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COMPARISON OF SEVERAL MICROBIOLOGICAL TOXICITY SCREENING TESTS

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ABSTRACT

Four short-term microbiological toxicity screening tests were compared using the following test chemicals: 3,5 dichlorophenol, cetyl trimethyl ammonium chloride, sodium lauryl sulfate, phenol, copper (II) sulfate, mercury (II) chloride, and zinc (II) sulfate. These seven chemicals represent a wide range of toxicity. The methods examined were Beckman's Microtox system, the Spirillum volutans motility test, inhibition of respiratory activity of activated sludge, and inhibition of activated sludge TTC-dehydrogenase activity.

The results obtained indicate that each method has its own toxicity sensitivity pattern, and among the substances tested, only mercury (II) chloride and phenol were ranked equally by the four methods as the most and the least toxic chemical, respectively. In a tentative ranking of the methods, according to sensitivity, the Microtox test came out as the most sensitive test, followed by the Spirillum test, which in turn appeared more sensitive than the two sludge tests. This ranking has meaning only in a statistical sense, however, and the variable nature of the results support the philosophy that for assessing toxicity more thoroughly, a battery of several tests is required.

Mechanisms for alerting monitoring and surveillance agencies about the presence of toxic chemicals in waters and effluents are inconsistent and chaotic. Due to increased industrialization over the past few years, as well as the increased demand for chemicals, both the developed and developing nations face increasing ecological and toxicological problems from the release of toxic contaminants to the environment. With the increasing awareness of the long term effects of chemicals discharged into various national water systems, research efforts are being directed at short term bioassay tests, in an attempt to alert dischargers as well as monitoring agencies of toxic conditions. In many countries, there are objectives for aquatic species' tolerance to effluents as well as for river, lakes, ocean and mixing zones within these waters. A direct bioassay using native or laboratory aquatic species is superior in many ways to the analysis for all of the constituents (many unknown and unsuspected) in the effluents and natural water bodies (Kohn, 1980).

As industrial effluent pollutants and also such toxicants as herbicides, pesticides, fertilizers, car emissions etc. affect aquatic biota systems at different levels and in many ways it is acknowledged that the battery approach utilizing several different short term biological indicators would be preferred in any monitoring scheme. At present, the most singularly used aquatic toxicity testing system is based on the 24 or 96-hr reactions of fish in static or flow through systems. The wide acceptance of the fish bioassay procedure is

probably due to the knowledge that fish are indigenous to water and are effected by adverse conditions, though the species used may be foreign to the waters being tested. The fish may also act as bioaccumulators and the results or end point of the tests are easily visible to the untrained eye. However, present technological needs are for indicator systems which can assess the toxicant levels of effluents in as short a time frame as possible. One of the reasons for this time emphasis is that some effluents may be able to be stopped or contained for short periods for extra treatment, if necessary, but volume problems would make it unrealistic to attempt a 24-hr, much less a 96-hr containment. Also, by rapid assessment of changes in effluent quality, it may be possible to modify treatment before too great an environmental impact has occurred.

Several of the newer short term biological techniques to assess toxicity in water or effluents such as methods utilizing the inhibition of TTC-dehydrogenase activity (Ryssov-Nielsen, 1975) and respiratory activity of activated sludge microorganisms, the Microtox system (Beckman Instruments, Inc.) and Spirillum volutans (Bowdre and Krieg, 1974) have the potential of providing information on toxicant levels within one to three hours of sample collection. Procedures for the use of these short term biological systems will be described and their sensitivities to several toxicants will be compared.

METHODS AND MATERIALS

Inhibition of respiratory activity of activated sludge ("respiration test")

A working group within the International Standards Organization (ISO, 1981) has recently drafted a proposal for a standardized method for assessing the inhibitory effect of a test material (chemical substances, mixtures thereof or wastewater samples), on the respiratory activity of activated sludge microorganisms. The test is performed by adding activated sludge to a series of vessels with synthetic sewage medium according to OECD, (1976) and different concentrations of the test material. The concentration of sludge in the final test mixture is about 1.5 g/l of suspended solids. The test mixtures are aerated continuously to at least 80% oxygen saturation. The respiratory activity of each mixture is then measured after a 30 minute contact time between sludge and test material by stopping the aeration and recording the rate of decrease of dissolved oxygen by means of an oxygen electrode. The rate of decrease of dissolved oxygen which is proportional to the die off rate of the organisms is estimated most conveniently by means of a recorder. The time required for a measurement is about 5 to 10 minutes. The procedure can be repeated after a contact time of 3 hours.

