Turbidity and Color Correction in the Microtox™ Bioassay

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Abstract: We propose a new method of correcting for the effects of color and turbidity on bacterial light output measurements in the Microtox™ bioassay. Using our method, toxicity assessment involves clearly defined calculation factors, based on light loss measurements using a modified double-cuvette.

An existing color-correction method requires additional spectrometer measurements and over-corrects for turbidity. For a range of clear, colored fluids our new method and the existing method gave similar, corrected EC50 values.

Our new method needs no separate equipment and is likely to provide valid toxicity assessments of whole fluids, without any need for sample clarification. It accounted for > 90 % of the variance in loss of measured bacterial light caused by color and turbidity of a range of test fluids.

Key Words: Turbidity, Color, Microtox, Bioassay.

In the Microtox™ bioassay, aliquots of the luminescent marine bacterium *Vibrio fischeri* are mixed with serial dilutions of the test fluid in 2 % NaCl. Any resulting fall in measured bacterial light intensity from its initial value indicates sample toxicity.

Light readings taken after 5 or 15 min. lead to toxicity assessments that generally correlate well with those from more time-consuming methods using other biota [1].

However, the Microtox test has the disadvantage that sample turbidity and color cause uncertainty in light output measurements. Red-brown coloration absorbs the blue light given off by *V. fischeri*, thus affecting toxicity assessment of many wastewater samples.

In such cases, the initial toxicity assessment must be modified by correcting data from the regular procedure, which goes as follows:

BASIC CALCULATIONS

A light loss factor, Gamma (the ratio of light lost to light I_t remaining after time t), is defined [2, 3] at each serial dilution level, as follows:

$$Gamma_t = (I_o - I_t) / I_t = (I_o/I_t) - 1 \quad (1)$$

To allow for natural drift in bacterial light output during the test each initial, pre-mixing sample light intensity reading I_o is adjusted, using corresponding readings for a control aliquot in pure salt solution:

$$Gamma_t = [\ Control\ (I_t/I_o)\ x\ Sample\ (I_o/I_t)\ ] - 1 \quad (2)$$

Plotting $Gamma_t$ values from Eq. (2) against sample concentration provides a toxicity assessment, for example an EC50(t) value, the concentration of test fluid at which light intensity was halved, after time t. This EC50 value (corresponding to $Gamma_t = 1$) is valid for clear, colorless test fluids. Often, a log-log plot is used, the EC50 then being the antilogarithm of the intercept at $\log_{10} Gamma_t = 0$.

LIGHT-ABSORBING TEST FLUIDS

Two color-correction methods are given in Microtox™ User Manuals [2, 3]. Both methods adjust for sample light absorbance by multiplying the initial sample I_o at each sample concentration by a transmittance factor T (< 1), by means of the following equation:

$$Gamma_t = [\ Control\ (I_t/I_o)\ x\ Sample\ (I_o.T/I_t)\ ] - 1 \quad (3)$$

Later light intensity readings I_t at each sample dilution level are thus compared against an initial I_o reading that has been adjusted downwards for light absorbance, as well as corrected for any drift in bacterial light output. The procedure in effect estimates measured light intensity I_o immediately after mixing, as lowered by light absorbance alone, before sample toxicity has had time to affect bacterial light output (it being impracticable to make this actual measurement).

Corrected $Gamma_t$ values from Eq. (3) are then plotted against concentration to obtain a color-corrected EC50(t) value. For a clear, non-absorbing sample, T = 1 and Eq. (3) reverts to Eq. (2). The inherent validity of Eq. (3) is not in question, but uncertainty exists regarding appropriate values for the factor T.

TRANSMITTANCE FACTOR T

Evaluating T is a two-stage process. Both User Manuals [2, 3] without elaboration instruct the user to calculate T at each sample dilution level c from an absorbance factor A, using the following equation:

$$T_c = (1 - e^{-Ac}) / A_c \quad (4)$$

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Absorbance Factor A

The two User Manuals describe different methods for obtaining the factor A.

The Microbics method [2] requires a double-walled test cuvette, where an aliquot of *V. fischeri* in an inner tube is kept separate from the sample during light intensity measurements. The user is directed to calculate A_c at serial dilution level c from light readings I_f and I_e made respectively with and without a selected concentration c′ of sample in the outer compartment.

$$A_c = 3.1 \log_e (I_e/I_f) \times c/c' \quad (5)$$

In marked contrast, the Azur method [3] for evaluating the factor A requires a separate spectrometer, and involves reading the optical absorbance of the test fluid at 490 nm, the wavelength of maximum emission from *V. fischeri*.

