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Toxic Effects of Organic Solvents on the Growth of *Chlorella vulgaris* and *Selenastrum capricornutum*

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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 unavoidable since many pesticides and organic pollutants have low water solubilities and need to be dissolved in organic solvents prior to addition into experimental systems. So, one area of concern with laboratory bioassays is the stress imposed on test organisms by organic solvents (Bowman et al. 1981). Most reports on the comparative toxicity of solvents towards test organisms deals with the effects of solvents on fish and aquatic invertebrates (Majewski et al. 1978; LeBlanc and Surprenand 1983) with some data available for blue-green algae (Stratton 1987) and green algae (Stratton and Smith 1988). The US 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) but, in the literature, the nature of the solvent and the final concentration used vary among the different authors and are often higher than EPA limits due to problems associated with the use of small test volumes and toxicant solubility. Organic solvents can cause toxic effects on their own, but it has been also reported that they can interact with pesticides to alter toxicity (Stratton et al. 1980; Stratton and Corke 1981). The first step in choosing a solvent for use in bioassays should be a detailed screening to identify solvents with inherently low toxicity to the test organism (Stratton 1987), followed by an interaction study (pesticide and solvent interactions) to choose the best concentration to use (Stratton et al. 1982).

The purpose of this study is to compare the inhibitory effects of four solvents commonly used in pesticide bioassays towards growth of two green algae, Chlorella vulgaris and Selenastrum capricornutum.

MATERIALS AND METHODS

Two green algae were used as test cultures; Chlorella vulgaris was isolated from the Lake Geneva (Switzerland, France) by M. Feuillade in 1980; Selenastrum capricornutum (Printz) was obtained from the Environmental Protection Agency (EPA, Corvallis, Oregon). Both cultures were axenic and were grown in the same AAP (Algal Assay Procedure 1971) medium with the following modification: $NaHCO_3$ was increased to 15 mg/l to give sufficient carbonate for growth in

capped tubes, and K_2HPO_4 was increased to 12 mg/l. The stock cultures were grown in 500 ml flasks with 250 ml of algal suspension media (with a sterile air bubbling system) and incubated at 21° +/- 1°C (standard temperature of the culture room) under continuous illumination of 100 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The solvents tested included ethanol (95%), methanol (100%), dimethyl sulfoxide (DMSO, 99.9% HPLC grade), and dimethyl formamide (DMF, 99.9% HPLC grade). All solvent concentrations are given as percent (%) volume/ volume.

During solvent bioassays experimental test organisms were cultured in test tubes (20 x 125 mm) in both the presence and absence of solvent, as outlined below. Growth in the test systems was monitored by following the increase in chlorophyll (a) content over time for 5 days using a Turner Model 111 Fluorometer equipped with a blue light source, blue excitation, and red emission filters. For each alga, calibration curves were drawn between fluorescence (arbitrary units) and chlorophyll (a) content (µg/l). The correlation coefficients were higher than 0.99 for both calibration curves. The tubes were placed on a rotative incubator to prevent clumping and to obtain the same illumination as the stock cultures. The experiments were conducted until the algae reached their stationary phase. Each solvent, tested at a number of 5 concentrations ranging from 0.05 to 1%, was assayed towards the growth of each of the chlorophyceae. Each solvent concentration was replicated three times. Appropriate control systems containing no solvent were included in each experiment. Bioassay systems contained 20 ml of growth medium, an appropriate volume of solvent, and an inoculum standardized to yield an initial chlorophyll (a) content of 5 µg/l. The tubes were kept at very low light intensity (1.5 $\mu E \cdot m^{-2} \cdot sec^{-1}$) 20 minutes before and also during the measurements of fluorescence to induce a steady state of the algae. In each experiment, chlorophyll (a) content was determined daily. Percent inhibition values, relative to growth in control systems, were calculated on day 4.