For each concentration of test substance, the percentage of inhibition is calculated as reduction in respiratory activity relative to a control sample (synthetic sewage without test substance), and a plot of percentage of inhibition versus concentration of test material is constructed.

From this plot estimates of EC_{50} (concentration of test substance causing 50% inhibition of respiratory activity) and other key figures (for example EC_{10} and EC_{90}) are calculated. (The figure EC_{10} which marks the beginning of inhibition, appears to be particularly useful as a supplement to EC_{50}). Brown et al. (1981) has recently described the use of a similar test to assess the inhibitory effect of a number of dye-stuffs.

Inhibition of activated sludge TTC-Dehydrogenase activity ("TTC-test")

The test used in this work has been described by Ryssov-Nielsen (1975), and is a modification of the method introduced by Bucksteig and Thiele (1959), which also appears in Deutsche Einheitsverfahren (1960). For review of the method refer to Klapwijk et al. (1974). The test is carried out by incubating (in centrifuge tubes) 5 mL activated sludge samples with a tris-buffer solution of the redox dye triphenyltetrazoliumchloride (TTC) and test material in an appropriate dilution series.

During incubation (60 minutes at 37°C and pH 7.5) TTC, which replaces oxygen as a hydrogen acceptor, is reduced by the aerobic cytochrome system of the sludge microorganisms to triphenylformazan (TF) which is a red coloured compound, insoluble in water. Under appropriate test conditions the amount of formazan formed is proportional to the "dehydrogenase activity" of the sludge, and inhibition of this enzyme activity results in a reduced formazan production.

The amount of formazan formed after 60 minutes is determined spectrophotometrically after centrifugation and ethanol extraction of the sediment. The percentage of inhibition is calculated as reduction of formazan production relative to a control without test material. A plot of percentage of inhibition versus log concentration of test material is constructed and interpreted as described for the respiration test.

Microtox Test

Beckman Instruments, Inc. have devised a test for acute toxicants in water in which specialized strains of luminescent bacteria (Photobacterium phosphoreum) are used as the bioassay organism. This test is functional because the metabolism of the luminescent bacteria is influenced by low levels of toxicants and, occasionally, stimulants. Any alteration of metabolism affects the intensity of the organisms' light output. By sensing these changes in light output,

the presence and relative concentration of toxicants can be obtained by establishing the EC_{50} levels from graphed data; EC_{50} being, in this case, that concentration of toxicant (or dilution of unknown) causing a 50% reduction in light from the base level (Beckman Instruments, Inc., Product Development Bulletin 6964). Basically, the test involves the addition of luminescent bacteria into a vial of precooled dilutent solution (15°C) and allowed to stabilize. Approximately 15 minutes later, a measured amount of sample or sample dilution is added to this vial, which is then transferred to a light-tight turret where it is exposed to a photomultiplier tube. Total light output is read, usually over a 5, 10 or 15-minute period, from a digital panel meter attached to an accessory recorder. By testing several concentrations of sample, an EC_{50} can be established. In earlier studies (Dutka and Kwan, 1981) it became apparent that the light output readings obtained after 5 minute incubation were often very difficult to reproduce because of the continuing action between sample and cells. In all comparisons, the 15 minute readings which were found to be more reproducible, are used, although 5 minute and 10 minute readings are presented for comparison.

Spirillum volutans Test

Spirillum volutans, a large aquatic bacterium with a rotating fascicle of flagella at each pole, was used to test the samples

for toxicity, following a modification of the procedure developed in 1974 by Bowdre and Krieg (Dutka, 1978). The procedure involved pipetting 0.1 mL of Defined Test Medium into 13 x 100 mm tubes, and adding 0.8 mL of the sample plus 0.1 mL of healthy bacteria from an overnight culture in logarithmic growth phase. The tube is swirled, a drop removed via Pasteur pipette and placed on a slide and quickly examined under a darkfield or phase contrast microscope (125X). This is the 0-minute reading. Samples from the tube were examined at 5, 10, 30, 60, 90 and 120-minute intervals. If, during any one examination, the reversing motility had been eliminated in more than 90% of the cells, a positive toxic effect was recorded. Negative controls in distilled water were routinely used to ensure inhibition was due to the tested sample. Positive controls were used only to verify percentages of lost motility in doubtful reactions.