In this case, the user calculates A_c from the spectrometer reading Abs at each serial dilution level, by means of the following formula:

$$A_c = 1.75 \times Abs \quad (6)$$

Both Manuals state that the respective multipliers 3.1 and 1.75 in Eqs. (5) and (6) were derived empirically and correct for the geometry of the system. Ownership of the Microtox™ test has changed hands several times since the 1980s. We have been unable to uncover publications or data, or to obtain expert advice related to the derivation and validity of Eq. (4) through (6).

On the topic of color correction, the current owners (SDI, Inc.) refer the user to the technically easier Azur method [3]. SDI do not supply double cuvettes; as a result the Microbics method [2] is obsolescent and will not be discussed further here.

The Azur color-correction method [3] does not take explicit account of sample turbidity; analysts must either remove turbidity [4], or else assume that suspended dark particles act to reduce light readings in essentially the same way as red-brown color [5].

In our laboratory we routinely assess drilling waste fluids, which must pass the Microtox bioassay before disposal on land in western Canada. These fluids can be dark brown, with turbidity that sometimes cannot be entirely removed even by high speed centrifugation. In any case, whole fluid testing would be preferable, since some toxicity may be associated with suspended particles.

The aim of our work was to develop a color-correction method that would avoid unexplained factors and formulas, and at the same time be capable of handling turbid samples.

EQUIPMENT

We used the standard Microtox™ analyzer (Model 500, available from SDI Inc.), which has thirty wells, arranged in six rows, maintained at 15 ºC. Batches of freeze-dried *V. fischeri*, Reconstitution water, Osmotic Adjustment solution (22 % NaCl) and Diluent (2% NaCl), were obtained from SDI via Osprey Scientific Inc., as well as the cylindrical cuvettes that fit the wells.

Cuvettes are automatically lowered past an optical shutter into the "Read" well of the Model 500, then returned after the reading has been made. Double-walled cuvettes were made from regular cuvettes, but modified for our study in that the inner tube is supported near its open end and stops 5 mm above the floor (Fig. **1**). All light from bacteria in the inner tube must pass through the outer compartment to the detector in the Read well.

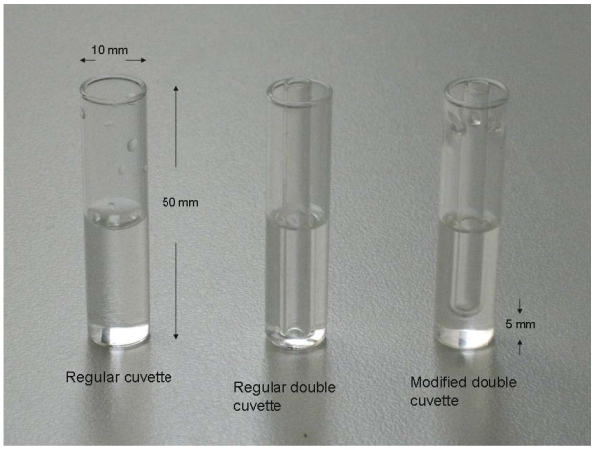

Fig. (1). Test cuvettes.

Separate light absorbance and turbidity measurements were also made on test fluids. Absorbance at 490 nm was measured in a 1 cm path length cell, using a DR/4000U spectrophotometer (Hach Instruments Inc.). Turbidity (90 degree scattering) was measured in 30 mL vials using a Micro 100 Turbidimeter (HF Instruments).

SAMPLE PREPARATION AND PROPERTIES

We tested various fluids, ranging in optical absorbance, turbidity and toxicity. Drilling waste fluids were samples submitted for routine testing by waste disposal contractors. Other clay suspensions were obtained by decanting 100 mL from settled whole soil suspensions (1 L) after particle size analysis by the hydrometer method [6].

