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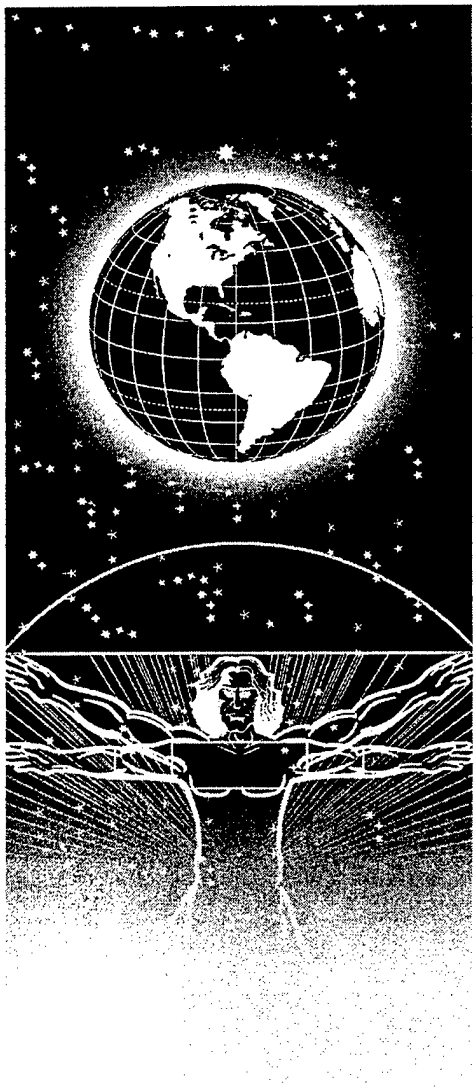

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Polarized Light Scattering as a Rapid and Sensitive Assay for Metal Toxicity to Bacteria

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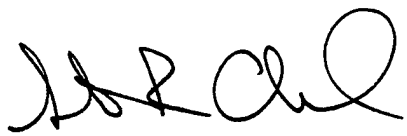

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A new method that utilizes the scattering of polarized light from a suspension of bacteria to assay the effect of toxins is evaluated. The method compares the time dependence of changes in an angular scattering pattern obtained from a suspension of Escherichia coli bacteria with no toxin exposure to the corresponding, but reduced, changes that occur when there is exposure to a small concentration of certain toxicants. The changes are due to growth of a specially prepared population of these bacteria. The changes in the pattern normally reflect a change in average bacterial size due to growth, whereas the reduction of the change in pattern occurs when there is rapid cessation of bacterial growth. The method was tested with varying concentration of the ions of five different heavy metals. The results using this method during the first few minutes after exposure to the toxicant were compared to the relative survival of colony-forming units of the bacteria. The graphs for the two methods were found to be approximately parallel for each of the five metals examined. This result indicates that the toxic effect of these metals takes place relatively quickly for these bacteria. These results were compared with results available from the literature for the same metals but using other methods for measuring the toxicity to bacteria.

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TABLE OF CONTENTS

FIGURES

TABLES

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Polarized Light Scattering as a Rapid and Sensitive Assay for Metal Toxicity to Bacteria†

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Key words: toxicity to bacteria; polarized light scattering; *E. coli*; heavy metals: rapid detection; metal toxicity; growth inhibition.

A new method that utilizes the scattering of polarized light from a suspension of bacteria to assay the effect of toxins is evaluated. The method compares the time dependence of changes in an angular scattering pattern obtained from a suspension of *Escherichia coli* bacteria with no toxin exposure to the corresponding, but reduced, changes that occur when there is exposure to a small concentration of certain toxicants. The changes are due to growth of a specially prepared population of these bacteria. The changes in the pattern normally reflect a change in average bacterial size due to growth, whereas the reduction of the change in pattern occurs when there is rapid cessation of bacterial growth. The method was tested with varying concentrations of the ions of five different heavy metals. The results using this method during the first few minutes after exposure to the toxicant were compared to the relative survival of colony-forming units of the bacteria. The graphs for the two methods were found to be approximately parallel for each of the five metals examined. This result indicates that the toxic effect of these metals takes place relatively quickly for these bacteria. These results were compared with results available from the literature for the same metals but using other methods for measuring the toxicity to bacteria.

