Bull. Environ. Contam. Toxicol. (2003) 70:1–8

DOI: 10.1007/s00128-002-0148-9

Bulletin of Environmental Contamination and Toxicology

Limitations of a Cosolvent for Ecotoxicity Testing of Hydrophobic Compounds

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Received: 29 March 2002/Accepted: 20 September 2002

Biological assays or sensors are a valuable complement to traditional chemical analysis for assessment of contaminated soils and sediments. Biological tests respond to the total range of pollutants found at a site, and give a single measure that can integrate the different contributions of the individual contaminants to the total toxic load (Pankhurst et al. 1998). Bioluminescent microbial bioassays are rapid, sensitive, and inexpensive, and well suited to high throughput analysis of many hundreds of samples. They are thus ideal for contaminated site characterization and monitoring (Boyd et al. 1997). The naturally bioluminescent marine bacterium *Vibrio fischeri* (the well-known Microtox® test) is widely used and most appropriate for ecotoxicity assessment of marine samples, whereas terrestrial bacteria that have been genetically modified to contain the *lux* genes for bacterial bioluminescence have been used for assessment of contaminated soils (Paton et al. 1997).

Toxicity testing of soils will frequently require some kind of extraction of the soil. A "solid phase" test could be used, in which the contaminated soil is shaken with water and mixed with the microbial strain to be used (Brouwer et al. 1990). However, if the soil were contaminated with hydrophobic organic compounds (HOCs), the bacterial toxicity test would be unlikely to "see" the contaminants. Several workers have reported that a solvent extract of contaminated sediments was able to elicit a greater toxic response than either an aqueous extract or elutriate or a solid-phase test (e.g. Demuth et al. 1993, Guzzella 1998). One sensible approach is to compare aqueous (or solid phase) and organic solvent extracts (Donnelly et al. 1991). A water-miscible cosolvent, or transfer solvent, may either be used to extract the soil directly, or to take up the residue after a previous extraction with another solvent. A large number of different studies have used dimethyl sulfoxide (DMSO) as a cosolvent (e.g. Wang and Bartha 1990, Ho and Quinn 1993, Bundy et al. 2001, Beg et al. 2001). Surprisingly, there is little information on how the use of a cosolvent may affect bioassay response, or on the selection of a co-solvent. A previous study focussed on the cosolvating ability rather than the effects on biological responses (Li et al. 1996). Sunahara et al. (1998) tested the effects of acetonitrile, DMSO, and acetone as cosolvents for ecotoxicity analysis of nitroaromatics using *V. fischeri*. However, only one

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compound (trinitrotoluene) was used for this comparison, and there was no attempt to explore the systematic effect of a cosolvent on a range of compounds. The aim of the work reported here was to evaluate the effect of using a model cosolvent, DMSO, on a *lux*-marked bacterial bioassay suitable for terrestrial ecotoxicity testing. We applied simple quantitative structure-activity relationships (QSARs) to provide a testable hypothesis of the systematic effects of a cosolvent on a series of compounds in ecotoxicity bioassays. Thus the null hypothesis was that the use of DMSO as a cosolvent would not affect the relationship between toxicity and log K_{ow}.

MATERIALS AND METHODS

The *lux*-marked bacterium *Escherichia coli* HB101 (pUCD607) was used, which expresses *lux* constitutively (Rattray *et al.* 1990). All assays were performed in triplicate. A freeze-dried vial was rehydrated in 10 ml of 100 mM KCl for 60 minutes (25 °C, 250 rpm). The rehydrated cell suspension (25 µl) was then added to the test solution (225 µl) in a microtitre plate, and luminescence measured after 15 minutes in a Lucy Anthos 1 luminometer (Labtech, Uckfield, UK). Luminescence measurements are expressed relative to controls, i.e. water for the aqueous test solutions and 10% DMSO for the cosolvent test solutions.

