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Toxic Effects of Organic Solvents on the Growth of Blue-Green Algae

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Bioassays are an important means of gathering data on the potential environmental impact of various pollutants. An increased awareness of the ecological ramifications of pollution and its possible legal implications has highlighted the importance of standardizing and improving bioassay methodology to ensure the acquisition of accurate, reproducible toxicity data with which to make in situ environmental predictions. One area of concern with laboratory bioassays is the stress imposed on test organisms by organic solvents (Bowman et al. 1981). Organic solvents can make their way into the environment as industrial wastes and components of pesticide formulations. In laboratory bioassays, the use of organic solvents is often unavoidable, since many pesticides and organic pollutants have low water solubility and must be dissolved in organic solvents prior to addition into experimental systems.

Relatively few reports have been published on the comparative toxicity of solvents towards test organisms, and these deal primarily with fish and aquatic invertebrates (Majewski et al. 1978; Bowman et al. 1981; LeBlanc and Surprenant 1983). Information for microbial systems are more limited (Stratton 1985), with some data available for algae (Rowe et al. 1982) and slightly more for fungi (Stratton 1985). Aside from direct toxic effects of their own, solvents can interact synergistically and antagonistically with the toxicant in solution. This problem has been well documented with pesticides (Stratton et al. 1982; Stratton 1985), and a procedure has been developed to identify and eliminate these effects from bioassays (Stratton et al. 1982). With solvent-pesticide interactions, both the type of response and its magnitude are dependent upon the solvent and solvent concentration used (Stratton 1985). Although the U.S. Environmental Protection Agency recommends maximum allowable limits of 0.05% solvent for acute tests and 0.01% for chronic tests (U.S.E.P.A. 1975), such guidelines are often inappropriate for microbial bioassays. Microbial tests involving agar growth media, or aqueous or soil systems, often employ solvent concentrations up to 1.0%, due to problems associated with the use of small volumes of test compounds, toxicant solubility, and other technical limitations. The first step in

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choosing a solvent for use in microbial bioassays should be a detailed screening to identify solvents with inherently low toxicity to the test organism (Stratton 1985), followed by an interaction study to choose the best concentration to use (Stratton et al. 1982). The purpose of the present study was to compare the inhibitory effects of six solvents commonly used in pesticide bioassays towards five species of blue-green algae (cyanobacteria), in order to identify solvents with low toxicity for use in bioassays.

MATERIALS AND METHODS

Five species of blue-green algae were used as test cultures. Anabaena sp. ATCC 27899, A. cylindrica ATCC 29414, A. variabilis ATCC 29413, and Nostoc sp. ATCC 29105 were obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. A. inaequalis was obtained from the Department of Botany and Genetics, University of Guelph, Guelph, Ontario, Canada. Stock cultures were grown in 250 mL Erlenmyer flasks sealed with cotton bungs and containing 150 mL of an inorganic, nitrogen-free medium (Stratton et al. 1980). Flasks were incubated at 25±1°C and a light intensity of 7 klux on a 12 hour light-dark cycle.

The solvents employed included acetone, methanol, hexane (glass distilled, pesticide grade, Caledon Laboratories, Georgetown, Ontario, Canada), ethanol (absolute, Commercial Alcohols Ltd., Gatineau, Quebec, Canada), dimethyl sulfoxide (DMSO), and N,N-dimethylformamide (DMF) (reagent grade, Fisher Scientific, Fair Lawn, New Jersey, U.S.A.). All solvent concentrations are given as percent (%) volume/volume.

During bioassay experiments test organisms were cultured in test tubes (150 x 25 mm O.D.) in both the presence and absence of solvent, as outlined below. Growth in test systems was monitored by following the increase in optical density over time for 10 to 14 days using a Turner model 330 spectrophotometer equipped with a universal test tube adaptor and appropriate filters. All test tubes were optically standardized prior to inoculation. Test tubes were incubated in racks inclined at a 45° angle under the same environmental conditions as the stock cultures. The most suitable wavelength to use for monitoring the growth of each culture was determined using the method of Sorokin (1973). This was 600 nm for Anabaena sp., A. inaequalis, and Nostoc sp., and 660 nm for A. cylindrica and A. variabilis. Standard curves of optical density, adjusted for deviations from Beer's Law (Toennies and Gallant 1949), plotted against cell numbers, determined by direct microscopic counts (Burnham et al. 1973), yielded straight lines with correlation coefficients of 0.996, or better, for each culture.