Chemicals

The following chemicals were used to compare the sensitivities of the short term microbial bioassay tests: 3,5 dichlorophenol, cetyl trimethyl ammonium chloride, sodium lauryl sulfate, phenol, copper (from $CuSO_4$), mercury (from $HgCl_2$) and zinc (from $ZnSO_4 \cdot 7H_2O$). Where possible, based on the concentration tested, Microtox and Spirillum volutans samples were tested at pH 6.5 to 6.7. The

respiration test with activated sludge and the TTC test were carried out at pH 7.5 ± 0,2 and pH 7.5 ± 0,5 respectively.

Activated Sludge

The activated sludge samples used for the respiration test and the TTC-tests were collected from Rungsted municipal treatment plant, Denmark. The plant deals almost exclusively with domestic sewage. For practical reasons several different sludge samples were used. Normally, the samples were used on the day of collection. However, in a few cases the respiratory or dehydrogenase activity was considered too low and was brought up to a suitable level by feeding the sludge overnight with OECD synthetic sewage. The samples were kept aerobic by means of aeration.

Results and Discussion

Table 1 presents a summary of all the chemicals tested for their acute toxicity effects as measured by the Microtox test, after 5, 10 and 15 minutes incubation, the Spirillum volutans test, the inhibition of respiratory activity of activated sludge test and the inhibition of activated sludge TTC-dehydrogenase activity test. In Table 1, it can be seen that although the majority of chemicals tested via the Microtox procedure have an increasing effect with increased incubation (contact), 3,5 dichlorphenol appears to have a fairly

stable toxicity effect over the 15 minute period and phenol decreases in toxicity with increased incubation. One plausible explanation for the "recovery" of the Microtox system when tested with phenol, is that the reaction between phenol and the organisms emitting light is almost instantaneous and is not progressive. Thus although the light emission of the control and test sample decrease with time the proportion of the organisms emitting light in the test remain constant and show up as a slight recovery when the data are plotted. Similar responses can be seen in the "respiratory activity test" where cetyl trimethyl ammonium chloride has a fairly stable toxic effect from 0.5 hours to 3 hours of contact and phenol decreases in toxicity with prolonged contact.

One of the most striking features of Table 1 is the wide ranges of chemical concentrations which produce toxicity effects. Although the metal data are reported as ppm of the formal concentration of the ionic form of the metal, the concentrations of free, toxic metal species may be many fold less and will depend on the complexing capacity of the medium. Similarly, pH may have an effect. The respiration test with activated sludge and the TTC-dehydrogenase test were carried out at pH 7.5 while the Microtox and Spirillum tests were tested at pH 6.7, as this is the pH required by the Microtox test. The same pH (6.7) was used for Spirillum tests for convenience as laboratory studies have shown that distilled water samples augmented with acid to a pH of 4.5 were not toxic with the Spirillum test. It

is conceivable, that if the incubation temperature was increased or the pH of the test were varied where possible and different test media used; a different set of toxicity patterns could emerge from the four tests and thus change the character of Table 1.

Figure 1 presents an example of dose response curves for cetyl trimethyl ammonium chloride which were used to derive EC_{50} or in the case of S. volutans test EC_{90} values.

The data presented in Table 1 and Figure 1 are condensed and summarized in Table 2 and in Figure 2 to produce a ranking of the chemical's toxicity as indicated by the four testing procedures. There is total agreement on only two of the seven tested chemicals, the most toxic being Hg^{++} and the least toxic, phenol. All the other chemical rankings indicate the variety of sensitivities, microbiological testing systems have, to toxic substances. Thus from Tables 1 and 2 and the literature i.e. Dutka-Kwan, 1981, U.S. EPA Quality Assurance Newsletter (1981), it is very obvious that no single microbiological or biological testing procedure can predict the presence of all toxicant levels which might effect aquatic organisms or eventually be bioaccumulated and affect their predators or man.