A range of non-mineral, essentially clear, colored solutions was also tested. These included two water-soluble red-brown dyes ("Rust" and "Seal") obtained from fabric stores. A sample of caramel, made by heating moistened sucrose at 105 ºC, was re-dissolved in de-ionized water. Tea, coffee and red beet powder were steeped in hot de-ionized water, allowed to cool, and filtered before testing the extract. Soy sauce was diluted ten-fold in de-ionized water. Apple juice was brought to pH 7 by adding dilute NaOH. An acidic cola sample was treated with dilute NaOH, aerated at pH 11 to remove CO_2, then brought to pH 8 with dilute HCl.

The pH of test samples was in a range (5-8) that in itself has little effect on *V. fischeri* [7]; moreover, any pH-related toxicity is unrelated to light absorbance.

The soil suspensions and drilling waste fluids (15 in all) ranged in optical absorbance (Abs) from 0.6-2.8 units and in turbidity from 60-900 NTU. The other 12, non-mineral test solutions had Abs from 0.3-3.1 and were less turbid (1-250 NTU).

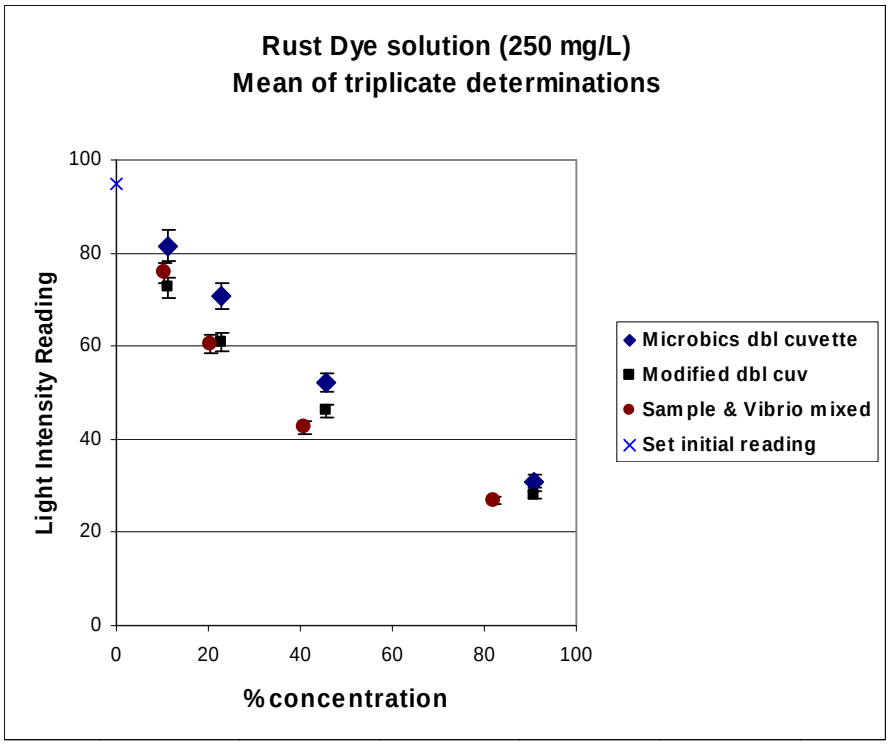

Fig. (2). Initial bacterial light intensity (Rust dye).

BIOASSAY PROCEDURE

Following the so-called increased sensitivity assay (ISA) version of the Microtox™ bioassay [7], each fluid was first mixed (10:1) with 22 % NaCl osmotic adjustment solution in one cuvette, then serially diluted (1:1) into 2 % NaCl in three others, giving 90.9, 45.5, 22.7, and 11.4 % concentrations of the raw test fluid in 2 % NaCl. After equilibration to 15 ºC, to start the bioassay 900 µL portions were transferred to four other cuvettes containing 100 µL aliquots of bacterial suspension, thus giving 81.8, 40.9, 20.5 and 10.2 % concentrations of the test liquid.

Aliquots of bacterial suspensions and test fluids were added to cuvettes and mixed by autopipet, and withdrawn from double cuvettes using a teat pipet fitted with 10 cm of plastic tubing (2 mm ext. dia.).

To check bacterial quality, a reference toxicity standard was made from A.C.S. grade phenol solution (88 %, EMD Chemicals) diluted in de-ionized water to 94 mg/L. An EC50(5) in the range 13-26 mg phenol/L is specified for batches of *V. fischeri* from SDI Inc. Selected solutions were also tested using a 48 h *Daphnia magna* bioassay [8].

