Bull. Environ. Contam. Toxicol. (2004) 72:1187–1194

DOI: 10.1007/s00128-004-0369-1

Bulletin of Environmental Contamination and Toxicology

Evaluation of the Shk1 Activated Sludge Bacterial Luminescence Inhibition Assay: Narcotic Chemicals

S. Ren,[1,3] T. W. Schultz,[2,3] P. D. Frymier[1,3]

[1] Department of Chemical Engineering, 419 Dougherty Engineering Building, Knoxville, TN 37996-2200, USA
[2] Department of Comparative Medicine, 2407 River Drive, Knoxville, TN 37996-4543, USA
[3] Center for Environmental Biotechnology, 676 Dabney Hall, The University of Tennessee, Knoxville, TN 37996-1605, USA

Received: 15 October 2003/Accepted: 15 April 2004

The use of luminescent bacteria as sensor organisms for monitoring toxicity to wastewater treatment facilities (WWTFs) has been reviewed in a Water Environment Research Foundation report (Love and Bott 2000). While a toxicity monitoring system that operates in an online and continuous manner is preferred, a system that operates in a batch mode may be sufficient and appropriate in some cases. However, currently available single species aquatic toxicity assays such as Microtox® are deemed inappropriate as they are more sensitive to acute toxic insults than consortium-based endpoints such as activated sludge respiration inhibition assays. In an effort to address this discrepancy, researchers in the Center for Environmental Biotechnology at The University of Tennessee (UT-CEB) have been developing systems based on genetically-engineered luminescent bacteria. A continuous system that employs the luminous bacterium "Shk1" has been described (Ren and Frymier 2003). The parent strain of Shk1 was an activated sludge bacterium isolated from an industrial WWTF (Kelly et al. 1999). Compared to other luminescent organisms, Shk1 is considered a "significant advantage" for assessing toxicity to activated sludge because of its origin (Love and Bott 2000). Parallel to the continuous system, Lajoie et al. (2002) developed a batchwise assay based on Shk1 for toxicity assessment of grab samples.

In the present study, we determined the toxicity of twenty narcotic chemicals in batch mode with an assay protocol modified from the one described in Lajoie et al. (2002) using the Shk1 strain. The quality of the resultant toxicity data was evaluated by the development of quantitative structure-activity relationships (QSARs). While the primary function of ecotoxicity QSARs is to predict toxicity, QSARs can also be used to provide assurance of the quality of experimental data (Comber et al. 2003). After confirming data quality, the appropriateness of this strain as a surrogate for wastewater treatment facilities was examined with the toxicity data of the Shk1 assay being compared with data of two other assays frequently used for assessing wastewater toxicity (*Vibrio fischeri* luminescence inhibition assay and activated sludge respiration inhibition assay).

MATERIALS AND METHODS

The Shk1 bacterium was obtained from the culture collection of UT-CEB. Nutrient broth (NB) (Fisher Scientific, Atlanta, GA, USA) was used as the growth

Correspondence to: S. Ren, Gradient Corporation, 238 Main Street, Cambridge, MA 02142, USA

medium. As Shk1 is tetracycline-resistant (Kelly et al. 1999), NB medium was amended with 25 mg/L tetracycline after sterilization and before use.

The assayed compounds (Table 1) consisted of eleven nonpolar narcotic and nine polar narcotic chemicals (Sigma-Aldrich, Milwaukee, WI, USA). These chemicals were selected based on the following criteria: they span a range of hydrophobicity sufficient for QSAR analysis, toxicity data are available from other assay systems for comparative purposes, and they are common wastewater constituents. All chemicals were of sufficient purity and further purification was not needed. Stock solutions were prepared in de-ionized water. For some phenols with low solubility, acetone was used as the carrier. Two-fold dilutions of the stock

Table 1. Organic chemicals, hydrophobicity, and toxicity ($\log 1/EC_{50}$, mM^{-1}).

