Bull. Environ. Contam. Toxicol. (2001) 67:526–534

DOI: 10.1007/s00128-001-0155-2

Bulletin of Environmental Contamination and Toxicology

Use of Microtox for Assessing Heavy Metal Complex Formation With the Organic Solvents Acetontrile and Dimethyl Sulphoxide: A Preliminary Study

C. Cooper

Cranfield Centre for EcoChemistry, Cranfield University, Shardlow Hall, Shardlow, Derby DE72 2GN, United Kingdom

Received: 3 January 2001/Accepted: 24 June 2001

Natural waters are more prone than ever to contamination by chemicals of anthropogenic origin. The concentrations of these pollutants, are controlled by biogeochemical processes such as organometallic interactions, adsorption onto particle surfaces and biological uptake (Vasseur *et al* 1988). In natural water bodies such as surface waters, estuaries, marginal seas and coastal zones pollutant concentrations tend to be variable and are governed by a wide variety of rapidly occurring reactions. Thus, any pollutant discharged into the aquatic environment undergoes a series of complex reactions before its assimilation into the sediments or the food web (Vasseur *et al* 1988). The pollutants may be present adsorbed on particulates or colloids, as humic acid-pollutant sorbed complex or as "inert" compounds (irreversibly bound). Each of these three forms can react differently in natural waters and their distributions between sediment, water and biota is both highly varied and dynamically controlled. It is the chemical speciation of these compounds, which holds the key to the understanding of their geochemical and biochemical reactivities. Therefore, it is important to measure their equilibrium behaviour and the "bioavailable" form(s) which characterise their toxicity. The development of biosensors has allowed determination of the bioavailability of anthropogenic chemicals in various environmental phases. Screening of water sample toxicity with the marine bacterium *Vibrio fischeri*, especially in combination with toxicity identification evaluation procedures, is a useful and realistic alternative to more conventional toxicity tests (Bitton, 1983).

This study is concerned with the interactions of organic and inorganic contaminants in order to assess the potential complex formation between solvents and metals. Pesticides are contaminants of treated soils and are often found in aquatic environments by the means of soil leachates. Solvents and metals are commonly released into surface waters with industrial effluents. Their combined effects are worthwhile to consider since living organisms are exposed to multiple contaminants in natural ecosystems. The combined effects of toxic substances may be additive in the simplest case, antagonistic in the optimal one, but also synergistic. This last situation is most feared by toxicologists as it results in important damage on life (Vasseur *et. al* 1988).

MATERIALS AND METHODS

The chemicals used for the tests were all supplied by BDH Merck:
$NiCl_2.6H_2O$, $CdCl_2$, $ZnSO_4.7H_2O$, $K_2Cr_2O_7$ - all 99% pure or greater.
Acetonitrile, HPLC Grade, 99+% purity - BDH
Dimethyl sulphoxide, DMSO (98+ purity)

The metals were chosen due to their occurrence in industrial wastewaters. The solvents were chosen due to their low toxicity towards the test organism at particular concentrations (Cooper, unpublished data, Kaiser & Palabrica, 1991) and they contain three major chelating atoms, nitrogen, oxygen and sulphur between them.

All stock solutions for use in the toxicity tests were prepared by dissolving the chemical of interest in 2% NaCl (Microtox® Diluent) solution. This prevented any osmotic adjustments having to be performed prior to the assays. All test were performed at pH 7.7-7.9

The Microtox® Test is a commercially available biological assay, which involves evaluating the decrease of the bioluminescence of the marine bacterium *Vibrio fischeri* when toxic substances are added to the bacterial suspension (full details are given in the Microtox® user manuals, supplied by AZUR Environmental). The test organism has been well studied and the toxic response towards many chemicals is well documented (Kaiser & Palabrica, 1991). The ease of the test makes it a good choice to try to evaluate the possible interactions of metals with simple organic compounds and how these interactions may affect the observed toxic response.
Briefly the Microtox® test involves:

- Rehydration of the freeze-dried luminescent bacterium, *Vibrio fischeri* (Microtox® Acute Reagent) in 1mL of distilled water.
- 10µL of the reconstituted bacterial suspension is added to 0.5mL of 2% NaCl and incubated for 15 minutes. The initial (I_o) measurement of light output was measured after this incubation period.
- After the initial measurement 0.5mL of sample was added to the bacterial suspension, giving the desired test concentration. The luminescence was measure in a Deltatox™ photometer (AZUR Environmental).
- Residual bioluminescence was measured after 15 minutes of exposure.