In spite of the inconsistent ranking of most chemicals and the ascertained different sensitivity pattern of each test, the results show, nevertheless, that the Microtox test (15 min incubation)

with only one exception (Cu^{+2} in TTC test) comes out with the lowest EC_{50} figure, and may accordingly be classified as the most sensitive method. Also the Spirillum test most frequently gave lower figures than the two activated sludge tests. The two activated sludge tests on the other hand did not show similar systematic differences. The relative sensitivity of the tests are compared in Table 3 which includes the results of a tentative non-parametric statistical analysis (carried out according to Brondum and Monrad, 1975) of log (EC_{50}) values obtained in the various tests compared two by two, and by assigning a ranking number to each of the seven chemicals examined.

As judged from a non-parametric regression analysis, the correlations between the log (EC_{50}) figures obtaine in the various tests are significant at least at a 90% level, except for the Spirillum versus the activated sludge respiration test. A quite narrow correlation (significance level greater than 99.9%) is found between the Microtox and the Spirillum tests.

A Wilcoxon's test for two random samples was carried out in order to examine differences in sensitivity. It turned out that the Microtox test gave significantly (95%) lower EC_{50} values, i.e. was more sensitive than the sludge tests. The differences in sensitivity between the Microtox and the Spirillum test, and between the Spirillum test and the two sludge tests, respectively, were significant only at

a 90% level, while no difference (90% significance) could be ascertained between the respiration and the TTC-dehydrogenase activated sludge tests.

The above statistical analysis could be criticized because the data is too limited and that the chosen 90% level of significance is arbitrary. However, the results appear to be consistent with a qualitative examination of the data and are considered to provide at least some additional information.

The Microtox toxicity testing procedure, which is now undergoing a very thorough review by many North American laboratories, has, in the laboratory of one of the authors, shown to have some problems with reproducibility. For instance in Table 4, five toxicants are compared with only two toxicants (Hg^{++} and phenol) having similar results in the two laboratories. Another example of this type of reproducibility problem is shown by various EC_{50} values for ethanol. In one study, performed by the U.S. Environmental Protection Agency laboratory in Duluth, a 5 minute EC_{50} of 56,706 ppm ethanol was found and in another study by the same laboratory an EC_{50} value of 44,000 was obtained (Curtis et al., 1981). For comparison, the 5 minute EC_{50} obtained for ethanol by the Beckman Instruments laboratory was 31,000 ppm (Bulich et al., 1981) and by Chang et al., (1981) 47,000 ppm. However, LC_{50} values for fish toxicity tests and other biological tests are also known to show similar and greater reproducibility problems, if the procedures used are not strictly standardized.

However, realizing the variability of biological testing systems, the results presented herein indicate that the Microtox system is a sensitive toxicity assaying procedure which has as its major benefit, a quick turnaround time which makes it an ideal member of a battery of screening tests and also a potential "early warning" test.

Reviewing the data from the four toxicity assessing techniques, it is obvious that each procedure has its own toxicity sensitivity pattern and cannot readily be correlated with other procedures. There are concurrences as well as wide divergences in toxicant sensitivity. Similar results are reported in an U.S. government EPA sponsored project (EPA Quality Assurance Newsletter, Vol. 4:2, April 1981) of an effluent study comparing 24-hour fathead minnow and Daphnia pulex LC_{50} tests with the 5 minute Microtox test. It was found that the Microtox test indicated the presence of toxicity in 81% of the effluents that were toxic to the fathead minnows, and the Microtox test also indicated the presence of only 62% of the samples which were toxic to Daphnia pulex.

In a study reported by Dutka and Kwan (1981), the toxicity of the effluents from steel mills were assessed by the Microtox procedure, Spirillum volutans and fingerling rainbow trout. The authors found that although the concentrations of potential toxicants were too

low to elicit consistent responses from Spirillum volutans and the fingerling rainbow trout, the Microtox procedure proved to be the most sensitive to sample fluctuations, providing indications varying from growth enhancement to no effect and to acute toxicity.