Each solvent was assayed towards the growth of each of the blue-green algae tested at a minimum of 10 concentrations ranging from 0.1 to 10% for acetone, 0.1 to 8% for ethanol, 0.6 to 10% for methanol, 1.0 to 14% for hexane, 0.1 to 6% for DMSO, and 0.02 to 5% for DMF. Each solvent concentration was replicated five to ten times and all experiments were repeated three to five times.

Appropriate control systems containing no solvent were included in each experiment. Bioassay systems contained 9.5 mL of growth medium, the appropriate volume of solvent, and 0.5 mL of inoculum standardized to yield an initial cell concentration of 1.0×10^6 cells/mL. Screening experiments indicated that any effect noted in the bioassays was due to the solvent and not the dilution of growth medium by the ranges of solvent levels employed. Incubation parameters are outlined above.

In each experiment, percent inhibition values, relative to growth in control systems, were calculated daily for all solvent-culture bioassay systems using spectrophotometric data. This yielded 10 to 14 inhibition values for each solvent concentration tested. EC_{50} values (the solvent concentration required to cause a 50% reduction in growth) were calculated using linear regression analysis (percent inhibition versus solvent concentration) and the StatPac microcomputer statistics analysis package (Walonick Associates, Minneapolis, MN, U.S.A.). All correlation coefficients for linear regression, using % inhibition values and % solvent levels, were >0.900, with the majority >0.96. Significant differences within any given solvent-culture system were determined using a Dunnett's t-test at P=0.05 (Winer 1971). Significant differences between cultures or solvents were determined using an analysis of variance procedure followed by a Duncan's multiple range test at P=0.05.

RESULTS AND DISCUSSION

The solvents used as test toxicants in this study were chosen because of their widespread use in pesticide bioassays, or their presence in commercial pesticide formulations. The blue-green algal cultures chosen are common inhabitants of both aquatic and terrestrial ecosystems. Data for the effects of organic solvents towards the growth of blue-green algae are summarized in Tables 1 and 2. DMF was always the most toxic solvent tested, with EC_{50} values generally <0.05% (Table 1). However, the exact order of toxicity of test solvents was dependent upon the culture used (Tables 1 and 2). DMF caused a total inhibition of culture growth at levels >2.0 to 5.0% with all cultures except Anabaena sp., where this was observed at DMF concentrations above 0.1%. Usually the next most toxic solvent was ethanol, with EC_{50} values of 0.08 to 2.87%, but was occassionally DMSO or acetone (Table 1). Ethanol stimulated growth at levels below 0.1 to 0.4% with all of the Anabaena species, and below 1.0 to 1.5% with Nostoc. Total inhibition was observed at ethanol concentrations >1.5 to 3.0% with all cultures other than Nostoc sp., where total inhibition occurred only above 8.0% ethanol.

With most cultures, DMSO and acetone were intermediate in toxicity, yielding EC_{50} values between 0.36 and 4.38% (Table 1). Acetone stimulated growth at concentrations less than 0.1 to 1.0% with all cultures except Anabaena sp., where levels above 0.7% caused total inhibition. Total growth inhibition occurred at acetone concentrations greater than 2.0% with A. cylindrica, greater than 4.0 to 5.0% with A. inaequalis, and greater than 8.0 to 10.0%

Table 1. Comparison of solvent effects towards growth of blue-green algae.