LIGHT INTENSITY MEASUREMENTS.

To allow for natural decline in light output from bacteria during the hours following reconstitution of a batch from the freeze-dried state, light intensity readings (displayed by the Model 500) are automatically given an arbitrary value (95) by having the detector "set" one aliquot at the start of a test [2, 3]; light output varies a little among aliquots and drifts naturally during a run.

In our tests, initial light readings were always in the range 80-105. Readings were closely similar, whether a 100 µL aliquot was in a regular cuvette (just covering its floor), or in the inner tube of a modified double-walled cuvette (6-15 mm above the floor).

Light readings of course fell when light-absorbing fluids were placed in the outer compartment of the latter, though not when 2 % NaCl was used.

Light intensity I_e with an empty outer compartment was equated to the mean of two readings taken before and after a reading I_f made after adding test fluid to the 15 mm level. The 1.25 mL needed to fill the compartment to that level, so as to envelop 100 µL in the inner tube, was transferred from a regular cuvette containing 1.5 mL at 15 ºC. Filling to 20 mm rather than 15 mm did not affect I_f.

Light Loss Quotient, Q

Our modified color-correction procedure involves a new factor (> 1) which we will call Q, defined as:

$$Q = I_e / I_f \quad (7)$$

After measuring Q, each dilution level of the raw test fluid was returned to its regular cuvette. Each dilution and a control were then tested, following the ISA procedure described above. In addition to pre-mixing readings, light readings were taken starting within 30 s of mixing sample and bacteria, and continuing for up to 15 min.

When test solutions were mixed with bacteria, readings fell from their pre-mixing levels very sharply at first, but changed more slowly over the next few minutes. Initial post-mixing light readings were usually much lower than the corresponding readings made in modified double cuvettes. Lower readings for the mixtures indicated that, as well as absorbing some emitted light, the fluid was toxic to *V. fischeri*.

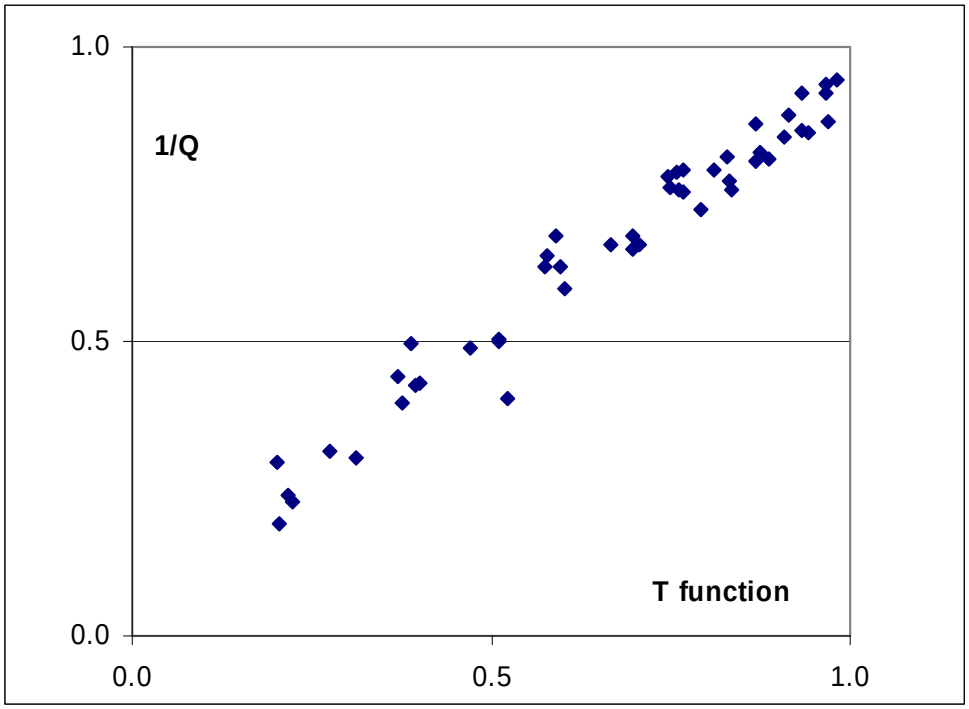

Fig. (3). Inverse relationship of Q and T (non-turbid fluids).

