Cadmium
5-3	P	Water-Blank	5L	P	Cyanide
6-3	P	4-NQO	6L	P	Phenol
7-3	P	Paraoxon	7L	P	Cadmium
8-3	N	Water-Blank	8L	P	Arsenic
9-3	P	4-NQO	9P	P	Phenol
1-4	P	Mustagen	10L	P	Cadmium
2-4	P	Buffer-Blank	11L	P	Arsenic
3-4	P	2,4-D	12L	P	Cyanide
4-4	P	Paraoxon	13L	P	Paraoxon
5-4	N	Buffer-Blank	14L	P	4-NQO
6-4	P	2,4-D	15L	P	Water-Blank
7-4	P	Mustagen	16L	P	Paraoxon
8-4	N	Buffer-Blank	17L	P	4-NQO
9-4	P	2,4-D	18L	N	Water-Blank
			19L	N	Paraoxon
			20L	P	4-NQO
			21L	N	Water-Blank

The unknown samples were all assayed using the slightly inhibitory REMEL BHI broth. Growth rates were inferior to those obtained by SRI using fresh BHI broth prepared at their laboratories. Had we used better BHI broth for our stock culture medium, we believe that our results would have been improved further.

Referring to the statistical program defined by McDonald (Sec. 6, Test 4) for the midrange levels, we note that the null hypothesis was not confirmed by the 53rd sample. Thus to accept H_0: $p = 0.95$ by the 53rd sample would require that

$$50 \text{ (correct)} > -3.0124 + 0.9277 \text{ (53 trials)} = 52.1805 \tag{24}$$

But the alternative hypothesis H_1: p 0.90 also failed since this requires

$$50 < -3.8683 + 0.9277(53) = 45.2998. \tag{25}$$

The statistical design required that additional tests, up to a total of 60, be performed. Such samples were not prepared. However, it is evident from the results presented above that the accuracy of the identification of unknown samples was consistent with a null hypothesis H_0: $p = 0.92$ or greater.

For the low level test, a null hypothesis H_0: $p = 0.88$ seems reasonable.

8. CONTAMINANT EFFECTS

During the 1984 to 1986 period, studies were made of the effects of contaminants upon the various scoring algorithms. From a statistical point of view, very few measurements were made, yet some general results and trends were evident. In addition, the data processing software and instrument electronics were less refined than at present, and the culture lyophiles were often inconsistent. On the basis of a single set of measurements on various municipal waters and defined contaminants, the data of Tables 21 and 22 were generated. The changes following filtering (see Table 22) are quite interesting as they show for some waters that the filtering process removes some inhibitory compounds or, more probably, reduces turbidity thereby improving the test's ability to detect otherwise masked toxicants. Multicontaminant water contained 700 mg/l of total dissolved solids, 0.1 mg/l of the surfactant sodium dodecylsulfate, 20 mg/l of sodium acetate, 5 mg/l of residual chlorine, and 3 mg/l of sodium thiosulfate.

TABLE 21. SCORING FOR SOME BOTTLED AND MUNICIPAL WATERS

WATER[a]	TAU/TAUC WT	TAU/TAUC fh	AVGDEL	SKOR3	WT66TC	WTFH66	WT6WT66	FH6FH66
DI Control	1.00	1.00	—	—	—	—	—	—
Sparklett's Crystal Fresh	0.68	0.94	4.2	46	1	58	31	48
Sparkletts Purified	0.85	1.04	3.3	26	1	52	26	35
Santa Barbara Tap	1.18	1.65	5.7	36	10	75	41	16
Eagle Rock Tap[b]	1.56	1.98	4.0	86	7	47	28	17
Owens-Reseda Tap[b]	1.37	1.77	5.1	114	8	37	19	17

[a] analysed without additional filtering
[b] from Los Angeles

Table 21 shows the scoring results of two commercially bottled and three municipal tap water samples. (Refer to Sec. 5, Table 1 for definitions of various scoring algorithms.) The store-purchased Sparklett's water is better in quality as indicated by the Tau/TauC (0.68 / 0.94) being near or below 1.00 for both bacterial strains. The Los Angeles tap waters are not as good in quality and, since the Tau/TauC (1.56 / 1.98 and 1.37 / 1.77) is near 2.0 and the SKOR 3 values are high (86 and 114), there is an indication of high chlorine levels being present. (Sodium thiosulfate was not added before testing began.) Other characteristics of these unfiltered tap water samples, being normal, however, and suitable for drinking, are indicated by the AVGDEL being small and slightly positive, WT66T.C being 10 or less, and WTFH66, WT6WT66 and FH6FH66 being numbers generally well above 15. Water samples are usually filtered to remove artifacts due to naturally occurring particulates.