Chemical	MOA[1]	log K_{ow}[2]	Shk1	*Vibrio fischeri*[4]	Activated sludge[5]
1-propanol	1	0.25	-2.22	-2.22	-2.26
1-butanol	1	0.88	-1.94	-1.44	-
1,2-dibromoethane	1	1.96	-1.09	-0.59	-0.83
1-hexanol	1	2.03	-1.05	0.68	-
benzene	1	2.13	-0.81	1.59	-1.10
trichloroethylene	1	2.42	-0.50	0.13	-0.77
1,2,3-trichloropropane	1	2.50[3]	-0.45	0.90	-
1,4-dichlorobutane	1	2.81[3]	-0.66	0.31	-
ethylbenzene	1	3.15	0.053	1.29	-0.32
1,5-dichloropentane	1	3.30[3]	0.13	0.95	-
1,2-dichlorobenzene	1	3.43	0.36	1.73	0.48
3-cresol	2	1.96	-0.038	1.20	-0.61
4-chlorophenol	2	2.39	1.26	2.13	0.09
4-bromophenol	2	2.64	0.66	2.63	-
2,6-dichlorophenol	2	2.75	0.69	1.23	0.39
1-naphthol	2	2.85	0.73	1.59	-
2,4-dichlorophenol	2	3.06	1.04	1.85	0.78
4-chloro-3-methylphenol	2	3.10	1.48	2.72	-
3-phenylphenol	2	3.23	1.12	3.23	-
2,4,5-trichlorophenol	2	3.72	1.85	2.22	0.93

[1] Mechanism of action: 1 = nonpolar naoprcosis, 2 = polar narcosis.

[2] Hansch and Leo (1995).

[3] Calculated with $LogK_{ow}$ (KowWin), Syracuse Research Corporation, Syracuse, NY.

[4] Kaiser and Devillers (1994). Data are based on 5-minute tests.

[5] Data taken from Sun et al. (1994).

solutions of assay compounds were prepared to achieve suitable test concentrations after range-finder experiments. At least five concentrations of each assay compound in addition to a control (de-ionized water) were tested. Four replications for each of the compounds were conducted.

Shk1 was grown in 1 L flasks with 100 mL NB medium in an oscillating incubator at 30°C and 200 rpm. When optical density (OD) measured with a spectrophotometer (DU® 520, Beckman Coulter Inc., Fullerton, CA) at 600 nm reached a value of 1.0, cell solutions were transferred to centrifuge bottles. Cells were then separated from the NB medium by centrifugation (Beckman J2-21, Beckman Coulter, Inc.) at 14,000*g* and 4°C for 20 minutes. The liquid supernatant after centrifugation was discarded. The cell pellets were re-suspended in 4°C 0.1 *M* potassium chloride solution to reach an OD of 1.0. The re-suspended cells were stored in a refrigerator at 4°C overnight. Note that the original protocol described in Lajoie et al. (2002) does not require separation between cells and NB medium. However, this was conducted in the present study because of concerns of interference that might be caused by the dark color of NB medium. The effects of color on bioluminescence-based assays had been investigated by Lappalainen et al. (2001).

Refrigerated Shk1 cells were activated prior to use by placing cells in a waterbath set at room temperature for 20 minutes. A volume of 0.1 mL activated cells was mixed with 0.9 mL of an assay compound solution to achieve a total test volume of 1.0 mL. A Wallace Victor Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA, USA) was used for luminescence measurements performed at room temperature. Luminescence of the control was measured before and after a 7-minute exposure. Data were recorded for further analysis only if the control luminescence did not shift more than 5%.

The toxicological endpoint was luminescence repression (*LR*) calculated according to Eq. [1].

$$LR = \frac{Lc - Lt}{Lc} \qquad \text{Eq. [1]}$$

In Eq. [1], *Lc* is luminescence of cells exposed to de-ionized water (i.e., control luminescence), and *Lt* is luminescence of cells exposed to an assay compound (both measured after the seven-minute exposure time). The results from the four replicates were combined and *LR* was plotted versus assay compound concentration to produce concentration-response curves. EC_{50} values (in m*M*) were obtained by a least squares regression of the relatively linear portion of the concentration-response curves. Toxicity data were reported as log ($1/EC_{50}$).

QSARs based on the logarithm of the octanol/water partition coefficient (log K_{ow}) were developed for compounds with the nonpolar and polar narcosis mechanism, respectively. As such, regressions were conducted using toxicity data as the dependent variable and log K_{ow} (shown in Table 1) as the independent variable. JMP 5.0 (SAS Institute, Cary, NC, USA) was used for this purpose. A compound

with a semi-studentized residual of greater than four was judged to be an outlier (Neter et al. 1996). After eliminating outliers (if any), regression was repeated and the final model was obtained with number of observations (n), coefficient of determination (r^2), and root mean square error (RMSE) noted. Considering the small data size, validation of the QSAR models was conducted by the leave-one-out cross-validation method. The values of coefficient of determination for cross-validation (r^2_{cv}) were reported as a measure of model validity.