Thus from the study data and the reported data we believe that it is unwise to try and assess the presence of toxicants in waters or effluents by a single species or single biochemical process test. The battery approach, encompassing two or three genera and involving two to four species is recommended, to more thoroughly assess the potential presence of toxicants. Furthermore biochemical process tests have a place in, and should be considered as part of, the battery approach.

CONCLUSIONS

In summary, reviewing the data from the four toxicity assessing techniques, it is obvious that each procedure has its own toxicity sensitivity pattern, although also some general trends and systematic differences can be seen. Based on the limited data obtained, the methods could be ranked broadly according to sensitivity as follows: (in order of decreasing sensitivity) Microtox test Spirillum test activated sludge respiration test and TTC-dehydrogenase test. There are also wide divergences between the test results.

Results likewise showing both concurrences and divergences between different methods are reported in a U.S. government EPA sponsored project (EPA Quality Assurance New Letter, 1981).

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Table 1. Sensitivity of four acute toxicity screening procedures to various chemicals

Chemical	Concentration in ppm to Give Typical Endpoint Reaction to Toxicants						
	Microtox			S. volutans	Inhibition of Activated Sludge		
	5 min EC_{50}	10 min EC_{50}	15 min EC_{50}	90% inhibition 2 hr	Respiratory Activity 0.5 hr	3 hr	TTC-Dehydrogenase Test 1 hr
3,5 dichlorphenol	3.2	3.0	2.9	5.0	38	22	80
Cetyl trimethyl ammonium chloride	1.35	0.98	0.86	1.45	5.8	5.5	18.5
Sodium lauryl sulfate	3.19	2.1	1.8	4.15	188	135	48
Phenol	28	31.9	34.3	300	740	1000	1400
Copper (Cu^{++})	19.5	9.4	3.8	10	34	17	2.1
Mercury (Hg^{++})	0.064	0.049	0.046	1.0	1.3	0.96	1.5
Zinc (Zn^{++})	13.8	6.1	3.45	11.6	6.1	5.2	24

Table 2. Ranking of test chemicals in order of increasing toxicity based on four microbiological toxicity screening procedures.

Chemical	Microtox	*S. volutans*	Inhibition of Respiratory Activity	Inhibition of TTC-dehydrogenase Activity
Hg^{++}	1	1	1	1
Cetyl trimethyl ammonium chloride	2	2	3	2
Cu^{++}	6	5	2	4
Sodium lauryl sulfate	3	3	5	6
3,5 dichlorophenol	4	4	6	5
Zn^{++}	5	6	4	3
Phenol	7	7	7	7

Table 3. Ranking of tests according to relative sensitivity as assayed by seven test chemicals and as expressed by log (EC_{50}) values.

Procedures Compared		Number of assays out of 7 in which test 1 was more sensitive than test 2	Non-parametric regression analysis of log (1) versus log (2) Significance of correlation (F-test)	Wilcoxon's test for two random samples Hypothesis: log (1) log (2) level of significance
(1)	(2)			
Microtox (15 min EC_{50})	S. Volutans (EC_{90} 2 hr)	7	99.9%	90%
Microtox (15 min EC_{50})	Inhibition of Respiratory Activity (0.5 hr)	7	90%	95%
Microtox (15 min EC_{50})	TTC-Dehydrogenase Test (1 hr)	6	95%	95%
S. Volutans (EC_{90} 2 hr)	Inhibition of Respiratory Activity (0.5 hr)	6	no significance at 90% level	approx. 90%
S. Volutans (EC_{90} 2 hr)	TTC-Dehyrodgenase Test (1 hr)	6	90%	approx. 90%
Inhibition of Respiratory Activity (0.5 hr)	TTC-Dehydrogenase Test (1 hr)	5	95%	no significance at 90% level

Table 4. Comparison of Microtox EC_{50} values obtained in two laboratories.