An aqueous solution of the "Rust" dye (optical absorbance = 3.10) was the only colored fluid tested that gave similar light intensity readings in the Model 500, whether diluted samples were mixed with an aliquot of bacteria or kept separate in a modified double cuvette. The two sets of resulting data points (Fig. **2**) indicate little toxicity of the Rust dye to *V. fischeri*, in the initial minutes following mixing. At the same concentration (250 mg/L) it was mildly toxic to *Daphnia magna* (IC50 = 70 %) after 48 h.

The same serial dilutions of Rust dye in the outer compartment of a regular double cuvette [2] gave consistently higher light readings (Fig. **2**) suggesting that, with an inner tube attached at the floor of the cuvette, bacterial light can leak to the detector through the base.

Modified Color-Correction Formula

Judging from the neighboring data points in Fig. (**2**), light loss in a modified double cuvette matches that portion of loss due solely to light absorbance, when sample and bacteria are mixed in a regular cuvette. Support for this assumption is in Fig. (**3**), which shows that, for our 12 mainly clear fluids, values of T at each dilution level (calculated from spectrometer absorbance by the Azur method using Eqs. 4 & 6) were essentially the same as the reciprocal of the corresponding values of Q.

We surmised that I_o values could therefore be adjusted for light absorbance as in the customary Eq. (3), but by dividing I_o by the determined factor Q instead of multiplying it by the evaluated factor T. Corrected values of Gamma (also corrected as usual for any change in control light output during the run) would then be calculated as follows:

$$Gamma_t = [\ Control\ (I_t/I_o) \times Sample\ I_o/(Q.I_t)\ ] - 1 \qquad (8)$$

Table **1** includes color-corrected EC50 values for various red-brown fluids, obtained by plotting Gamma values calculated from Eq. (8) using values of Q, as well as from Eq. (3) using values of T obtained by the Azur method [3]. For fluids with low turbidity, both sets of corrected EC50 values agreed well.

Effect of Fluid Turbidity

A turbid clay suspension (300 NTU) made from pristine subsoil was tested in the way described above for Rust dye solution. Light loss (Fig. **4**) was again similar, whether sample dilutions and bacteria were mixed, or kept separate in a double cuvette. This finding again supports the view that the modified double cuvette gives readings that accurately match light loss caused by absorbance, when sample and bacteria are mixed. When centrifuged, the clay suspension was almost colorless (490 nm absorbance = 0.08) and caused little light reduction when mixed with bacteria (all readings > 85), confirming that it was non-toxic to *V. fischeri*. The raw clay suspension affected light readings far less than the Rust dye solution, despite giving a slightly higher absorbance reading (= 3.19). This result indicates that light absorbance due to turbidity cannot be corrected for in the same way as that due to color.

The detrimental effect of high turbidity on the Azur method of color-correction is illustrated by results for drilling waste A, a brown, turbid fluid with Abs = 2.85 and turbidity = 415 NTU (Table **1**). In this case, light intensity readings in regular cuvettes (with sample and bacteria mixed) were only about half the corresponding readings obtained with modified double cuvettes, indicating considerable toxicity.

Despite this, the Azur method of correcting Model 500 data resulted in Gamma values < 1 at all dilution levels of this fluid (thus all $\log_{10}$ Gamma values < 0) and a very weak correlation ($r^2 = 0.27$) between log Gamma and log concentration (Fig. **5**). Such plots are obtained with genuinely non-