Throughout this communication the term « significance » refers to a Student T-test analysis for a comparison of chlorophyll (a) mean values at the confidence level of $P = 0.05$.

RESULTS AND DISCUSSION

The four solvents selected as tests toxicants in this study were chosen because of their widespread use in pesticide bioassays, or their presence in commercial pesticide formulation. The five solvent levels studied were chosen because they are widely used in bioassays. The two chlorophyceae chosen are commonly used as test algae in ecotoxicological bioassays and have been studied in the laboratory since 1980. Chlorella vulgaris is also a common inhabitant in aquatic ecosystems.

Table 1. Comparison of solvent effects towards growth of Chlorella vulgaris and Selenastrum capricornutum.

	Chlorella vulgaris				Selenastrum capricornutum			
Solvent	ethanol	methanol	DMSO	DMF	ethanol	methanol	DMSO	DMF
0.05%	37*	7	-1	-7	14*	7	10	-5
0.1%	54*	4	-5	-29*	19*	5	9	-14*
0.2%	69*	33*	1	-8	26*	23*	10	7*
0.5%	86*	69*	-4	7	37*	21*	13	38*
1%	95*	83*	1	32*	48*	42*	12	76*

Table entries are mean percent inhibition values of growth calculated from chlorophyll (a) content in the control on day 4. The percent inhibition values that are followed by a star significantly differ from the control (P = 0.05, using a Student test).

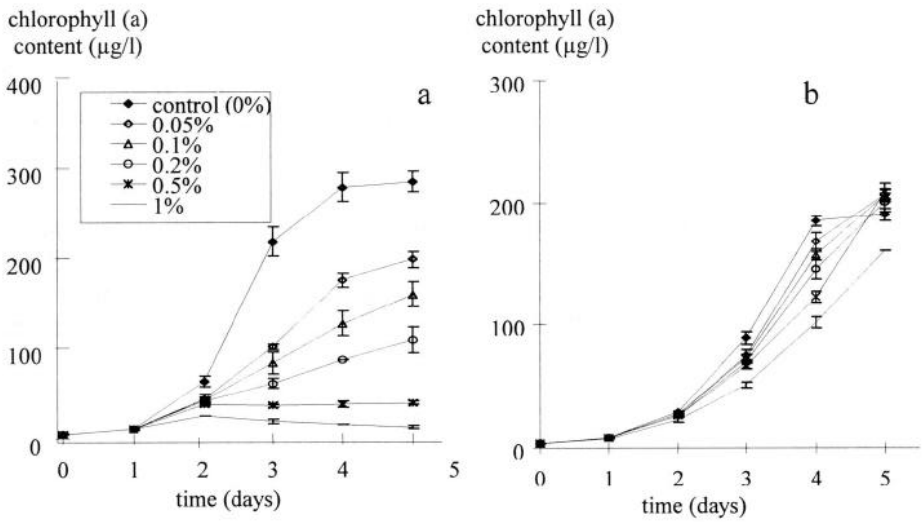

Figure 1. Growth response of Chlorella vulgaris (a) and Selenastrum capricornutum (b), expressed as chlorophyll (a) content after treatment with ethanol. Means are plotted with error bars that indicate confidence interval (P = 0.05, using a Student test).

Ethanol was the most toxic of the solvents tested for both algae. At 0.05% ethanol, the inhibition of growth in C. vulgaris was of about 37%; 86% and 95% inhibitions were observed at concentrations of 0.5 and 1%, respectively (Figure la and Table 1). S. capricornutum was least affected where the lowest concentration of ethanol caused a 14 % inhibition of the growth (Table 1) and little inhibition at highest concentrations (Figure lb). Maximum inhibition of S. capricornutum growth was 48% with 1% level of ethanol.