Toxicant	5 minute EC_{50} (ppm)	
	Bulich et al*	Dutka-Nyholm-Petersen
Hg^{++}	0.065	0.064
Sodium lauryl sulfate	1.6	3.19
Zn^{++}	2.5	13.8
Cu^{++}	8.0	19.5
Phenol	25.0	28.0

* A.A. Bulich, M.W. Greene, D.L. Isenberg (1980)

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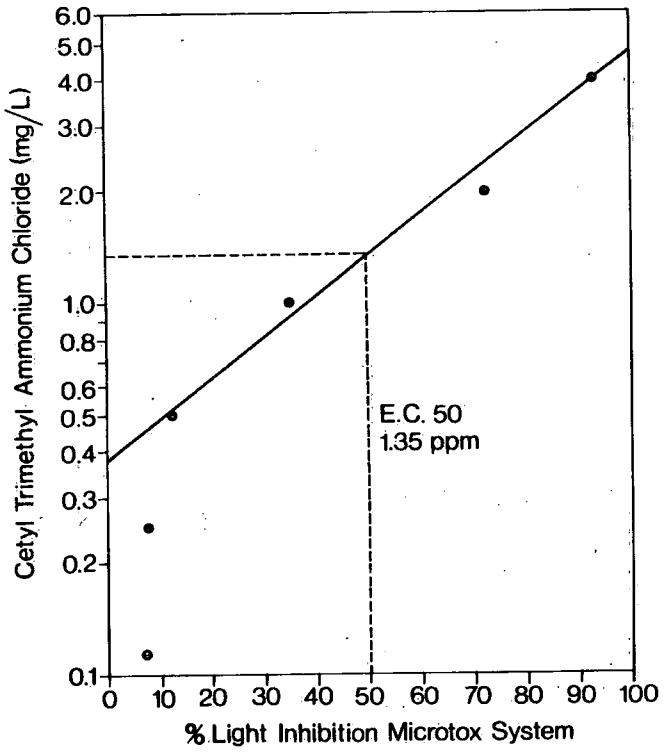

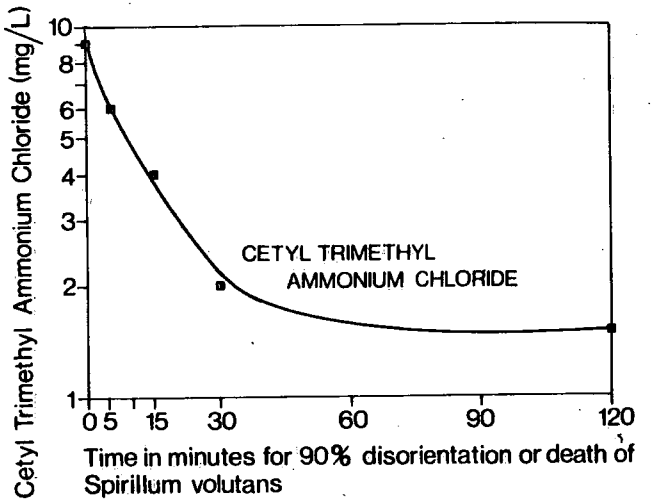

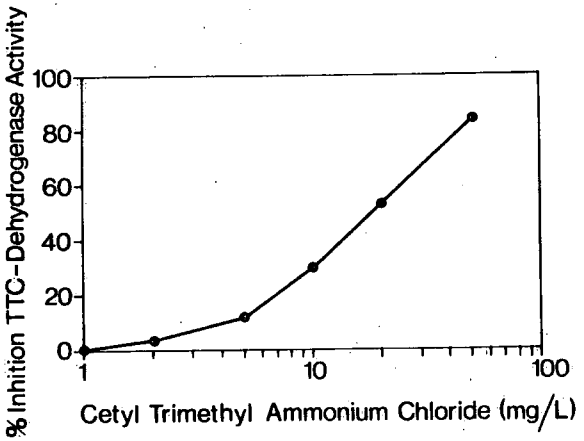

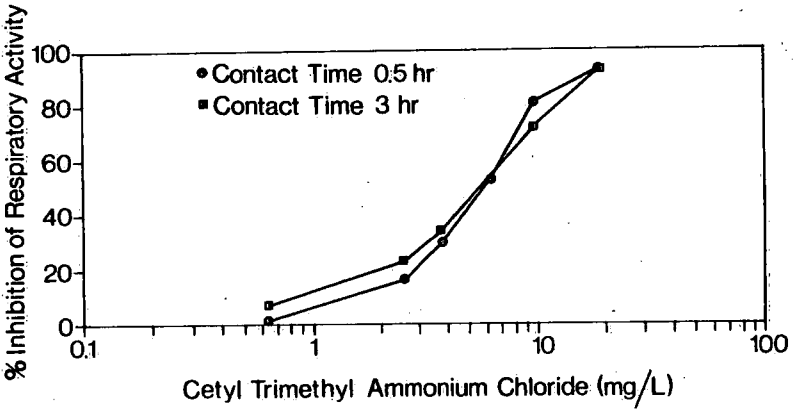

Figure 1 Example of dose response curves obtained from Cetyl Trimethyl Ammonium Chloride