Referring to Table 1, we see that the WT6WT66 and FH6FH66 algorithms compare the shapes of the WT and fh scattering curves respectively at the beginning (6 minutes) to grow well during their incubation. Were a culture "frozen" at the beginning of the incubation period due to the presence of toxicants, then the shapes would be identical with an associated score = 0. The larger these values, the better the cultures have grown. (Compare the 6 and 66 minute curves of the control cultures in Fig. 5.)

WT66T.C represents a comparison algorithm for the test and control curves of the WT strain at 66 minutes. Since all curves are represented by the analysis programs in terms of Chebyshev polynomials, the expansion coefficients C_n for n greater than 0, will be identical if the two curves have an identical shape. An identical *shape* of test and control curves after 66 minutes is indicative of the absence of toxicants affecting this strain. This corresponds to a small value of WT66T.C.

AVGDEL represents the average curve length change of *both* WT and fh strains during the 6 to 66 minute incubation period in the test medium relative to the same curves in the control (DI water) medium. Uninhibited, free growing cells *decrease* in size during the incubation period. Thus the length of the scattering curve *decreases* and the change over the 60 minute incubation period is a negative number. Inhibited growth generally results in this difference being of smaller magnitude. Thus the *control* change should be more negative than any other change. The difference between the control and test changes should be slightly positive and close to zero if the test samples are free of toxicants. Large negative values (< -10) generally correspond to the presence of contaminants.

TABLE 22. SCORING FOR VARIOUS UNFILTERED AND FILTERED WATERS

WATER[a]	TAU/TAUC		AVGDEL	SKOR3	WT66T.C	FH66T.C	WTFH66	WT6WT66	FH6FH66
	WT	fh							
Unfiltered									
DI Control	1.00	1.00	–	–	--	--	--	--	--
SB	1.47	1.35	-1.7	41	16	3	84	47	27
LT	0.99	0.83	0.6	30	7	2	85	45	51
ALA	1.43	1.05	0.3	110	10	26	114	43	31
SD	1.10	0.99	1.00	23	7	3	81	44	42
Well	1.54	1.38	2.9	56	11	4	80	44	22
Muddy	45.06	90.09	-12.0	235	299	290	2	1	2
Multicontaminant	1.01	0.92	-0.5	48	8	5	48	27	34
Filtered									
DI Control	1.00	1.00	–	–	–	–	--	--	–
SB	0.88	0.99	-1.4	26	18	2	99	54	28
LT	0.69	0.82	4.3	41	16	8	106	66	59
ALA	0.65	0.88	4.1	45	16	5	106	59	52
SD	0.78	0.89	1.9	38	12	7	103	59	47
Well	0.81	1.07	4.0	21	7	2	89	45	29
Muddy	0.75	1.02	6.5	104	6	8	56	42	38
Multicontaminant	0.73	1.03	5.4	73	6	5	45	29	40

[a]SB, Santa Barbara; LT, Lake Tahoe; ALA, Alabama; SD, San Diego

Table 22 shows comparisons of some scoring results for unfiltered and filtered water samples. All of the unfiltered samples except for Lake Tahoe (LT) and multicontaminant water had Tau/TauC values significantly higher than 1.0. Filtration reduced the range of the Tau/TauC values to the range 0.65 to 1.07 for all samples including muddy water which contains many naturally occurring contaminants. Only the unfiltered muddy water gave a near zero low negative AVGDEL(-12); very high SKOR3, WT66T.C, FH66T.C (235 to 299); and (1 to 2) WTFH66, WT6WT66, FH6FH66 . These dramatically different values for the muddy water compared to the municipal waters also indicate the direction the values would be expected to go for contaminated water samples. Filtration of the muddy water