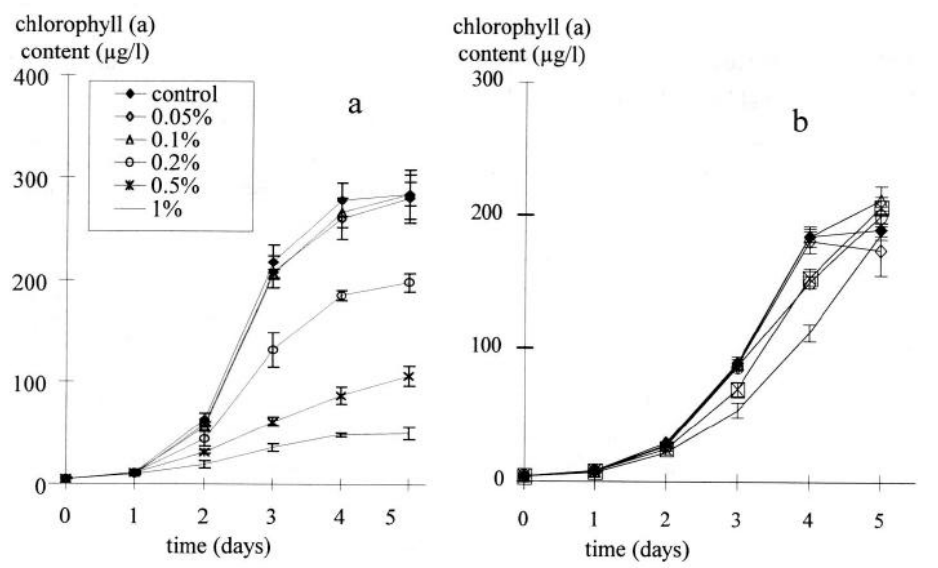

Figure 2. Growth response of Chlorella vulgaris (a) and Selenastrum capricornutum (b), expressed as chlorophyll (a) content after treatment with methanol. Means are plotted with error bars that indicate confidence interval (P = 0.05, using a Student test).

Methanol was intermediate in toxicity with both cultures. The growth of C. vulgaris and S. capricornutum was not significantly affected at concentrations less than 0.1% (Figure 2). As with ethanol, S. capricornutum was least sensitive to methanol; inhibition of growth was of 83% for C. vulgaris and 42% for S. capricornutum at 1% methanol (Table 1).

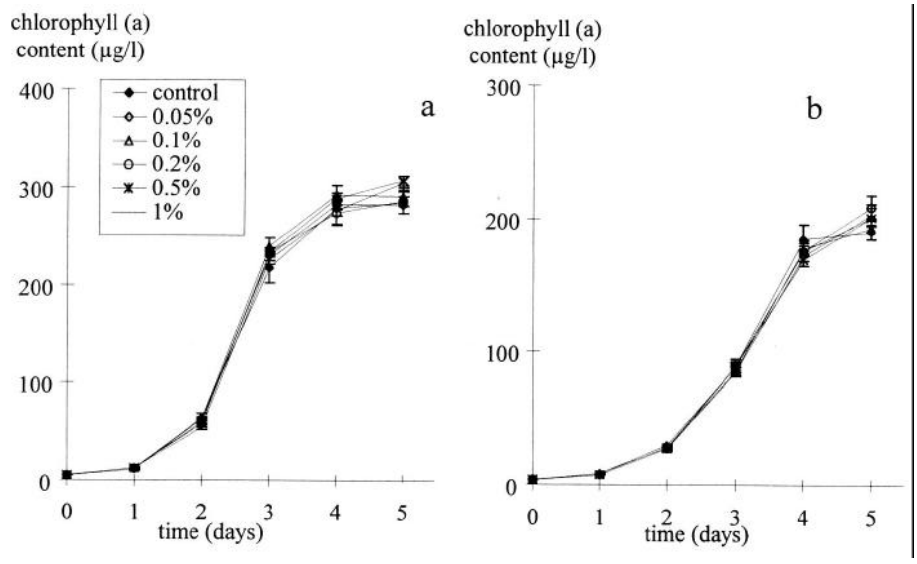

Figure 3. Growth response of Chlorella vulgaris (a) and Selenastrum capricornutum (b), expressed as chlorophyll (a) content after treatment with dimethyl sulfoxide. Means are plotted with error bars that indicate confidence interval (P = 0.05, using a Student test).