The assays were carried out in either in water, or in a 10% v/v DMSO/water solution. Standards were made up at nominal concentrations for the aqueous solutions by dissolving the compound below its aqueous solubility limit. Less soluble compounds (1,2-dichlorobenzene, decanone, and *n*-butylbenzene) were made up by shaking an excess of compound with water in a vial with a polytetrafluorolethylene (PTFE)-lined septum, and allowing the two layers to separate with the vial inverted. The test solution was then drawn off from the lower aqueous layer using a syringe. The compounds were assumed to be at their maximum aqueous solubility limit. Solutions in DMSO/water were made up by dissolving the compound in DMSO at 10 times the required concentration followed by dilution with water. At least five concentrations spanning the appropriate range plus control were used for each compound, and the concentration at which luminescence was inhibited by 50 per cent (EC_{50}) was calculated by exponential regression.

Log K_{ow} values are measured values and were taken from the ClogP for Windows v1.0 software, and are given in Table 1. The narcotics were selected such that they spanned a range of log K_{ow} values at regular intervals. Maximum aqueous solubility values are measured values and were taken from the online Syracuse Research Corporation PhysProp database (http://esc.syrres.com/interkow/physdemo.htm) and are given in Table 1 for the three compounds made up at their maximum aqueous solubility limit as described above.

RESULTS AND DISCUSSION

The toxicity values for the different compounds in the two different solvent systems are presented in Table 1. It can be seen that above a certain hydrophobicity cut-off, it is no longer possible to record an EC_{50}. Use of a co-solvent increases this cut-off: the most hydrophobic compound which elicited 50% reduction in luminescence for aqueous solutions alone was 1,2-dichlorobenzene, with a log K_{ow} of 3.38. Using DMSO as a cosolvent enabled values to be determined for an additional two compounds, *n*-decanone and *n*-butylbenzene, with log K_{ow} values of 3.77 and 4.38 respectively. More hydrophobic compounds were tested (dibutylphthalate, pyrene, and *n*-pentylbenzene, with log K_{ow} from 4.50 to 4.90) but failed to elicit an EC_{50} in either aqueous or cosolvent systems.

Two separate issues must be addressed for ecotoxicity testing of solvent extracts of soils and sediments. Firstly, different solvents will extract different proportions of the organic contaminants present, thus giving different toxic responses (Ho and Quinn 1993). Secondly, some kind of water-miscible solvent must be used as a cosolvent to allow toxicity testing of the extracts, and it is this problem that we have focussed on in the current study. It is important to use some kind of formal scheme in order to interpret the effects of the cosolvent on the bioassay response. The most obvious is simply to compare toxicity – i.e. $\log(1/EC_{50})$ – values directly for aqueous and DMSO solutions:

$$\text{toxicity}_{DMSO} = 0.97 \times \text{toxicity}_{aq} - 0.57 \quad (1)$$
$$(n = 7, R^2 = 0.96)$$

There is an excellent linear relationship between the two sets of data, as shown by the high R^2 and the slope close to unity. The toxicity was on average 0.56 log units higher for the aqueous solutions compared to the DMSO solutions; the only exception was anisole, which was slightly more toxic in the cosolvent than aqueous solution (Table 1). However, it would be valuable to have an additional systematic measure of cosolvent effects on biosensor response, and how these effects are related to the physical properties of different compounds.

Quantitative structure-activity relationships (QSARs) are widely used in environmental sciences to predict biological endpoints such as toxicity. They can also be used as tools for probing mechanism (Hermens 1991), or interrogating data for outliers (Lipnick 1991). One research focus in current environmental QSAR is prediction of toxicity for a wide range of compounds belonging to different chemical classes, for example by using neural networks (Niculescu *et al.* 2000). However a more basic QSAR is easier to interpret and thus more suitable for explaining trends in data. The traditional "class/mode of action"-type QSAR

Table 1. Toxicity of narcotic compounds to bioluminescent bioassay, expressed as $\log(1/EC_{50})$, with EC_{50} given in mM.

Compound	Toxicity (water)	Toxicity (cosolvent)	log K_{ow}	Solubility (mg l^{-1})[a]
ethanol	-2.91	-3.50	-0.31	na
butanone	-1.65	-2.33	0.29	na
pentanone	-0.85	-1.73	1.31	na
pentanol	-0.59	-1.23	1.51	na
anisole	-0.36	-0.29	2.11	na
heptanol	0.69	0.06	2.62	na
1,2-dichlorobenzene	0.70	0.12	3.38	156
2-decanone	nt	-0.23	3.77	76.8
n-butylbenzene	nt	-1.04	4.38	11.8

nt: not toxic, i.e. 50% luminescence inhibition not observed at maximum soluble concentration.

na: not applicable.