reversed the direction of the values in the scoring indicating that toxic chemicals were not present, at least in a soluble state. Note that the scoring algorithms FH6FH66 and WT6WT66 (see Table 1) compare the shapes (via the Chebyshev expansion coefficients) of the fh and WT curves, respectively, at 6 and 66 minutes of incubation. If the culture's scattering characteristics were identical at 6 and 66 minutes, it would not have grown signficantly in that time period and the algorithm would yield a zero value. Thus a small value of either scoring algorithm is indicative of the presence of a toxicant. Further studies with defined waterborne contaminants indicated that toxic chemicals could be detected even in the presence of turbidity, TDS, color, surfactant, and/or five chemicals.

Turbidity. Figure 19 shows that N/ZERO values increased with increasing concentrations of bentonite clay. This is due entirely to the increasing particulate load in the sample. For bioassay experiments, water samples are routinely filtered (0.2 µm membrane) to remove particulate matter including clay. This makes the sample more similar to tap or deionized water, and there was minimal or no interference in the bioassay.

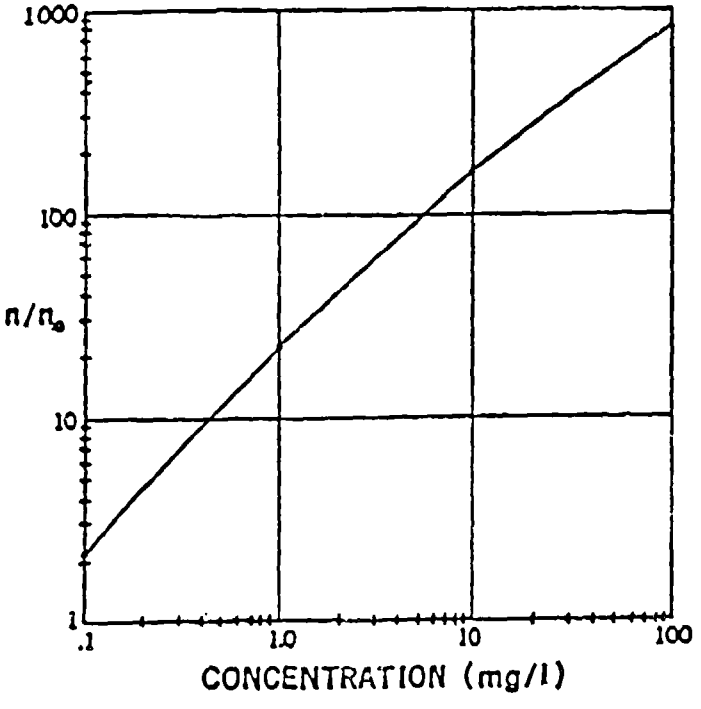

Fig. 19. Effects of bentonite clay (0.1, 1, 10, and 100 mg/l) on N/NZERO using SKORDATA and scoring vs. DI water.

TDS. The effects of total dissolved solids (TDS) at 800 and 1500 ppm were investigated briefly. In general, these high levels of TDS accelerated growth and yielded TAU/TAUC values less than 1.0, characteristics of growth stimulation or enhancement.

Color. Various concentrations (10^{-5}, 5×10^{-4}, and 10^{-4}) of Schillings' food coloring were made and scoring values were obtained in the absence of bacterial cells. Using SKORDATA and scoring against the deionized water control, the N/ZERO values increased with color concentrations and ranged from 1.5 to 3.9 for the red, yellow, green and blue colors. Again, these changes are due to absorption or scattering by the dye pigments.

The 10^{-4} dilution of each color was chosen for two bioassay experiments. In the first experiment, cultures WT and fh grew well in the four color solutions. In the second experiment, the two bacterial strains were grown in the four color solutions and also in the blue and yellow solutions containing formaldehyde (10 mg/l). As indicated by the results shown in Table 23 for the blue and yellow solutions with and without formaldehyde, the Tau/TauC, SKOR3, AVGDEL, WT66WT.C, FH66T.C, WT6WT66, and FH6FH66 values are significantly different. The inhibition by formaldehyde, is evident even in the blue and yellow solutions.