A control was run simultaneously in order to evaluate the physiological variance of luminescence. This variation is expressed as the Blank Ratio (BR):

$$BR = I_t/I_o \text{ for the control medium}$$

The toxicity in the toxic medium is calculated from the ratio of the residual luminescence, β, to the initial luminescence, corrected by BR (Vasseur *et al*, 1988):

$$\beta = I_t/(I_o \times BR)$$

We chose to use a toxic unit approach to joint toxicity action primarily for ease of use and understanding. In the toxic unit (TU) model, a value of 1 TU is assigned to the 50% effective concentration (EC50) value of a contaminant (Pape-Lindstrom & Lydy, 1997). A summation of TU contributed by each component describes the toxicity of a mixture, which is always unity:

$$\Sigma TU = (Cw_1/EC50_1) + (Cw_2/EC50_2) + \ldots\ldots..(Cw_i/EC50_i)$$

Where Cw_i = the concentration of the chemical in the mixture and $EC50_i$ = is the EC50 for that respective component. The experimentally measured toxicity can then be compared to the expected toxicity (predicted by ΣTU and is unity*). When the EC50 of ΣTU (the mixture) occurs at less than unity, the mixture is exhibiting greater than additive toxicity (synergism).* Determination of *less than additive toxicity is made when the EC50 is at values greater than unity (i.e. greater than predicted ΣTU).*

In our scenario one component is used below its No Effective Concentration (NOEC), and is arbitrarily given a contribution value of zero. Therefore, if it does not interact with the toxicant being tested the observed toxicity of the single component solution and the binary mixture should not be significantly different to one another. If however, there is an interaction between the chemicals, then this should be reflected in a significant difference between the single component solution toxicity and the binary solution toxicity.

RESULTS AND DISCUSSION

Table 1 gives the concentrations of the toxicants used in the tests for both single component and binary mixtures and summarises the observed effect of acetonitrile on toxicity, the toxic units are the mean values of a series of 8 replicates. Table 2 gives the concentrations of the toxicants as for table 1 and summarises the effects of a sulphur and oxygen bearing solvent, dimethyl sulphoxide (DMSO) on the observed toxicity.

The histogram of Figure 1a shows the toxicity data for both single component heavy metal solutions towards the Microtox® organism and binary mixtures containing the solvent, acetonitrile plus a heavy metal. Acetonitrile was used at a concentration ≤ 1% v/v, which is below its no effective concentration (NOEC). Figure 1b shows the amount of toxicity contributed by the "free" aqueous metal ions and organo-metallic species. The amount of organo-metallic contribution has been calculated from the percentage increase or decrease in observed toxicity as

the single component toxicity is solely due to "free" metal ions and this is used as the benchmark value to calculate any changes. It is apparent, from the data that the toxicity of zinc and chromium is markedly reduced by the presence of acetonitrile, 47% & 95% for metal concentrations of 10ppm and 25ppm respectively. Therefore it is postulated that there is a 47% & 95% reduction in free metal ion concentration. Moreover, when the concentration of metal and acetonitrile are halved the reduction in toxicity is less and the relative percentages of free metal ions are increased, showing that metal to solvent ratios are obviously important (Figure 1b). The observed toxicity of cadmium and nickel is not significantly affected by the presence of acetonitrile i.e. there is very little change in metal ion concentration. Only one concentration for Cd and Ni are given, although higher metal concentrations were tried, the results were very similar as the amount of acetonitrile had to be kept below its NOEC limit the ratio of metal to solvent was incredibly large.

Table 1. Effect of acetonitrile on the observed toxicity of heavy metals towards the bacterium *Vibrio fischeri*.

Toxicant	*Concentration /ppm*	*Acetonitrile conc/% v/v*	*Average toxicity TU*	*Effect of Acetonitrile*
Zinc Sulphate	10.0	0.0	2.24	-
Zinc Sulphate	10.0	0.5	1.07	Antagonistic
Zinc Sulphate	5.0	0.0	1.20	
Zinc Sulphate	5.0	0.25	0.98	
Potassium dichromate	25.0	0.0	20.2	
Potassium dichromate	25.0	0.5	1.09	Antagonistic
Potassium dichromate	12.5	0.0	1.77	-
Potassium dichromate	12.5	0.25	1.05	Antagonistic
Cadmium Chloride	50.0	0.0	1.66	
Cadmium Chloride	50.0	0.5	1.47	No Interaction
Nickel Chloride	50.0	0.0	1.27	-
Nickel Chloride	50.0	0.5	1.13	No Interaction

Table 2. Effect of di-methyl sulphoxide (DMSO) on the observed toxicity of nickel and cadmium towards the bacterium *Vibrio fischeri.*