[a]maximum aqueous solubility, used for calculating nominal concentrations.

divides compounds *a priori* into different classes with different toxic mechanisms of action (Bradbury 1995). Of these classes, simple or non-polar narcotics give rise to baseline toxicity. The baseline toxicity of narcotics is typically highly correlated with log K_{ow}; this relationship holds for a wide range of different aquatic test systems employing different organisms (Cronin and Dearden 1995). Essentially all organic compounds exhibit at least baseline toxicity, i.e. are as least as toxic as would be predicted on the basis of log K_{ow} alone. (Compounds belonging to different chemical classes are usually more toxic than a narcotic of equivalent log K_{ow}.) Thus, previous workers have validated the response of a genetically modified bacterial bioassay by demonstrating that a linear relationship holds between log K_{ow} and toxicity of a series of narcotics (Layton *et al.* 1999).

The relationship between log K_{ow} and toxicity for the two different test systems, aqueous and cosolvent, is shown in Figure 1. There is an excellent linear relationship ($R^2 = 0.97$) between toxicity towards *E. coli* HB101 (pUCD607) and log K_{ow} of a series of narcotics made up as aqueous solutions. Therefore the bioassay response to toxicants behaves similarly to a large number of aquatic toxicity tests for wholly aqueous solutions. Conversely, the relationship between toxicity and log K_{ow} for the compounds dissolved in 10% DMSO solution is quite different (Figure 1). A clear toxicity maximum is reached for compounds with an intermediate log K_{ow} of around 3. More hydrophobic compounds actually have lower toxicity: for the three compounds with log $K_{ow} > 3$ that elicited an EC_{50}, there is a negative correlation with log K_{ow}. This effect is highlighted by the use of simple QSARs such as the one shown in Figure 1. One possible explanation for

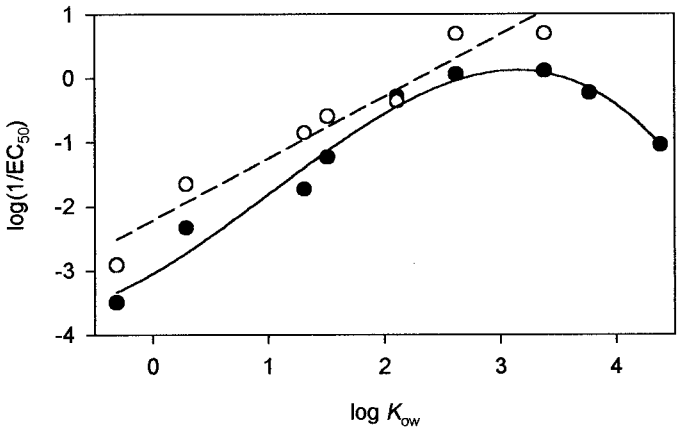

Figure 1. Relationship between hydrophobicity and toxicity for a series of narcotic chemicals. ○ aqueous solutions; ● chemicals tested in 10% DMSO solution.

this response of the compounds in DMSO solution is that it is not really a cosolvent effect. Biological activity is commonly found to have a quadratic relationship with log K_{ow}, particularly in pharmacological QSAR modelling (Hansch and Clayton 1973). This is because there is an optimum value for penetration into biological systems: the ability of a compound to pass through cell membranes increases with log K_{ow} up to a point, but if they are too hydrophobic the compound sequesters in the membrane and becomes less bioavailable at the active site. However, we do not believe that this is likely to be the correct explanation in the present study. Simple narcotic compounds are believed to act largely through partitioning into membranes (van Wezel and Opperhuizen 1995), and hence would not be expected to show a hydrophobicity maximum. This is particularly likely to be the case with a prokaryotic test system, where there is no question of the compound needing to penetrate to a target organ or tissue. An alternative explanation could be that the test compounds are not as bioavailable in the DMSO/water mixture as if they were genuinely dissolved. DMSO can form solvated or micellar complexes, analogous to the use of surfactants for pseudo-solubilization of HOCs. Several previous studies have reported that addition of surfactant can actually reduce biodegradation of HOCs (e.g. Laha and Luthy 1991), although the mechanism is likely to be more complicated than simple sequestering in micelles (Volkering et al. 1997). Finally, the possible effect of the cosolvent on the bioassay organism should also be considered. The dose-response curve for *E. coli* HB101 (pUCD607) to DMSO is given in Figure 2. DMSO has a noticeable stimulatory effect on bioluminescence, with an increase in light output of around 75 percent at below toxic concentrations. The exact reason for this is not known, although it has been suggested that it is caused by an increase of free intracellular fatty acids as a consequence of membrane disruption, which then act as substrates for the luciferase pathway resulting in an increase in luminescence