TABLE 23. EFFECTS OF BLUE AND YELLOW COLOR SOLUTIONS (10^{-4} CONCENTRATION) IN THE ABSENCE OR PRESENCE OF FORMALDEHYDE (10 mg/l)

WATER SAMPLE	TAU / TAUC WT	fh	AVGDEL	SKOR3	WT66T.C	FH66T.C	WT6W66	FH6FH66
DI Control	1.0	1.0	-1	25	1	0	67	48
Blue	1.9	1.8	-2	361	12	6	82	94
Blue + formaldehyde	9.6	9.3	-13	832	37	55	146	66
Yellow	1.2	1.2	-6	30	1	1	74	44
Yellow + formaldehyde	3.3	3.2	-12	310	20	10	31	30

Surfactant. Sodium dodecylsulfate (0.1 mg/l) had no effect on the growth or morphology of the WT or fh strains. This surfactant did not interfere with the bioassay.

Magnesium sulfate and sodium nitrate. In preliminary experiments, it was determined that magnesium sulfate (250 mg/l) was stimulatory and sodium nitrate (10 mg/l) was inhibitory to the growth of both WT and fh strains.

As shown by the results in Table 24, formaldehyde (10 mg/l) inhibited bacterial growth and caused significant changes in DLS patterns as indicated by the underlined values. In this experiment, sodium nitrate (10 mg/l) was slightly inhibitory as shown by the SKOR3 value (40) being higher than the deionized water score (24).

TABLE 24. EFFECTS OF FORMALDEHYDE, MAGNESIUM SULFATE AND/OR SODIUM NITRATE IN DI WATER ON *B. SUBTILIS* 168 WT AND *B. SUBTILIS* fh 2006—7 (12101985)

Water Sample	Chemical Concentration (mg/l)	TAU/TAUC WT	fh	AVGDEL	SKOR3	WT56T.C	FH66T.C	WT6W66	FH6FH66
DI Control	0	1.0	1.0	-0.7	24	1	0	36	61
$MgSO_4$ $7H_2O$	250	0.8	0.8	-0.8	44	17	4	24	50
$NaNO_3$	10	0.8	1.0	1.1	40	4	2	37	78
$MgSO_4$ + $NaNO_3$	250 + 10	0.8	0.9	1.1	35	13	4	22	38
Formaldehyde + $MgSO_4$	10 +250	7.1	4.5	-8.9	258	25	23	11	20
Formaldehyde + $NaNO_3$	10 +10	23.1	5.2	-9.6	266	32	13	40	48
Formaldehyde + $MgSO_4$ + $NaNO_3$	10 +250 +10	4.3	4.5	-6.2	251	32	7	21	28

Sodium acetate (20 mg/l) was inhibitory to the bacterial strains.

Free available chlorine disinfectant and neutralization with sodium thiosulfate. According to *Standard Methods for the Examination of Water and Wastewater*, 13th edition, 1971, page 657, "0.1 ml of a 10% solution of sodium thiosulfate will neutralize a sample containing 15 mg/l residual chlorine." Therefore, 10 mg of sodium thiosulfate neutralized 15 mg of residual chlorine which is a 2:3 ratio. Neutralized residual chlorine produced no effect on the bioassay.

In summary, these studies showed that the presence of a toxic chemical can be determined if a majority of the scoring algorithms indicate both inhibition and meaningful DLS pattern changes relative to the control.

9. CONCLUSIONS AND RECOMMENDATIONS

The laser bioassay technique incorporating only two isogenic strains of *B. subtilis* has been shown to be an effective and statistically reproducible method for screening a broad range of toxicants in drinking waters. Earlier studies had shown that such results could be extended to waters containing modest levels of a variety of non-toxic background contaminants, but more extensive testing would be required to establish limits of diminished responsivity to toxicants produced by each contaminant class. Simple protocols and kits for the treatment of water samples preparatory to testing could be improved. Current preparation protocol includes chlorine neutralization with sodium thiosulfate, pH neutralization with 7 mM phosphate buffer, and filtration with 0.2 μm Nuclepore filters. A broader study of contaminant effects on the bioassay must be undertaken if low levels of toxicants are to be detected in the presence of such contaminants.