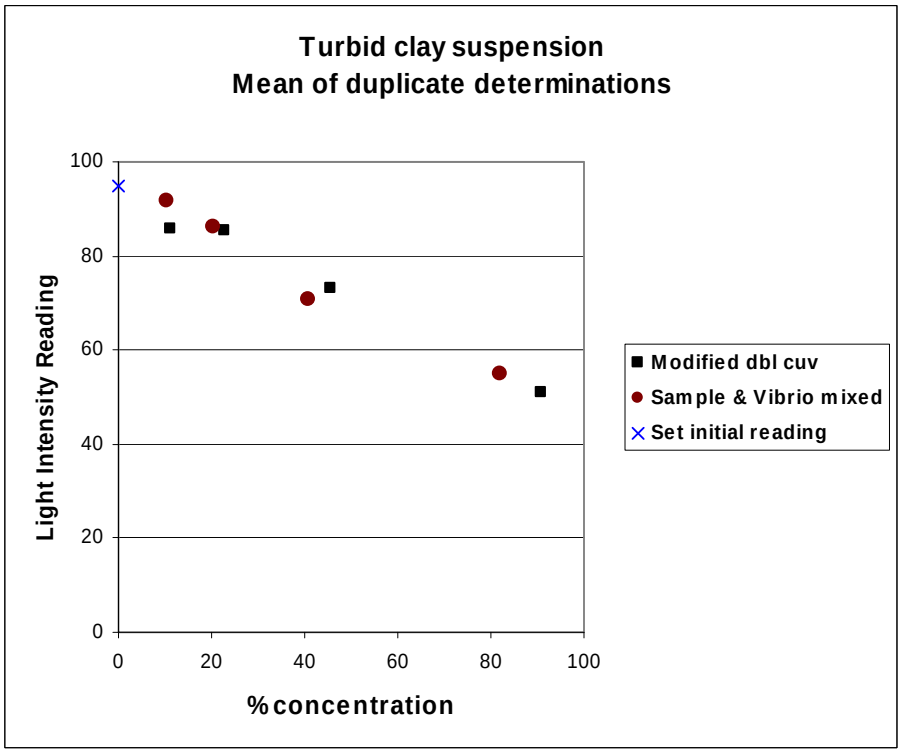

Fig. (4). Initial bacterial light intensity (turbid suspension).

Table 1. Toxicity of Fluids to *V. fischeri*, Corrected by Two Methods

Test fluid	Raw Fluid Properties		Microtox EC50 Values (%) [a]			48 h Daphnia IC50 (%)
	Abs (490 nm)	NTU	Uncorrected	Corrected		
				Eq. 3	Eq. 8	
"Seal" dye	3.05	< 5	12	27	22	19
Beet extract	2.87	250	10	24	21	25
Caramel	0.35	< 5	56	66	65	61
"Rust" dye	3.10	< 5	20	> 100 [b]	> 100 [b]	70
Tea	0.93	115	8	8	9	Not determined on these fluids
Coffee	1.93	70	2	1	2	
Apple juice	0.33	< 5	47	67	71	
Soy sauce	2.80	46	4	3	5	
Cola	0.97	13	41	> 100 [b]	> 100 [b]	
Drilling waste A	2.85	415	10	> 100 [b]	24	
Drilling waste B	1.48	180	12	14	13	
Soil suspension	1.75	65	34	> 100 [b]	> 100 [b]	

[a] At 5 min., except for tea, coffee & apple juice (1 min.); [b] All Gamma values < 1 in these cases.

toxic, non-absorbing samples (e.g. pure water) but, as noted, this fluid was undoubtedly toxic.

In these cases SDI's Omni™ software (which uses the Azur method) prints a default message that, due to the weak correlation, no EC50 value can be reported. In contrast, Gamma values corrected using Eq. (8) remained strongly correlated with concentration (r^2 = 0.97), resulting in a plausible EC50 intercept at Gamma = 1 (log Gamma = 0) indicating significant toxicity.

The Azur method fails with turbid samples, apparently because light scattering by suspended particles reduces spectrometer transmittance at 490 nm, so that the calculated