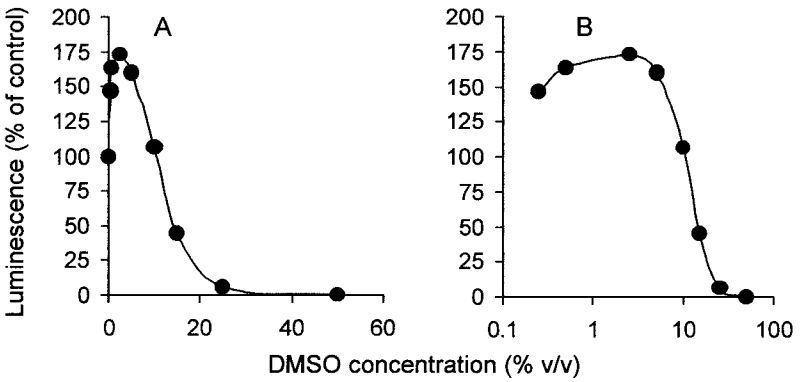

Figure 2. Dose-response curve showing effect of DMSO on luminescence of *E. coli* HB101 (pUCD607). A: linear scale. B: logarithmic scale.

(Heitzer et al. 1998). Organic solvents can exert a range of toxic effects on bacteria (Segura et al. 1999), so the possibility cannot be ruled out that some of the results observed were caused by toxic stress of DMSO on *E. coli* HB101 (pUCD607). However, given that EC_{50} values were generally higher in 10% DMSO solution than in water, we believe that the most likely explanation is that DMSO reduces the bioavailability of HOCs. This was particularly noticeable for compounds that were insufficiently water-soluble to allow a determination for a pure aqueous solution.

The implications for ecotoxicity testing of soils and sediments are intriguing. The results imply that hydrophobic compounds with very high log K_{ow} values extracted from sediments may not make a major contribution towards the toxicity of organic solvent extracts tested using a cosolvent. Although use of a cosolvent certainly does allow solubilization and toxicity testing of compounds with log K_{ow} only slightly too high to exert toxicity at the maximum aqueous solubility limit. Possibly it is the actual solvent extraction step that extracts more of the total toxicity. It would be an interesting experiment to compare the toxicity of (a) an aqueous extract of a contaminated soil, (b) an organic extract with the solvent removed and the residue taken up in DMSO and added to an aqueous test, and (c) an organic extract with the solvent removed and the residue redissolved in water, as far as possible. It would also be valuable for future work to repeat the experiments described in this paper using different concentrations of DMSO and different water-miscible cosolvents.

REFERENCES

Beg MU, Saeed S, Al-Muzaini K, Beg KR, Al-Obaid T, Kurian A (2001) Extraction, fractionation, and toxicity determination of organic contaminants in sediment from coastal area receiving industrial effluents in Kuwait. Bull Environ Contam Toxicol 67:881-888

Boyd EM, Killham K, Wright J, Rumford S, Hetheridge M, Cumming R, Meharg AA (1997) Toxicity assessment of xenobiotic contaminated groundwater using *lux* modified *Pseudomonas fluorescens*. Chemosphere 35:1967-1985

Bradbury SP (1995) Quantitative structure-activity relationships and ecological risk assessment: an overview of predictive aquatic toxicology research. Toxicol Lett 79:229-237

Brouwer H, Murphy T, McArdle L (1990) A sediment-contact assay with *Photobacterium phosphoreum*. Environ Toxicol Chem 9:1353-1358

Bundy JG, Campbell CD, Paton GI (2001) Comparison of response of six different bacterial bioassays to bioremediation of five contrasting oils. J Environ Monit 3:404-410