INTRODUCTION

During recent years, the use of single-celled organisms has been studied and is now well established as an alternative means of testing for toxicants. Bacteria have been used both in axenic culture and in complex heterogeneous cultures such as sludge, with a variety of methods to assay for toxicity.[1]

The use of laboratory animals is often necessary in testing for toxicity. This always entails sacrifice, expense and some suffering of these animals. In those experiments where bacteria can be used, some advantages are realized in addition to the elimination of the foregoing disadvantages of animal use. The expense of maintaining and assaying the animals sometimes limits their use in practical experiments to a few animals per data point. This can lead to statistical uncertainty in interpreting data. With bacteria, this difficulty is greatly reduced. Millions of bacteria can be tested for each data point. In addition, bacterial tests are generally much faster, which allows more testing and more variation of the test parameters. Of course, animal experimentation remains quite necessary in many procedures (e.g. determining organ specific toxicity).

Generally, in this type of experiment, the presence of toxicant is monitored by the decrease in some measurable function of the bacteria or other single-celled organism. Some examples follow. In the Microtox assay one measures the reduction of light production by a bioluminescent marine bacterium.[2,3] Another assay utilizes the inhibition of respiration using a mixed bacterial culture isolated from wastewater.[4] A third assay detects the inhibition of biosynthesis of β-galactosidase in *Escherichia coli*.[5] Still other assays include the growth of bacterial populations as measured by optical density, the microscopic estimation of lengths of filamentous bacteria and changes of motile and swimming pattern of *Spirillum volutans*. All these have been used as indicators to monitor the presence of toxicants.[1] Our new assay, likewise, depends on a physiological response of a known organism to the presence of a toxin. In this case the response is the absence of a change that occurs normally when *Escherichia coli* bacteria are changed from a poor medium to one rich in nutrients.

In a previous publication[6] we showed that the angular graph of a polarized light-scattering function is quite sensitive to changes in bacterial growth patterns during time intervals as short as 5–15 min. Based on this observation, we suggested[7] that one would expect these changes to be affected by the presence of toxins and this would give a new rapid method for assaying for toxicity. In the present paper we show that the method is useful by applying the assay to study the effect of several different heavy metal ions on the growth of the bacterium *Escherichia coli*.

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MATERIALS AND METHODS

Chemical

The following chemicals were used in the toxicity studies: $HgCl_2$ (Hg^{2+}), $AgNO_3$ (Ag^-), $CuSO_4 \cdot 5H_2O$ (Cu^{2+}), $Pb(NO_3)_2$ (Pb^{2+}), $ZnSO_4 \cdot 7H_2O$ (Zn^{2+}) All these chemicals were obtained from Sigma Chemical Company, St Louis, MO. The toxin treatments all took place at pH 6.8. except in the case of Cu^{2+} for which tests were done at pH 5.0 as well as at pH 6.8.