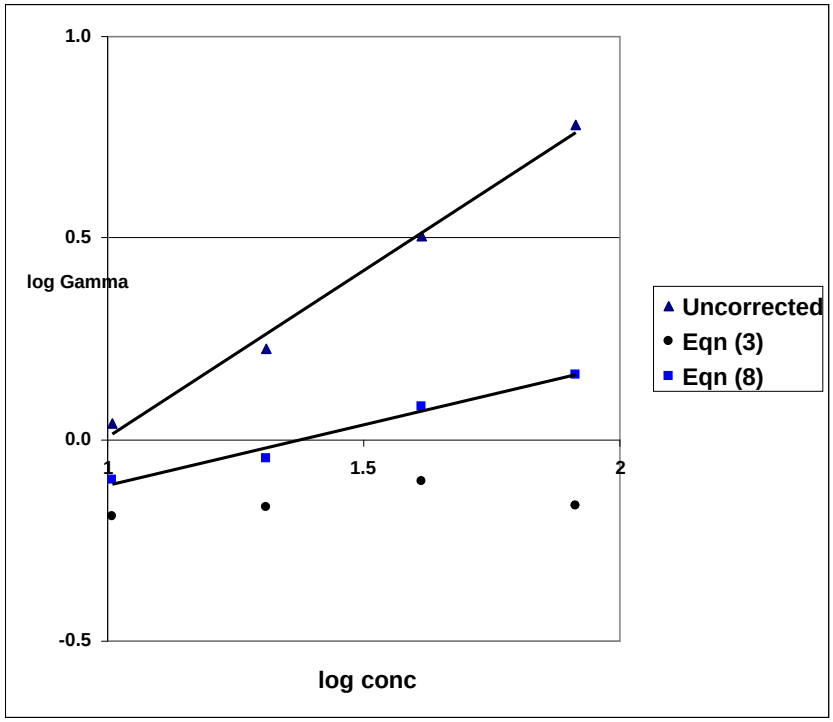

Fig. (5). Microtox™ data for a colored, highly turbid sample.

transmittance factor T is too small, resulting in over-correction of Gamma values using Eq. (3).

Combined Effect of Color and Turbidity On Light Reduction

Using multiple regression (Microsoft Excel™), values of the light loss quotient Q, at each of the four ISA dilution levels, were regressed against corresponding Abs and turbidity (NTU) values measured separately for all 27 tested fluids. The resulting intercept of 1.05 was forced to unity (with virtually no change in R^2) by regressing values of (Q-1) and putting the constant = 0, so as to have Q = 1 exactly (i.e. no light reduction) for a clear, colorless sample. The equation thus obtained was:

$$Q = 1.00 + 1.10(Abs) - 0.0019(NTU) \quad (9)$$

Eq. (9) accounted for almost all of the variance in light reduction observed (R^2 = 0.93, n = 108), despite the wide-ranging nature, absorbance and turbidity of the test fluids. This correlation indicates that light loss using a modified double cuvette represents light loss due to absorbance alone, when sample and bacteria are mixed, for any combination of fluid color and turbidity. Such a strong correlation is unlikely to be a mere artifact of cuvette design.

The negative NTU co-efficient in Eq. (9) indicates that when part of the overall absorbance is due to turbidity, light reduction in the Model 500 will be less than for a clear, colored fluid with the same measured Abs value; to assume that turbidity acts essentially like color in reducing light readings [5] will lead to over-correction, as noted.

Clear Fluids

The effect of turbidity is small for test fluids with < 100 NTU. The equation Q = 1.00 + 1.12(Abs) was a close fit (r^2 = 0.91, n = 48) to double-cuvette data for the 12 non-mineral fluids, ten of which had < 100 NTU (Fig. **6**). This strong correlation leads to the near-equivalence of values of T and 1/Q previously noted for these fluids, and illustrates the relationship seen with clear, colored fluids between spectrometric light transmittance at 490 nm and loss of detectable light in the Model 500, which underlies the Azur color-correction procedure [3].

CONCLUSIONS

The Azur color correction method [3] cannot accurately adjust for light loss due to sample turbidity, which has a different effect on bacterial light absorbance than red-brown color.

Light intensity measurements made with a weakly toxic, red-brown clear solution and an apparently non-toxic, minimally colored turbid suspension indicated that the modified double cuvette adequately predicts bacterial light loss due to optical absorbance alone, when such fluids are mixed with *V. fischeri* in a regular cuvette, in the Model 500.

Using multiple regression, values of the light loss quotient Q obtained using the modified double cuvette for a wide range of fluids, with both color and turbidity, were very strongly correlated with their measured optical absorbance and NTU values. This result suggests that light readings made with the modified double cuvette also adequately predict bacterial light loss caused by fluids having any combination of color and turbidity.

Confirmation of this assumption will require toxicity assessment of whole fluids, using other biota besides *V. fischeri*. The ability to correct for the effects of significant turbidity as well as color will allow toxicity assessment of whole fluids, which is more appropriate than testing a clarified sample and indeed avoids the need for sample clarification [4].