The statistical test designs confirmed that the laser bioassay system is an accurate and reproducible procedure for the detection of 10 of the 11 toxicants studied. The few inconsistencies were due to lack of sufficient data because of time constraints or mistakes in preparing the experiments properly. A large fraction of the time spent on this statistical confirmation program was devoted to elements not originally anticipated. Foremost among them were the unexpected problems with commercially prepared broths, growth problems with lyophile-initiated cultures of *B. subtilis* (clumping of bacteria for many hours) insufficient data to complete statistical analyses in some cases, poor attention to instrument alignment and calibration, and inattention to coordinating suitably the work between the two laboratories. Both SRI and WTC had major staffing changes during the course of this contract causing lost time and poor continuity. Despite these myriad difficulties, the results of the study overwhelmingly confirm the power of the technique. Both hardware and software have been improved continuously, even after the formal end of the project, with the result that instrumentation (with software) can detect problems with the protocols (such as culture ready or too old criteria) and alert the operator to potential inconsistencies associated with inferior reagents, erroneous protocols, and major human errors in preparing samples.

The four major results of the study, confirmed by the extensive statistical tests and analyses were:

1) The efficacy of essay cultures for their ability to detect toxicants could be evaluated prior to their use by measurement and quantitative analysis of their light scattering characteristics.
2) For 10 of the 11 toxicants tested, the important scoring algorithms developed yielded a linear dose-response for each toxicant and spanning levels considered potentially dangerous for ingestion.
3) Detection instruments (laser light scattering photometers) could be fabricated to yield the same results from the same types of samples with different instruments.
4) Starting with high quality water, toxicant-containing samples can be detected at mid-range levels (potentially dangerous) and low levels (safe) with an accuracy in excess of 90%.

A field portable system should be developed now which would maintain the basic elements of the DAWN bioassay instrument and contain a few "off-the-shelf" modifications including a single board, ROM programmed computer, well insulated battery powered incubation modules, and other battery powered components. Since all measurements last only a few seconds and require generation of the *ratios* of scattered intensities to the incident laser beam intensity, an instrument using a small duty cycle, lower power laser would be sufficient. Development by WTC of variations of the DAWN system for liquid chromatography has already resulted in a new detector system with a sensitivity 100 to 200 times greater than the standard DAWN systems used in this program. On this basis, the laser source used by the systems may be reduced in output power by at least a factor of 10,

with a corresponding reduction in input power. The field system would have, therefore, very small physical dimensions and be powered by a small, lightweight battery pack.

Expansion of detectible toxicant classes will involve introduction of a few simple preparative steps. Specifically, certain important toxicants (such as T-2 mycotoxin, xylene, various chemical agents, pesticides, etc.) require solubilization or metabolic activation, or both. Felkner[19] has already developed the preliminary "cocktails" needed to achieve aqueous deliverable toxicants or their metabolic products to the test organisms. The utilization of this emulsifier / dispersant solution in an aqueous system is expected to minimize sample preparations for analysing soil samples, as well, since it can be used to extract insolubles. Following filtration (a necessary preparative step to remove spurious particulates), such a sample could be assayed directly.

More work must be directed to reducing the time lag between culture initiation and the culture ready condition. Other members of the isoset, or related strains, must be examined in terms of their filament formation during early exponential phase. Felkner[19] still claims that spore-initiated cultures should be useable in less than an hour following heat shock induced germination. This has yet to be confirmed.

Some software modifications would be required for the field units. Sample processing would include a new series of prompt menus on a small screen, together with acoustical signals. Each sample could be bar coded and the program designed to read the sample ID before running. In addition, the program should keep track of all samples run, their agitation and reading schedules, and the results of their scoring subsequent to measurement. Scoring algorithms should be expanded to include effects of contaminants on the scoring. Although each sample is measured against itself, high background levels (relative to solvent offset levels) tend to mask actual growth rates. Simple algorithms which take into account sample background scattering before inoculation (relative to the pristine DI controls) may be incorporated easily into the programs and will aid in interpreting heavily pigmented samples.

In summary, therefore, the method viability has been confirmed as a rapid, inexpensive bioassay technique for the detection of a variety of toxicants at levels for which warnings are needed. The additional work required to make the system practical for field testing and deployment is straightforward, well defined, and achievable in the very near future.

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