Solvent[a] & culture	Estimated EC_{50}[b]	SE regression[c]	95% limits[d] lower	95% limits[d] upper
A. variabilis				
acetone	3.69[e]	0.611	2.49	4.89
ethanol	1.27	0.180	0.92	1.62
methanol	3.13[e]	0.504	2.14	4.12
hexane	6.58	0.724	5.16	8.00
DMSO	3.57[e]	0.638	2.32	4.82
DMF	<0.05	-	-	-
A. inaequalis				
acetone	2.75[e]	0.352	2.06	3.44
ethanol	1.02	0.078	0.87	1.17
methanol	2.68[e]	0.134	2.42	2.94
hexane	1.70[f]	0.188	1.33	2.07
DMSO	1.71[f]	0.241	1.24	2.18
DMF	0.60	0.124	0.36	0.84
A. cylindrica				
acetone	0.36[e]	0.162	0.04	0.68
ethanol	0.97[f,g]	0.060	0.85	1.09
methanol	2.57[h]	0.120	2.33	2.81
hexane	2.31[g,h]	0.816	0.71	3.91
DMSO	0.84[e,f]	0.303	0.25	1.43
DMF	<0.05	-	-	-
Anabaena sp.				
acetone	0.56[e,f]	0.132	0.30	0.82
ethanol	0.80[e]	0.071	0.66	0.94
methanol	3.12[g]	0.264	2.60	3.64
hexane	2.18[g]	0.375	1.44	2.92
DMSO	0.39[f]	0.138	0.12	0.66
DMF	<0.05	-	-	-
Nostoc sp.				
acetone	4.38[e,f,g]	1.057	2.31	6.45
ethanol	2.87[e,g]	1.251	0.42	5.32
methanol	5.48[e]	0.500	4.50	6.46
hexane	8.00[f]	1.146	5.75	10.25
DMSO	4.02[g]	0.196	3.64	4.40
DMF	<0.05	-	-	-

[a] DMSO=dimethyl sulfoxide; DMF=dimethylformamide

[b] Solvent concentration (% v/v) causing a 50% reduction in growth -calculated using regression equations of solvent concentration and percent inhibition

[c] Standard error of estimate for regression

[d] 95% limits for the estimated EC_{50} values, calculated using the standard error of estimate for regression

[e,f,g] Those EC_{50} values, within a culture grouping, that are followed by the same letter do not differ significantly at P= 0.05. Each culture is considered separately.

Table 2. Comparison of the solvent sensitivity of test cultures[a].

Test Culture	Solvent		
	acetone	ethanol	methanol
A. variabilis	3.69±1.20[b]	1.27±0.35[b,c]	3.13±0.99[b]
A. inaequalis	2.75±0.69[b]	1.02±0.15[b,d]	2.68±0.26[b]
A. cylindrica	0.36±0.32[c]	0.97±0.12[d]	2.57±0.24[b]
Anabaena sp.	0.56±0.26[c]	0.80±0.14[d]	3.12±0.52[b]
Nostoc sp.	4.38±2.07[b]	2.87±2.45[c]	5.48±0.98
	hexane	DMSO	DMF
A. variabilis	6.58±1.42[b]	3.57±1.25[b]	<0.05
A. inaequalis	1.70±0.37[c]	1.71±0.47	0.60±0.24
A. cylindrica	2.31±1.60[c]	0.84±0.59[c]	<0.05
Anabaena sp.	2.18±0.74[c]	0.39±0.27[c]	<0.05
Nostoc sp.	8.00±2.25[b]	4.02±0.38[b]	<0.05

[a] Tabular entries are the estimated EC_{50} (%v/v) values ± 1.96 times the standard error of estimate for regression (interval for the 95% limits; Table 1); DMSO= dimethyl sulfoxide; DMF= dimethylformamide

[b,c,d] Those entries in each column that are followed by the same letter do not differ significantly at P= 0.05; each solvent is considered separately

with both A. variabilis and Nostoc sp. DMSO induced total growth inhibition with all of the Anabaena species at levels >1.5 to 4.0%, but Nostoc sp. required DMSO concentrations above 6.0 to 10.0% before a similar response was noted. Hexane and methanol were generally the least toxic solvents tested, with EC_{50} values up to 8.0% (Table 1). Methanol stimulated culture growth at levels below 1.0 to 2.0%, and elicited total growth inhibition above 4.0 to 6.0% with all cultures except Nostoc sp., where a concentration of 10.0% was required for complete growth inhibition. Hexane stimulated culture growth below 1.0 to 1.5%, and caused a total inhibition of growth at levels greater than 3.0 to 4.0% with both A. inaequalis and Anabaena sp., greater than 6.0 to 8.0% with A. cylindrica, greater than 12.0% with A. variabilis, and greater than 20.0% with Nostoc sp.