DMSO was the least toxic solvent examined for both chlorophyceae (Figure 3). Over the range of concentrations studied, there was no significant effect, stimulation or inhibition of either alga species.

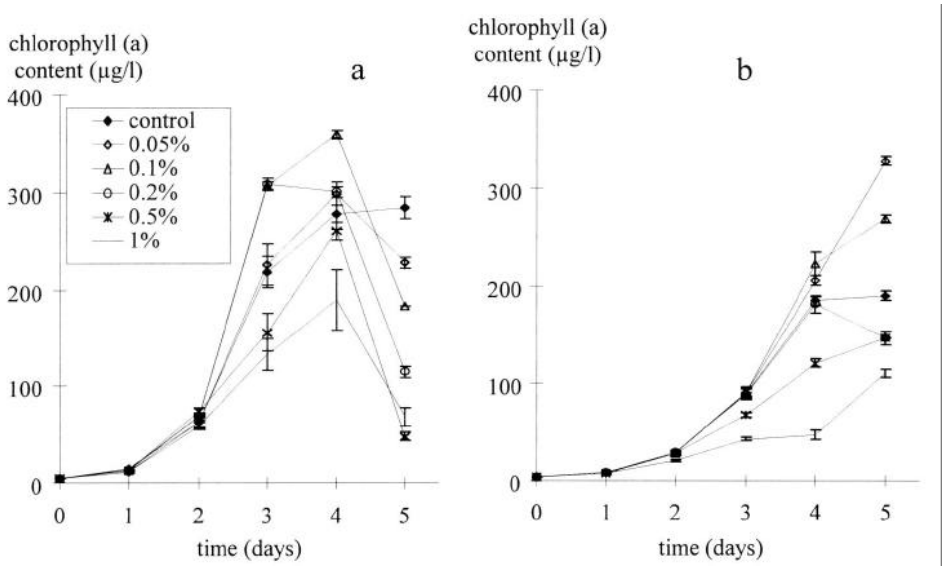

Figure 4. Growth response of Chlorella vulgaris (a) and Selenastrum capricornutum (b), expressed as chlorophyll (a) content after treatment with dimethyl formamide. Means are plotted with error bars that indicate confidence interval (P = 0.05, using a Student test).

DMF was the only solvent which stimulated growth compared to the control (Figure 4). This stimulating effect mostly appeared at 0.1% concentration for the two chlorophyceae, whereas the significant inhibiting effects appeared at 0.5%. The stimulation was of about 29% for C. vulgaris and 14% for S. capricornutum. The inhibiting effects were greater for S. capricornutum than for C. vulgaris. By day 5, the C. vulgaris control reached the stationary phase but the different treatments showed a net decrease of chlorophyll (a) content (Figure 4a); we observed a bleaching phenomenon correlated with chlorophyll destruction. The same observation was made for S. capricornutum on day 6 (data not shown).

Few data are available in the literature on the solvent sensitivity of chlorophycea. As shown in this study, the solvent effects vary depending on the concentrations selected as well as on the species used in the bioassay (Figures 1 and 2). Similar effects were also observed by Stratton et al. (1980) who showed a variability in the effects with different blue-green algae species grown in the presence of acetone.