As Ribo (1997) pointed out, one of the first steps in validating a new assay is to compare its results with the results of existing assays. Considering the applications of the Shk1 assay (wastewater toxicity assessment), the *V. fischeri* luminescence inhibition (i.e., Microtox®) and the activated sludge respiration inhibition assays were logical choices for comparison. To this end, toxicity data for these assays were obtained from the literature (shown in Table 1). For the activated sludge respiration inhibition assay, data availability is an issue and only a limited number of chemicals in Table 1 have respirometry data. For the *V. fischeri* luminescence inhibition assay, data quality should be considered. The toxicity data to *V. fischeri* shown in Table 1 are obtained from various sources in the literature. Concerns about the quality of such compiled data have been raised (Schultz and Cronin 2003). Taking into account data availability and quality, no quantitative comparisons between the Shk1 and *V. fischeri*/activated sludge respirometry assays (i.e., inter-species correlations), but rather, only qualitative comparisons were made.

RESULTS AND DISCUSSION

The toxicity of the twenty assay compounds (shown in Table 1) spans more than four log units. A plot of toxicity versus hydrophobicity (log K_{ow}) for the twenty assay compounds is displayed in Figure 1. Clearly, the twenty assay compounds form two "clusters" according to mechanism of action. Nonpolar narcotic compounds form a group based on which the baseline toxicity to Shk1 is later defined. Polar narcotic compounds form another group and show toxicity higher than the corresponding baseline toxicity.

Log K_{ow}-dependent QSARs for nonpolar and polar narcotic toxicity to Shk1 are established and shown below.

Nonpolar narcosis

$$\text{Log } (1/EC_{50}) = -2.57\ (0.13) + 0.81\ (0.05) \log K_{ow}$$

$$n = 11,\ r^2 = 0.96,\ r^2_{cv} = 0.94,\ \text{RMSE} = 0.16 \qquad \text{Eq. [2]}$$

Numbers in parentheses are the standard errors for the intercept and the slope, which are both statistically significant at a 5% significance level. No outliers were found to Eq. [2]. The high r^2 and low RMSE values suggest the high quality of fit. The high r^2_{cv} value indicates that the model is robust and offers high prediction

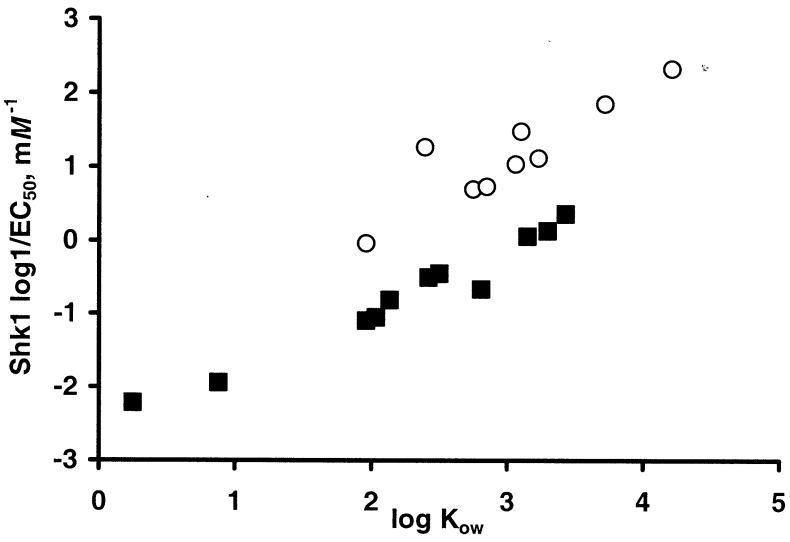

Figure 1. Observed toxicity to Shk1 versus hydrophobicity. Solid squares: nonpolar narcotics; open circles: polar narcotics.

accuracy. Therefore, Eq. [2] adequately provides a model for the baseline toxicity to Shk1.

Polar narcosis

$$\text{Log } (1/EC_{50}) = -2.18\ (0.31) + 1.07\ (0.10) \log K_{ow}$$

$$n = 8,\ r^2 = 0.95,\ r^2_{cv} = 0.94,\ \text{RMSE} = 0.17 \qquad \text{Eq. [3]}$$

Again the numbers in parentheses are the standard errors for the intercept and the slope, which are both statistically significant at a 5% significance level. 4-Chlorophenol was found to be an outlier and was not included in the development of the final model (Eq. [3]). The quality of model fit and prediction of Eq. [3] is similar to that of Eq. [2], with comparable r^2, r^2_{cv}, and RMSE values. Thus, Eq. [3] adequately describes the toxicity of polar narcotic compounds to Shk1.

The successful development of Eqs. [2] and [3] provides assurance that the experimental data obtained with the batchwise Shk1 assay are of good quality. Thus, expansion of the database with this assay is warranted.