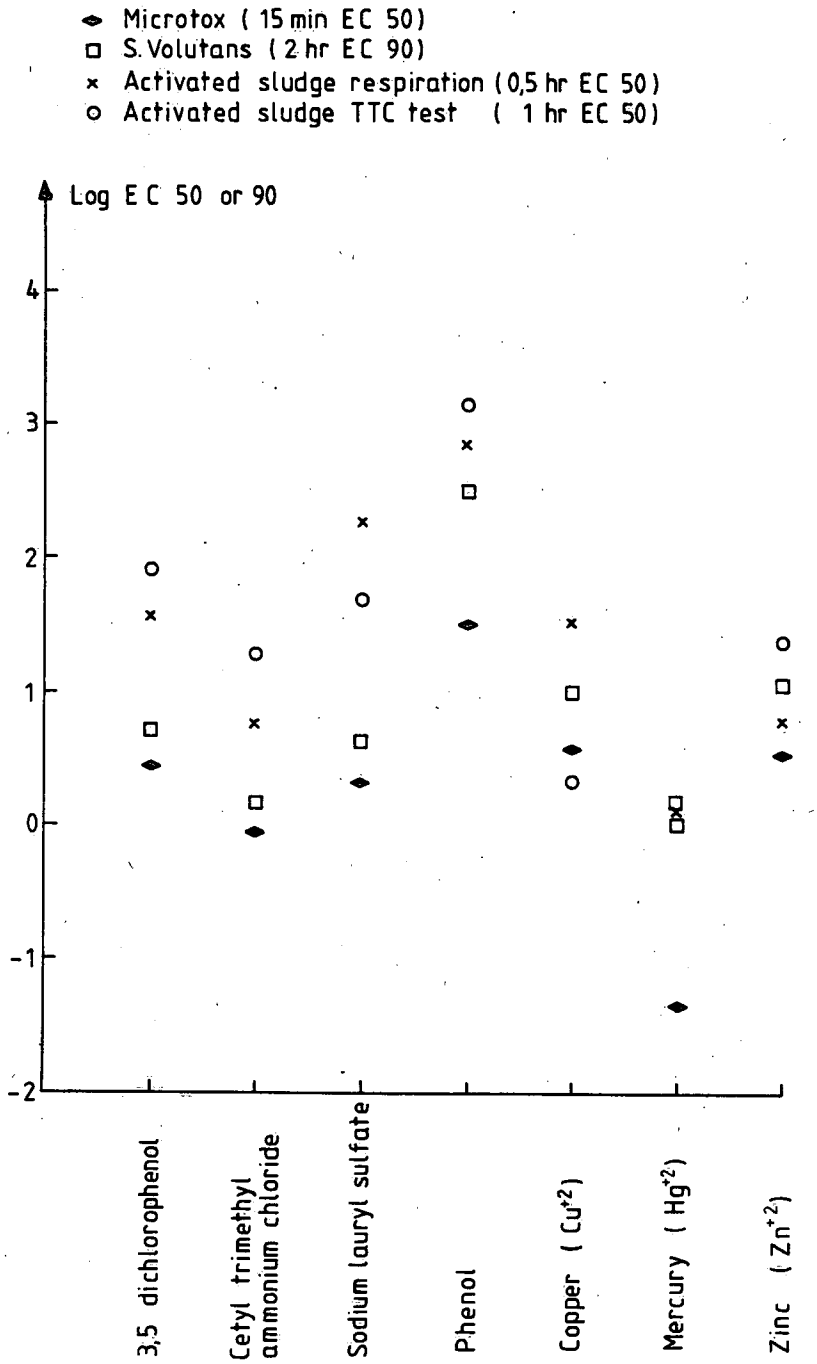

Figure 2 Graphical illustration of the ranking of seven chemicals by the four tests examined.