Biological

A single strain of *Escherichia coli* K12 (ATCC 49439) was used for these experiments. This strain was found previously[6] to give large and reproducible shifts in the graph studied (see below) when toxins were not present. The bacteria were grown overnight in LB broth (per liter of final volume) using distilled water and adding 10 g of NaCl, 10 g of tryptone (Difco, Detroit, MI) and 5 g of yeast extract (Difco). The bacteria were grown overnight (ca. 16–18 h) with vigorous aeration in a gyrating water bath at 30 ± 0.5°C. Bacteria were harvested, washed once in water (centrifuged to pellets and resuspended twice) and resuspended in distilled water at 2×10^7–3.0×10^7 colony-forming units (CFU) ml^{-1} (absorbance or OD at 600 nm = ~0.3) for testing the toxicity of the metal ions used. The toxicant in concentrated solution was diluted with thorough mixing into the bacterial suspension in a proportion that gave the indicated final concentration. The mixture was then incubated for 15 min at room temperature to allow reactions to occur. The suspensions were then centrifuged and washed twice in distilled water and then either diluted appropriately in 1/10 steps into phosphate-buffered saline (PBS, Sigma) at pH 7.4 for plating on LB agar (12 g agar l^{-1}) or finally resuspended in prewarmed PBS at an absorbance (600 nm) of 0.1 ± 0.02 and placed in the cuvette for scattering measurements. After the initial scattering measurement (zero time). an equal volume of prewarmed 2× concentration fresh LB was added to the cell suspension in the scattering cuvette and additional scattering curves were measured at timed intervals. The enriched medium causes immediate growth and a shift to the left of the oscillations of the scattering curves. with the peaks shifted to smaller angles when the cells are not inhibited by toxin. We note that we have checked the state of the bacteria with microscope measurements in these and previous experiments and that we have almost all individual bacteria with 1 or 2% of doubles and no clumps noted for the preparation described.

Light scattering

The theory and experimental set-up for measuring various scattering functions for micron-sized particles, including their 'Mueller matrix elements', as well as the definitions used here for the individual matrix elements are presented in detail in a readily available reference.[8] The application of this technique to microorganisms is described in Ref. 9. We present here only a brief overview on the scattering set-up. The set-up is shown in a simplified form in Fig. 1. In Ref. 10 we show that for light scattering from these bacteria the ratio of two of the Mueller matrix elements, S_{34}/S_{11}, gives rise to a graph like that of Fig. 2 as a function of the scattering angle θ. The graph is reproducible for a fixed set of experimental conditions but at the same time is very sensitive to the growth conditions that the bacteria experience in a particular experiment. (In our experience this ratio, i.e. the normalized S_{34} matrix element, most clearly shows the effect of the size change for a suspension of bacterial cells. The use of a normalized matrix element makes the measurement easier because it reduces the scale of the changes that otherwise are very large at small angles.) Light from a 670-nm diode laser is passed through a linear polarizer and then through a photoelastic modulator (PEM-80; HINDS International, Portland). The light is then scattered through the sample in the cuvette shown and detected in a photomultiplier tube (Hamamatsu R636), which is rotated about the cuvette on a movable arm to make measurements at 1° intervals between near-forward (20°) and rear-ward (120°) directions. The modulator that causes the emerging laser light to rotate between two oppositely rotating states of elliptical polarization (through circular polarization) at a frequency of 50 kHz allows the ratio S_{34}/S_{11} to be measured by means of a lock-in amplifier (5208 two-phase lock-in amplifier E G & G, Princeton, Applied Research Corp.). Signals are averaged over 1–3 s at each angular point.

We use the standard light-scattering coordinate system defined by Bohren and Huffman in figure 3.3 of Ref. 8. In that case, the polarization of the input beam is defined as parallel or perpendicular with respect to the scattering plane. With this definition, because the bacteria are randomly oriented, the matrix elements do