In our study, C. vulgaris was generally more sensitive to the solvents tested than S. capricornutum except for DMF. The data presented here indicated that DMF would not be a suitable solvent to use in toxicity tests involving either C. vulgaris and S. capricornutum, because it caused cell wall damage with chlorophyll destruction after a few days contact (5 to 6 days). Parasher et al. (1978) observed that cells of Chlorella pyrenoidosa treated with concentration of acetone above 3%

showed damage of the outer membrane of the cells leading to an increase of its permeability. Solvents like acetone, ethanol, methanol, DMSO, and DMF are often used for photosynthetic pigment extraction (Marker et al. 1980; Hains 1985; Suzuki and Ishimaru 1990) but usually at high concentrations (for example, 80 to 90% ethanol, acetone or methanol). However it seems possible that at lower concentration these solvents could cause cells injuries. DMF should be avoided with blue-green algae because of its high toxicity (Stratton 1987). DMF was found to be the most toxic solvent of six tested towards growth of soil fungi (Stratton, 1985). Hughes and Vilkas (1983) reported that the highest concentration tested that had no significant effect upon maximum standing crop (cell/ml or mg/l dry weight) for S. capricornutum exposed to DMF was 0.5 ml/l (0.05%) because 1 ml/l (0.1%) caused a significant increase in dry weight. The present study confirmed these last result. The data presented here also indicate that ethanol would not be a suitable solvent to be used in routine tests because of its high toxicity at concentrations as low as 0.05%, especially for C. vulgaris. Ethanol is often used in toxicity tests but is not effective as a solvent in pesticide systems (Hess 1980). The results strongly depend on the species considered. Indeed, Rowe et al. (1982) found that this solvent was least toxic than DMSO towards Chlorella pyrenoidosa and equal in toxicity towards Chlamydomonas eugametos. Stratton and Smith (1988) also indicated that ethanol would not be a suitable solvent to use in toxicity bioassay involving blue-green algae because of its high toxicity. There are two essential drawbacks when using ethanol in laboratory bioassays; first, it is not very effective as a solvent in pesticides, and secondly, as it is volatile, the pesticide concentration can increase with time.

The present study showed that methanol could be used at 0.05 or 0.1% level with C. vulgaris and S. capricornutum. Above these concentrations, methanol becomes toxic for both species. According to the literature, this solvent is often used in bioassays but would be a poor choice for use in toxicity bioassays because of its inferior solvent capabilities when compared to DMSO. There is a lack of data for ethanol and methanol effects on microorganisms.

Among the solvents used in this study, DMSO was found to be the most suitable to use in further experiments using C. vulgaris or S. capricornutum. Indeed, our results indicate that all the concentrations tested gave no toxic effects, suggesting that C. vulgaris and S. capricornutum are resistant to DMSO. According to Hess (1980), levels of DMSO greater than 1% are required to cause significant growth inhibition in Chlamydomonas eugametos and concentrations above 5% cause total inhibition. Stratton and Smith (1988) showed that DMSO was less toxic than ethanol or DMF towards C. pyrenoidosa. DMSO was also regarded to be an excellent solvent with blue-green algae. Contrary to ethanol, this solvent does not evaporate so there is probably less risk for an increase in pesticide concentration with time. However, when this solvent is mixed with herbicides, it becomes toxic for man as it facilitates penetration of herbicide into the skin. Additively, before using this solvent, we have to ensure that the interactions between this solvent and the compound tested did not give toxic effects.

These results confirmed that solvent choice in ecotoxocological assays is of great importance. Indeed, before each experiment using compounds which have to be dissolved in organic solvents, it is necessary to ensure that the solvent alone does not alter alga growth. Controls have to be prepared with the selected solvent, and it is recommended the lowest concentration of solvent be used. Based upon the present results, methanol at concentrations up to 0.1% and DMSO up to 1% would be suitable solvents to use in bioassays with Chlorella vulgaris and Selenastrum capricornutum.

As solvents can interact with pesticides, it is recommended to ensure that the toxicity of the pesticides is not altered by the solvent selected. Indeed, it is recommended that the solvent chosen be subjected to evaluation using an interaction technique (Stratton et al. 1982), in order to choose the solvent concentration that interacts additively with the test compound used. More research is required to provide sufficient informations about the effects of solvents on microorganisms.

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