Of note in comparing Eqs. [2] and [3] is that while Eq. [3] has a larger intercept, which is expected, Eq. [3] also has a larger slope than Eq. [2]. This is not consistent with most QSARs developed for other aquatic organisms such as fish (e.g., *Pimephales promelas*), protozoa (e.g., *Tetrahymena pyriformis*), and bacteria (e.g., *V. fischeri*) (Schultz et al. 1998). The distinction between nonpolar and polar narcosis has been an area of research. The toxicity of narcotic chemicals is elicited

by reversible physiological alterations at the cell membrane. Nonpolar narcotic chemicals are considered baseline toxicants. Their toxicity is proportional to their concentrations at the site of action and is caused solely by membrane perturbation (Veith and Broderius 1990). Polar narcotic chemicals, typified by most phenols and anilines, exhibit toxic potency higher than that estimated by their hydrophobicity due to the existence of polar substituents in the molecules. The polar groups give polar narcotic chemicals greater dipolarity and hydrogen bonding/donor capacity, which are considered to contribute to the increased toxicity of polar narcotics (Kamlet et al. 1986).

Despite clearly different fish acute toxicity syndromes exhibited by nonpolar and polar narcotic chemicals (McKim et al. 1987), some researchers suggest that the difference between nonpolar and polar narcosis in log K_{OW}-based QSAR modeling is simply an artifact of the inadequacy of log K_{OW} to quantify the partition between the cell membrane and the aqueous phase (Urrestarazu Ramos et al. 1998). Recently, Roberts and Costello (2003) proposed that nonpolar and polar narcoses are actually two separate mechanisms and the distinction can be attributed to differences in membrane partition.

Adding discussions to the topic of the distinction between nonpolar and polar narcosis was not the intention of the present study. However, the literature does suggest that membrane partitioning is a key factor for eliciting a narcotic effect. Membrane partitioning may help explain the inconsistency between the relative magnitudes of the slopes in Eqs. [2] and [3] and those in QSARs for other organisms. It is necessary to point out that, while not very common, a larger slope in a log K_{OW}-based QSAR for polar narcotic chemicals than that for nonpolar narcotic chemicals has also been observed for *Nitrobacter*, an activated sludge bacterium (Tang et al. 1992).

It is interesting to note that both Shk1 and *Nitrobacter* are representative of activated sludge organisms. Compared to other species such as *P. promelas*, activated sludge organisms have been subject to fewer toxicological studies. Consequently, many aspects of the toxicity to activated sludge organisms remain to be elucidated. It is possible, however, that the cell membrane of Shk1 (and *Nitrobacter*) differs in some way, e.g., fatty acid content, from that of other aquatic species, and this difference may affect the partition of narcotic chemicals (polar narcotics, in particular) and thus may account for the observed greater slope (and smaller intercept when applicable) in the log K_{OW}-based QSAR for polar narcotic chemicals. Naturally, assay conditions such as temperature may have an important impact on membrane composition and configuration. More research is certainly needed in this regard. Nevertheless, the quality of the Shk1 data has been assured, as discussed previously.

Data in Table 1 indicate that nearly all the chemicals tested exhibit higher toxicity to *V. fischeri* than to Shk1. The exception is 1-propanol that shows equal toxicity to these organisms. Thus, the Shk1 assay is in general less sensitive than the *V. fischeri* assay, which is desired for the purpose of wastewater toxicity monitoring

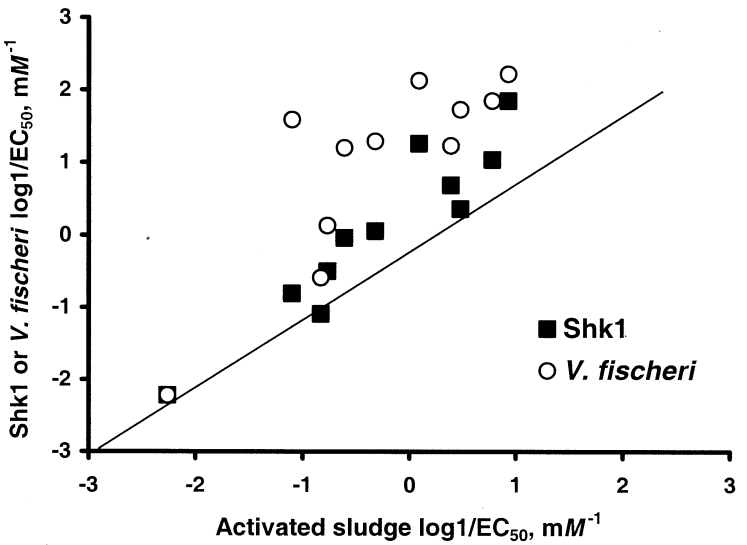

Figure 2. Comparison between Shk1/*V. fischeri* and activated sludge toxicity data.

as the latter assay has been found to be overly sensitive. Comparisons between the Shk1/*V. fischeri* luminescence inhibition and activated sludge respiration inhibition data are graphically displayed in Figure 2. Compared to *V. fischeri* data, most Shk1 data points are closer to the solid line in Figure 2 representing y = x. It is evident, therefore, that Shk1 data are more similar to activated sludge respiration inhibition data for these compounds, suggesting the appropriateness of the use Shk1 and the Shk1 assay for assessing wastewater toxicity to activated sludge.