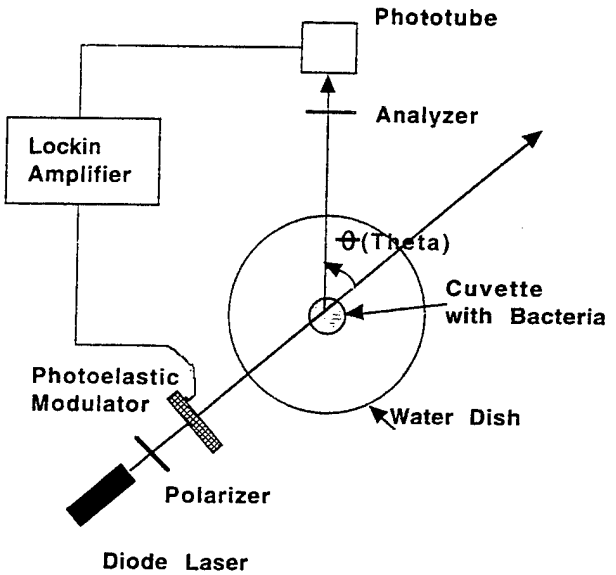

Figure 1. Simplified version of scattering set-up. A number of elements are omitted for simplicity. The bacteria contained in the cuvette as experimental scatters are *Escherichia coli* in liquid suspension. The cuvette is placed in a glass dish containing water to reduce reflections and improve the collection geometry.

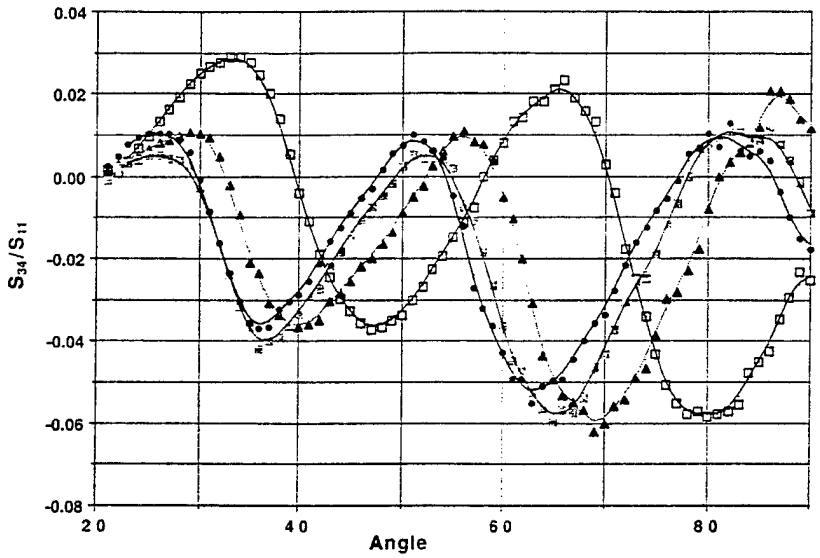

Figure 2. Graph of S_{34}/S_{11} vs angle for *E. coli* at various times after nutritional upshift with no toxicant present (i.e. control curve): (□) time zero; (▲) 9.1 min; (■) 18.2 min; (●) 22.3 min. The fitted curves were obtained with a two-point binomial smoothing routine from Grams/32 software (Galactic, Salem, NH).

not depend on any scattering (azimuthal) angle other than θ. (We note that this does not mean that the same scattering is observed at different azimuth angles for the same θ *unless* the polarization of the input beam is corrected in the experiment for the new scattering plane in accordance with figure 3.3 of Ref. 8.)

RESULTS

Measurement of toxicity

In Fig. 2 we show a timed sequence of the scattering curve for a control sample that was treated with distilled water but with no heavy metal toxin added. For every toxicant experiment, such control curves were run at zero time and at ca. 20 min. Polarized light scattering (PLS) curves are shown here for time zero and at subsequent times after adding fresh LB to the cell suspension (i.e. after nutritional upshift). Note that in each case a curve that oscillates with angle is produced, but that the peaks or maxima and minima of the curve are displaced towards smaller angles as time progresses after the 'upshift' to enriched medium. We showed previously that under the conditions of this experiment this peak displacement corresponds to an increased diameter of the bacteria.[10,11]

In this paper. we show that the presence of heavy metal toxins affects the scattering curve by reducing or halting the displacement of the curve peaks to the left, which otherwise occurs after a nutritional upshift. This suggests that the enlargement of the *E. coli* cells that ordinarily takes place after nutritional upshift is inhibited. It turns out that this inhibition is closely correlated with the reduction in the number of colony-forming units (CFU) that takes place.