Cronin MTD, Dearden JC (1995) QSAR in toxicology. 1. Prediction of aquatic toxicity. Quant Struct-Act Relat 14:1-7

Demuth S, Casillas E, Wolfe DA, McCain BB (1993) Toxicity of saline and organic solvent extracts of sediments from Boston Harbor, Massachusetts and the Hudson River-Raritan Bay Esturary, New York using the Microtox bioassay. Arch Environ Contam Toxicol 25:377-386

Donnelly KC, Brown KW, Anderson CS, Thomas JC (1991) Bacterial mutagenicity and acute toxicity of solvent and aqueous extracts of soil samples from an abandoned chemical manufacturing site. Environ Toxicol Chem 10:1123-1131

Guzzella L. (1998) Comparison of test procedures for sediment toxicity evaluation with *Vibrio fischeri* bacteria. Chemosphere 37:2895-2909

Hansch C, Clayton JM (1973) Lipophilic character and biological activity of drugs II: the parabolic case. J Pharm Sciences 62:1-21

Heitzer A, Applegate B, Kehrmeyer S, Pinkart H, Webb OF, Phelps TJ, White DC, Sayler GS (1998) Physiological considerations of environmental applications of *lux* fusions. J Microbiol Meth 33:45-57

Hermens JLM (1991) QSAR in environmental sciences and drug design. Sci Total Environ 109/110:1-7

Ho KTY, Quinn JG (1993) Physical and chemical parameters of sediment extraction and fractionation that influence toxicity, as evaluated by Microtox. Environ Toxicol Chem 12:615-625

Laha S, Luthy RG (1991) Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. Environ Sci Technol 25:1920-1930

Layton AC, Gregory B, Schultz TW, Sayler GS (1999) Validation of genetically engineered bioluminescent surfactant resistant bacteria as toxicity assessment tools. Ecotoxicol Environ Safety 43:222-228

Li A, Andren AW, Yalkowsky SH (1996) Choosing a cosolvent: solubilization of naphthalene and cosolvent property. Environ Toxicol Chem 15:2233-2239.

Lipnick RL (1991) Outliers – their origin and use in the classification of molecular mechanisms of toxicity. Sci Total Environ 109:131-153

Niculescu SP, Kaiser KLE, Schultz TW (2000) Modelling the toxicity of chemicals to *Tetrahymena pyriformis* using molecular fragment descriptors and probabilistic neural networks. Arch Environ Contam Toxicol 39:289-298

Pankhurst CE, Rogers SL, Gupta VVSR (1998) Microbial parameters for monitoring soil pollution. In: Lynch JM, Wiseman A (eds) Environmental Biomonitoring: the Biotechnology Ecotoxicology Interface, CUP, Cambridge, pp. 46-69

Paton GI, Rattray EAS, Campbell CD, Cresser MS, Glover LA, Meussen JCL, Killham K (1997) Use of genetically modified microbial biosensors for soil ecotoxicity testing. In: Pankhurst CE, Doube BM, Gupta VVSR (eds), Biological Indicators of Soil Health, CAB International, Wallingford, pp. 397-418

Rattray EAS, Prosser JI, Killham K, Glover LA (1990) Luminescence based non-extractive technique for *in situ* detection of *Escherichia coli* in soil. Appl Environ Microbiol 56:3368-3374

Segura A, Duque E, Mosqueda G, Ramos JL, Junker F (1999) Multiple responses of Gram-negative bacteria to organic solvents. Environ Microbiol 1:191-198

Sunahara GI, Dodard S, Sarrazin M, Paquet L, Ampleman G, Thiboutot S, Hawari J, Renoux AY (1998) Development of a soil extraction procedure for ecotoxicity characterization of energetic compounds. Ecotoxicol Environ Safety 39:185-194

van Wezel AP, Opperhuizen A (1995) Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity, mechanisms, and membrane burden. Crit Rev Toxicol 25:255-279

Volkering F, Breure AM, Rulkens WH (1997) Microbiological aspects of surfactant use for biological soil remediation. Biodegradation 8:401-417

Wang XP, Bartha R (1990) Effects of bioremediation on residues, activity and toxicity in soil contaminated by fuel spills. Soil Biol Biochem 22:501-505