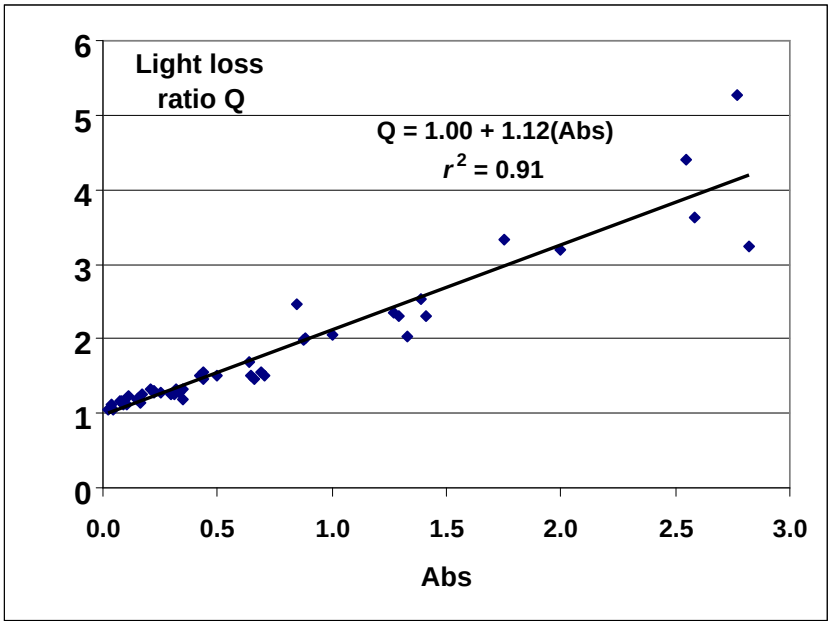

Fig. (6). Light reduction by 12 red-brown fluids with low turbidity.

Values of Q (= I_e/I_f) for insertion in Eq. (8) can be directly obtained using a modified double cuvette, avoiding complex formulas and empirical factors, and without needing additional spectrometric or turbidimetric measurements; toxicity assessment can therefore be done on-site.

APPENDIX: Q MEASUREMENTS

Values of Q at the actual sample concentrations tested (the ISA dilutions, defined above) were obtained from quadratic equations fitted to the values obtained at the four slightly higher pre-mixing concentrations. This approach allowed the same sample of fluid to be both assayed and color-corrected in one run, and was convenient with a wide range of test fluids, all needing color-correction.

In routine testing, with individual such cases, an alternative is to measure Q on a separate sample, using the actual test concentrations. When using the ISA procedure [7], mixing 2.22 mL of test fluid into a cuvette holding 0.25 mL of 2 % NaCl (Diluent) and 0.25 mL of 22 % NaCl (Osmotic Adjustment solution) provides almost exactly the highest ISA test concentration. Then for the other test levels, 1.30 mL portions can be serially diluted into cuvettes with 1.30 mL of Diluent.

As noted, to obtain Q, light intensity is read with and without 1.25 mL of each serial dilution present in the outer compartment of a modified double cuvette with 100 µL of *V. fischeri* in the inner tube. The detector can be re-set at the start of a set of readings at any dilution level, if I_e has decayed well below 95. It is important for all cuvette contents to be at 15 ºC, and to leave the modified double cuvette in the uncontrolled Read well only while actually taking or setting a reading.

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REFERENCES

[1] Munkittrick KR, Power EA, Sergy GA. The relative sensitivity of Microtox, Daphnid, Rainbow Trout and Fathead Minnow acute lethalitytests. Environ Toxicol Water Qual 1991; 6: 35-62.

[2] Microtox Manual. Microbics Corporation. Carlsbad: CA, USA 1992.

[3] Azur Environmental. Carlsbad: CA, USA. Omni™ Software for Windows. 1999. Available from: http://www.sdimicrotox.com

[4] Drilling Waste Management. Guide G-50. Energy Resources Conservation Board, Calgary: Alberta, Canada 1996

[5] Biological Test Method: toxicity test using luminescent bacteria. Report EPS 1/RM/24. Environment Canada. Ottawa, Ontario: Canada 1992.

[6] Kroetsch D, Wang C. Soil sampling and methods of analysis. In: Carter MR, Gregorich EG, Eds. Particle size distribution. Hydrometer method. Canadian Society of Soil Science. Ottawa: Canada 2008; pp. 720-2.

[7] Gaudet I, Ed. Standard procedures for microtox analysis. Alberta Environmental Centre: Vegreville, Alberta. AECV94-G1. Available from: http://www.wcmuc.com

[8] Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*, EPS 1/RM/14. 2nd ed. Environment Canada: Ottawa, Ontario, Canada 2000.

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