The amount of this inhibition depends on the dose of toxicant. For these experiments we measured the average rate of angular shift of the second maximum of the graph of S_{34}/S_{11} (initially near 66°) to the left during the first ca. 20 min after the medium upshift. In other experiments (not shown) we have found that this peak shift towards zero angle stops and reverses after times of >1 h because the bacteria again become smaller as the cells become more crowded and the stationary phase is approached.

We use the inhibition of this rate of shift (degrees min^{-1}) of the second maximum to measure toxicity. The ratio of this *shift rate* for a given dose to that for zero dose is our measure of relative activity. We call this the *shift ratio*. The shift of the second maximum for the graph of Fig. 2 was 14.7° during the first 22.3 min after upshift, so that the control *shift rate* for this experiment is ca. 0.66° $min,^{-1}$. In Fig. 3 we show the shift of the second maximum at various times after the upshift to enriched medium. We note that the maximum shifts continuously to smaller angles for times of <20 min.

In Fig. 4 we show four graphs, two of which are for zero time, and a shifted control graph after 22 min for zero dose for comparison. The graphs for non-zero dose of Hg^{2+} show lesser shifts after medium upshift at 21 min after the treatment with mercury. For the 0.05 mg l^{-1} dose the shift rate is 0.55° min^{-1}, and for the higher 0.075 mg l^{-1} dose the rate is 0.37° min^{-1}. In the present case the shift ratios (our measure of relative toxicity by light scattering) are 0.83 and 0.56, respectively, for the two doses of mercury, the higher dose gives the lower shift ratio, indicating more inhibition of growth.

The effective concentration for 50% toxic inhibition (EC_{50}) is defined for the scattering as the concentration at which the shift ratio for the treated sample is ~0.50. Likewise, for the survival experiments EC_{50} is the dose giving a CFU ml^{-1} value that is 50% of that obtained for a control experiment with zero dose of the toxin.

Toxicity results

In Fig. 5 we show a semilogarithmic graph comparing shift ratios and relative survival (relative CFU) for

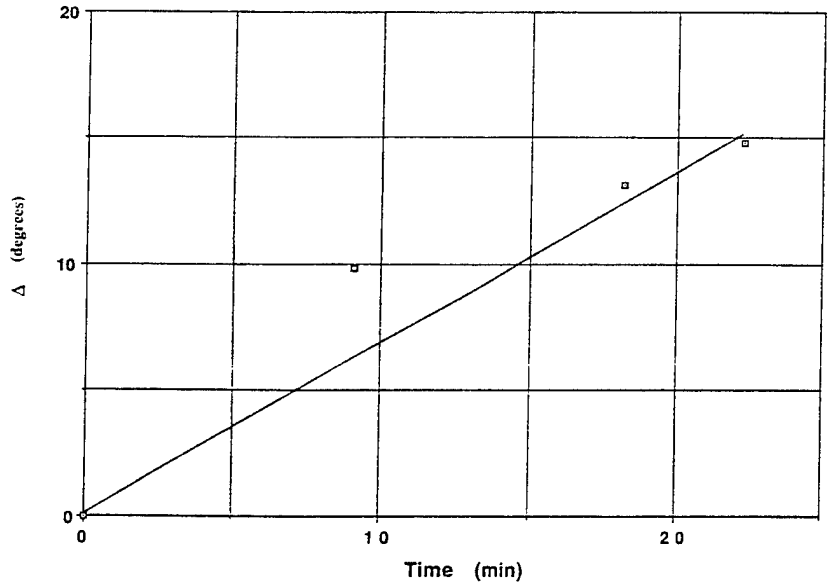

Figure 3. Change in angular position (Δ) of second maximum vs time for control curves