Toxicant	*Concentration /ppm*	*DMSO conc/% v/v*	*Average toxicity TU*	*Effect of DMSO*
Cadmium Chloride	50.0	0.0	1.48	
Cadmium Chloride	50.0	0.5	1.39	No Interaction
Nickel Chloride	50.0	0.0	1.23	-
Nickel Chloride	50.0	0.5	1.12	No Interaction

Figures 2a and 2b show similar plots for the heavy metals nickel and cadmium with a different solvent, di-methyl sulphoxide. These experiments were performed to see if the presence of a ligand containing sulphur and oxygen would produce a significant difference in the observed toxicity of these two metals. Again there appears to be little change in toxicity or free metal values. The reason sulphur and oxygen are chosen is that cadmium tends to behave as a "soft" or type B metal and has an affinity for sulphur based on the Hard Soft Acid/Base model. Nickel is known to behave as a "hard" or type A metal compared to cadmium and has an affinity for oxygen (Greenwood & Earnshaw, 1984).

The data for the effect on metal toxicity are quite interesting. The differences in the observed effects of zinc and chromium are can probably be explained in terms of aqueous coordination chemistry. Consideration of the co-ordination chemistry of zinc reveals that zinc will form stable complexes with *N*-donor ligand species (Greenwood & Earnshaw, 1984; Stumm & Morgan, 1996). The resulting aqueous complex lowers the amount of free metal ions in solution and resulting in lower observed toxicity towards *Vibrio fischeri* as can be observed in the significant decrease in observed toxicity. These results are similar to those of Morel *et al.*, 1988, who observed decrease in copper toxicity towards the Microtox® test organism with complexation to humic acids and root exudates.

Chromium is well known for forming stable complexes with all the common anions and virtually all species capable of donating an electron pair (Greenwood & Earnshaw, 1984). The carbon-nitrogen bond in acetonitrile is a triple bond, as a consequence, acetonitrile possesses abundant π-electron density, therefore, potentially, acetonitrile, can readily chelate to chromium by the donation of π-electron density to chromium in solution. The observed reduction in toxicity could be attributable to the formation of an organo-metallic chromium complex reducing the concentration of the free Cr species that exists in the Microtox® test medium of 2% NaCl.

Cadmium has a lower affinity for nitrogen than zinc (zinc has more "hard" character than cadmium) and also has a different mode of action.
Zinc is thought to affect cell membrane proteins in *Vibrio fishceri* (AZUR Environmental), but cadmium is actively transported into the cell via the manganese pump (Waite, *personal communication*). As cadmium has less affinity for nitrogen than zinc, only a small concentration of an organo- metallic complex may form, and leave enough cadmium to be transported into the cell resulting in very little change to the observed toxicity in the acetonitrile experiments.

Mazidji et al, 1992, however, reported that cadmium toxicity is reduced by use of the chelating agent EDTA, therefore the type of ligand or solvent is important depending on the metal under investigation. The lack of interaction of cadmium with DMSO is interesting. Cadmium has quite a high affinity for sulphur with form quite stable covalently bonded complexes with sulphur containing molecules (Greenwood & Earnshaw, 1984). However, under the experimental conditions, it appears that very little interaction occurs. This may be kinetic effect or an ionic strength effect. Further research into this will be required.

Nickel also has a tendency to undergo chelation reactions with *N*-donor ligands (Greenwood & Earnshaw, 1984) and is expected to behave quite similarly to zinc in this respect ("hard" metal character). However, nickel actually behave more like cadmium ("soft" metal character) in these experiments and doesn't interact with acetonitrile to any measurable degree, as observed by the insignificant change in toxicity towards *vibrio fischeri*. Nickel is known to co-ordinate with DMSO to form a stable octahedral complex. However, it would appear that this complex causes very little effect on the observed toxicity of nickel under these experimental conditions, indicating that the Ni-DMSO complex does not form to any significant degree under the conditions of the Microtox® test.

The addition of simple organic solvents to a solution containing the toxic metals zinc and chromium results in an observed significant decrease in toxicity of the metal towards the Microtox® organism, *vibrio fischeri*. The simplest explanation for this appears to be the formation of aqueous complexes between the solvent molecules and the metal ions.

However, other metal ions like cadmium and nickel behave differently. Both metals are known to undergo complexation reactions with nitrogen, oxygen and sulphur containing molecules and yet no change in their toxicity towards the test organism is observed. As suggested in the discussion this may be an indication of lack of complex formation. Other explanations could also be justified, such as, cadmium and nickel speciation is not important in the toxic response it elicits in the test organism and yet is of great importance for zinc and chromium toxicity towards *vibrio fischeri*.