Nostoc sp. was less sensitive towards test solvents than were any of the Anabaena cultures (Table 2). The least sensitive Anabaena species was A. variabilis. Anabaena sp. and A. cylindrica were generally the most sensitive cultures tested and yielded the lowest EC_{50} values (Table 2).

Few other data are available on the solvent sensitivity of blue-green algae for comparison with the results noted above. The data presented here indicate that DMF would not be a suitable solvent to use in toxicity tests involving blue-green algae, because of its high toxicity. DMF was also found to be the most toxic solvent

of six tested towards growth of soil fungi (Stratton 1985). However, DMF is considered an excellent solvent for use in tests with aquatic animals and has a low toxicity towards those organisms (Hughes and Vilkas 1983). DMF levels up to 0.1% also have no inhibitory effect on growth of the diatom Skeletonema costatum (Hughes and Vilkas 1983).

Acetone is the most common solvent used in toxicity tests involving algae (Adams et al. 1986) and is the solvent of choice in many other bioassay systems (Majewski et al. 1978). In the present study acetone had intermediate toxicity towards the test organisms. These data, together with the fact that acetone has superior solvent properties when compared with other solvents (Majewski et al. 1978), indicate that it would be a suitable choice for bioassays involving blue-green algae as long as it were used at levels well below those eliciting toxic effects. These data are comparable to those found in a similar study utilizing fungi as test organisms, where the EC_{50} for acetone varies from 2.0 to 12.0% v/v (Stratton 1985). Other studies on acetone effects yield data similar to those outlined here in Tables 1 and 2. Acetone levels <0.1 to 0.4% stimulate photosynthesis, nitrogen fixation, and heterocyst formation in Anabaena (Stratton et al. 1980; Stratton and Corke 1981a; Yee et al. 1985), and concentrations >1.0% are usually required for severe inhibition (Stratton et al. 1980; Stratton and Corke 1981a). Low levels of acetone (<0.2%) have no effect on growth of algae such as Chlamydomonas segnis (Yee et al. 1985), Chlamydomonas eugametos (Hess 1980), and Skeletonema costatum (Kleppel and McLaughlin 1980). Acetone concentrations of 3.33% can cause severe cytological damage in Chlorella pyrenoidosa (Parasher et al. 1978). Levels up to 1.0% have no effect on photosynthesis in C. pyrenoidosa and Scenedesmus quadricauda (Stratton and Corke 1981b).

DMSO was also found to be of intermediate toxicity in the present study and is regarded to be an excellent solvent. DMSO is considered to be a suitable substitute for acetone in fungal toxicity tests (Stratton 1985). This also appears to be the case here with blue-green algae. However, few data are available on DMSO effects towards algae and blue-green algae. Levels greater than 1.0% are required to cause significant growth inhibition in Chlamydomonas eugametos and concentrations above 5.0% cause total inhibition (Hess 1980).

Ethanol was quite toxic towards the test organisms (Tables 1 and 2) and would not be a suitable solvent to use in bioassays. Ethanol is sometimes used in fungal toxicity tests, but its widespread use in laboratories as a microbial biocide discourages its use in most bioassays. As well, ethanol is not very effective as a solvent in pesticide systems (Hess 1980). Ethanol is equal in toxicity to DMSO, on a percentage basis, when tested towards Chlamydomonas eugametos (Hess 1980), and less toxic than DMSO towards Chlorella pyrenoidosa (Rowe et al. 1982). Other data are limited. There is a similar lack of data for methanol and hexane effects on microorganisms. However, both would be poor choices as

a solvent in bioassays, primarily due to their inferior solvent capabilities when compared with acetone and DMSO.

In order to ensure that a given solvent does not interfere with a test toxicant's effects in bioassays, a procedure similar to that published by Stratton et al. (1982) must be followed. However, it is first necessary to choose a solvent which has low toxicity to the test organism used (Stratton 1985). Unfortunately, information on the comparative effects of solvents towards microbial test systems is limited and more research is required to provide these data. Based upon the results presented here, acetone or DMSO would be suitable solvents to use in bioassays with blue-green algae, provided that they did not interact with the particular toxicant being tested.

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