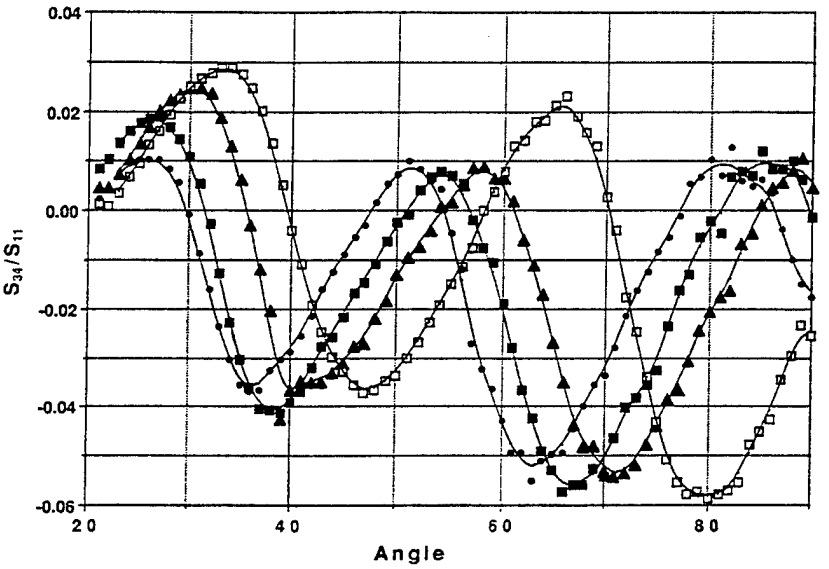

Figure 4. Graph of S_{34}/S_{11}, vs angle for treatments of *E. coli* with various doses of Hg^{2+} for 15 min: (□) zero time; (●) zero dose at 22.3 min after upshift; (■) 0.05 mg l^{-1} dose graph at 21.2 min; (▲) 0.075 mg l^{-1} dose graph at 20.75 min.

averages from three experiments in which we measured the toxicity of Hg^{2+}. In both cases the EC_{50} is at ca. 0.09 mg l^{-1} Hg^{2+}. We note that both measures fall relatively fast for low doses and then slower when the relative survival or shift ratio is lower than ca. 20%. We plotted graphs like this for each of the five metal ions tested, with similar-appearing graphs in each case. The decrease in shift ratio with increasing concentration for each of the heavy metal ions is rather similar to the decrease in relative CFUs for each case, at least at low doses where the shift ratio is easier to measure. In the case of Cu^{2+} we repeated both survival and upshift experiments at pH 5.0 as well as at pH 6.9, with very similar results.

In Table 1 we give averages from several experiments for *shift rates* for three treatment concentrations for each of five metal ions. The concentrations are stated in mg l^{-1} of the salt used.

In another experiment shown in Fig. 6, we plot the relative activity for a PLS experiment vs the time the cells are left in a toxicant treatment suspension containing 0.05 mg l^{-1} Hg^{2+} for various times before removing the mercury. We see that, as expected, the toxicant effectiveness increases monotonically with time, and that the increase is substantial for exposures longer than our standard treatment time of 15 min.

DISCUSSION AND CONCLUSION

In Table 2 we give the EC_{50} for the five heavy metal toxicants for both the PLS method as well as the