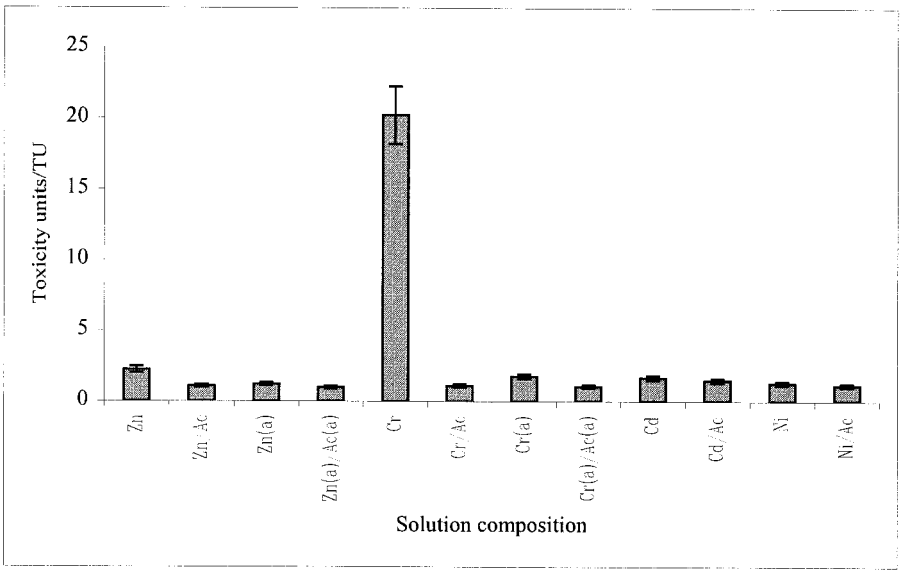

Figure 1a. Toxicity of solutions containing a toxic heavy metal and toxic heavy metal plus acetonitrile. The designation (a) indicates where the concentration of both the metal and the acetonitrile were halved. Error bars are the coefficients of variation on the 8 replicate samples.

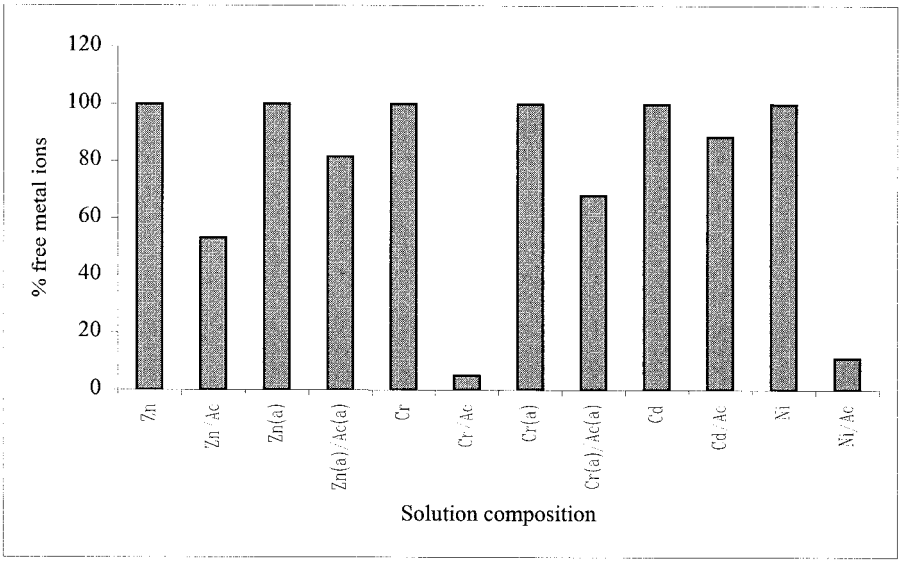

Figure 1b. Percentage of toxic "free metal ions" determined from percentage reduction in toxicity in solution in the single component and binary solutions.