Acknowledgements. Gratitude is expressed to Dr. Curtis Lajoie in the Department of Chemical Engineering and Materials Science at Syracuse University for discussions on the batchwise Shk1 assay protocol.

REFERENCES

Comber MHI, Walker JD, Watts C, Hermens J (2003) Quantitative structure-activity relationships for predicting potential ecological hazard of organic chemicals for use in regulatory risk assessments. Environ Toxicol Chem 22:1822-1828

Hansch C, Leo A (1995) Exploring QSAR: Fundamentals and Applications in Chemistry and Biology. American Chemical Society, Washington, DC

Kaiser KLE, Devillers J (1994) Ecotoxicity of chemicals to Photobacterium phosphoreum. Handbook of Ecotoxicology Data. Volume 2. Gordon and Breach Science, Longhorne, PA

Kamlet MJ, Doherty RM, Taft RW, Abraham MH (1986) Solubility properties in polymers and biological media. 7. An analysis of toxicant properties that

influent inhibition of bioluminescence in *Photobacterium phosphoreum* (The Microtox Test). Environ Sci Technol 20:690-695
Kelly CJ, Lajoie CA, Layton AC, Sayler GS (1999) Bioluminescence reporter bacterium for toxicity monitoring in biological wastewater treatment systems. Water Environ Res 71:31-35
Lajoie CA, Lin SC, Nguyen H, Kelly CJ (2002) A toxicity testing protocol using a bioluminescent reporter bacterium from activated sludge. J Microbiol Meth 50:273-283
Lappalainen J, Juvonen R, Nurmi J, Karp M (2001) Automated color correction method for *Vibrio fischeri* toxicity test. Comparison of standard and kinetic assays. Chemosphere 45:635-641
Love NG, Bott CB (2000) A review and needs survey of upset early warning devices. Water Environment Research Foundation, Alexandria, VA
McKim JM, Bradbury SP, Niemi GJ (1987) Fish acute toxicity syndromes and their use in the QSAR approach to hazard assessment. Environ Health Perspect 71:171-186
Neter J, Kuter MH, Nachtscheim CJ, Wasserman W (1996) Applied linear regression models. McGraw-Hill/Irwin, Chicago, IL
Ren S, Frymier PD (2003) The use of a genetically-engineered *Pseudomonas* species (Shk1) as a bioluminescent reporter for heavy metal toxicity screening in wastewater treatment plant influent. Water Environ Res 75:21-29
Ribo JM (1997) Interlaboratory comparison studies of the luminescent bacteria toxicity bioassay. Environ Toxicol Water Qual 12:283-294
Roberts DW, Costello JF (2003) Mechanisms of action for general and polar narcosis: a difference in dimension. QSAR Comb Sci 22:226-233
Schultz TW, Cronin MTD (2003) Essential and desirable characteristics of ecotoxicity quantitative structure-activity relationships. Environ Toxicol Chem 22:599-607
Schultz TW, Sinks GD, Bearden AP (1998) QSAR in aquatic toxicology: a mechanism of action approach comparing toxic potency to *Pimephales promelas*, *Tetrahymena pyriformis*, and *Vibrio fischeri*. In: Devillers J (ed) Comparative QSAR. Taylor & Francis, New York, p 51
Sun B, Nirmalakhandan N, Hall E, Wang XH, Prakash J, Maynes R (1994) Estimating toxicity of organic chemicals to activated-sludge microorganisms. J Environ Eng 120:1459-1469
Tang NH, Blum DJW, Nirmalakhandan N, Speece RE (1992) QSAR parameters for toxicity of organic chemicals to *Nitrobacter*. J Environ Eng 118:17-37
Urrestarazu Ramos E, Vaes WHJ, Verhaar HJM, Hermens JLM (1998) Quantitative structure-activity relationships for the aquatic toxicity of polar and nonpolar narcotic pollutants. J Chem Inf Comput Sci 38:845-852
Veith GD, Broderius SJ (1990) Rules for distinguishing toxicants that cause type I and type II narcosis syndrome. Environ Health Perspect 87:207-211