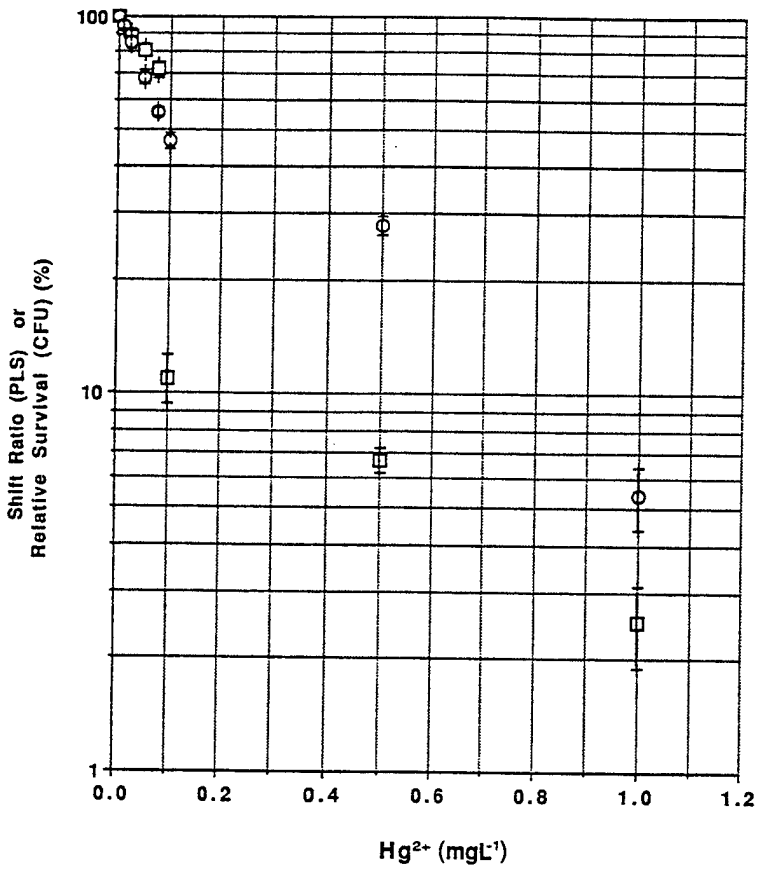

Figure 5. Semilog graphs for Hg^{2+} of the shift ratio in PLS graphs (see Fig. 4) (□) or relative CFU (○) expressed as percentage values. Data points are the mean ± SD from three experiments. At 2 mg l^{-1} there was zero shift and zero survival.

Table 1. Averaged upshift rates for five metal ions

	Number of experiments	Upshift rate ± SD (degrees min^{-1})
Control	15	0.62 ± 0.02
Ionic conc. (mg l^{-1})		
Hg^{2+}	3	
0.01		0.58 ± 0.02
0.05		0.52 ± 0.02
0.1		0.07 ± 0.01
Ag^{+}	3	
0.017		0.53 ± 0.01
0.17		0.33 ± 0.02
0.51		0.10 ± .01
Cu^{2+}	3	
0.5		0.58 ± 0.01
5.0		0.33 ± 0.02
50		0.11 ± 0.01
Pb^{2+}	3	
5		0.55 ± 0.01
20		0.42 ± 0.02
120		0.15 ± 0.01
Zn^{2+}	3	
2.5		0.52 ± 0.01
25		0.43 ± 0.04
200		0.18 ± 0.01

survival assay (CFU) for our experiments, as well as a concentration obtained from the literature for one or more other method. The methods compared to the present method are: Microtox;[3,13,15] movement of flagellate—Ecotox;[12] microtitration plate—MetPlate;[14] microcalorimetry.[16] When possible, we chose a published experiment with a toxicant treatment time close to that used here (15 min) for comparison with the PLS method.

We observe that in each case the PLS method gives a result that is quite comparable to that for the CFU method. The PLS method measures the phenomenon in which the average cell size grows rapidly larger when the bacterial population responds to a sudden enrichment of the medium encountered. From the similarity of this response to that for reproductive survival, we are led to believe that the metal ion toxicant stops growth at about the same number of molecules per bacterial cell as that required to kill the individual bacteria for the species used here (*E. coli*). This is not necessarily obvious *a priori*. It is not the case, for example, in some radiation-survival experiments where some cellular growth occurs, but reproduction of new cells may be sufficiently inhibited that the number of CFUs is greatly reduced.