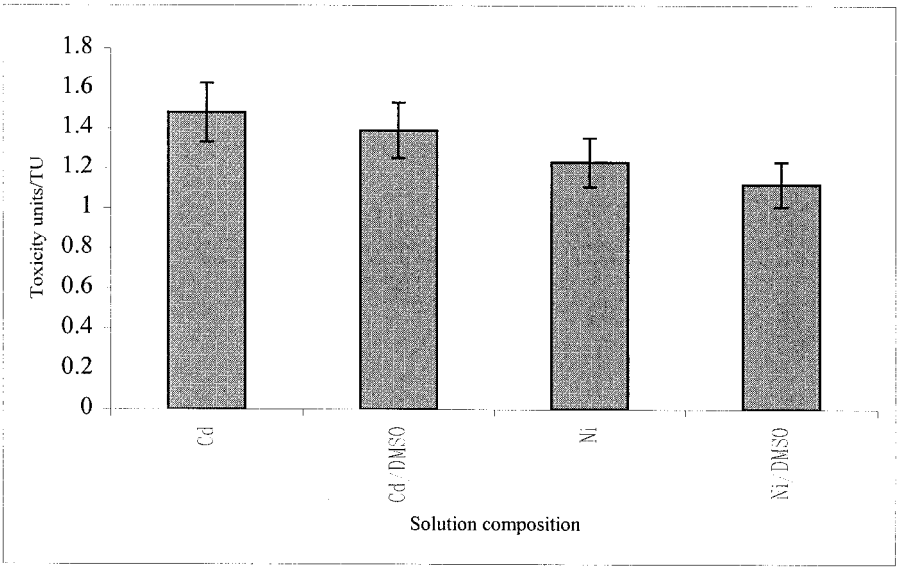

Figure 2a. Toxicity of solutions containing a toxic heavy metal and toxic heavy metal plus dimethyl sulphoxide (DMSO). Error bars are the coefficients of variation on the 8 replicate samples

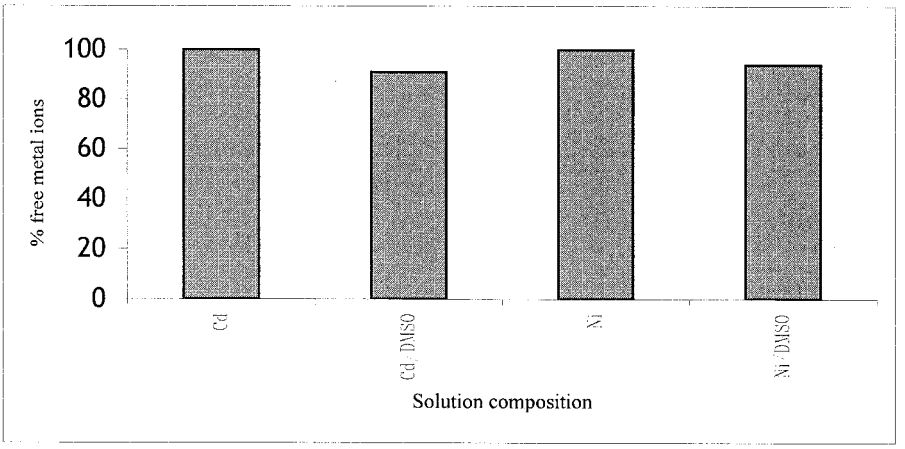

Figure 2b. Percentage of toxic "free metal ions" determined from percentage reduction in toxicity in solution in the single component and binary solutions.

This is only a preliminary study and further investigations are needed and warranted in order to explain fully the effects of speciation on observed metal toxicity towards *vibrio fisheri*. The author also realizes that although such an

assay cannot give an accurate description of the complex formation reactions, rapid information can be obtained with regard to the chemical forms under which toxic metals may be present in unknown solutions.

REFERENCES

AZUR Environmental Ltd. (1992) User Manuals, AZUR Environmental, Wokingham, Berkshire. UK

Bitton, G (1983) Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment – A review CRC Critical Reviews in Environmental Control 13: 51-67.

Greenwood, N N and Earnshaw, A (1984) Chemistry of the elements, Pergamon Press, Oxford, UK.

Kaiser, K L E and Palabrica, V S (1991) Photobacterium phosphoreum toxicity data index Water Pollut Res J Canada 26: 361-431

Mazidji, C N, Koopman, B, Bitton, G and Neita, D (1992) Distinction between heavy metal and organic toxicity using EDTA chelation and microbial assays, Environ Toxicol Water Qual 7: 339-353.

Morel, J-L, Bitton, G and Koopman, B (1988) Use of Microtox® for assessing copper complexation with organic compounds Arch Environ Contam Toxicol 17: 493-496

Pape-Lindstrom, P A and Lydy, M J (1997) Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition model, Environ Toxicol and Chem 16: 2415-2420.

Stumm, W and Morgan, J J (1996) Aquatic Chemistry: Chemical equilibria and rates in natural waters 3rd Ed Wiley-Interscience, New York.

Vasseur, P, Dive, D, Sokar, Z and Bonnemain, H (1988) Interactions between copper and some carbamates used in phytosanitary treatments Chemosphere 17: 767-782.

Villaescusa, I., Marti, S., Carme, M., Martinez, M and Ribo, J.M. (1997) Chromium (VI) toxicity to luminescent bacteria, Environ Toxicol Chem16: 871-874.