The comparison of the EC_{50} with the literature value for treatment times similar to those that we used turned out to be quite close for mercury and copper but not so close for silver: our experiments

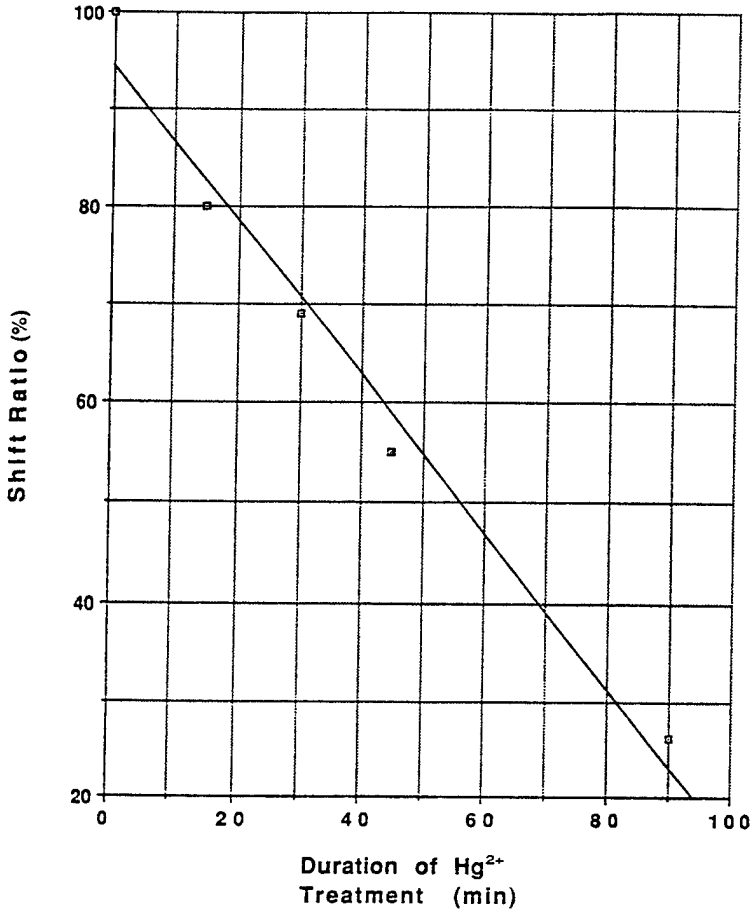

Figure 6. Shift ratio vs duration of exposure for treatment with 0.05 mg l^{-1} Hg^{2+}.

Table 2. Comparison of EC_{50} values (toxicant concentration[a]) (mg l^{-1}/μM l^{-1})

Metal ion	PLS method	CFU assay	Other method	Exposure time and Ref.
Hg^{2+}	0.07/0.26	0.09/0.33	0.05/0.2 Microtox	15 min (Ref.3)
			1.0/3.6 Ecotox	3 min (Ref. 12)
Ag^{+}	0.31/1.8	0.26/1.5	0.6/3.5 Ecotox	3 min (Ref. 12)
Cu^{2+}	5.3/21.2	2.9/11.6	3.8/15.3 Microtox	15 min (Ref. 3)
			3.2/12.9 Microtox	15 min (Ref. 13)
			8.0/32.1 Ecotox	3 min (Ref. 12)
Pb^{2+}	36/108.7	31/93.6	30.2/91.2 Microtox	15 min (Ref. 3)
			10/30.1 MetPlate	1 h (Ref. 14)
Zn^{2+}	71/247	67/233	64.7/235 Microtox	10 min (Ref. 15)
			50/174 Heat flow	10 min (Ref. 16)

[a]Salt concentration.

show greater sensitivity to the presence of silver ions. In the case of lead and zinc, our experiments fall within the fairly sizable spread of literature values. The EC_{50} values for lead, zinc and copper are fairly large, as expected, because these metals are rather 'feeble in germicidal action'.[17] We note, however, as confirmed in Fig. 6, that the toxicity of a given metal ion may vary substantially depending on the treatment protocol used.

We conclude that the new PLS method gives numerical values for toxic concentrations that are, in most cases, reasonably close to those obtained from other presently used methods. This was determined only for the limited case of the metal ions studied here, but is expected to hold true for any toxin that causes immediate cessation of growth. The present method gives a direct measure of the immediate cessation of growth that occurs in all the cases studied here, but is not necessarily always the case, as noted above.

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