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Effect of N,N-Dimethyl Formamide Used as Organic Solvent on Two Species of Green Algae *Chlorella*

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In order to make many water-insoluble chemicals, such as pesticides, PAHs and other organic compounds dissolve efficiently in experiment systems, organic solvents are widely employed as solubilizer in industry or laboratory bioassays. Most reports on comparative toxicity of solvents towards test organisms deal with the effects of solvents on fish and aquatic invertebrates (Leblance and Surprenant 1983), with some data available for blue-green algae (Stratton 1987) and green algae (Stratton and Smith 1988; EI Jay 1996; Hughes and Vilkas 1983). The US EPA recommends maximum allowable limits of 0.05% solvent for acute tests and 0.01% for chronic tests (USEPA 1975), but these recommendations apply to aquatic toxicity tests employing fish and macroinvertebrates. It is necessary to choose a proper organic solvent in experiments for lessening the influence of organic solvent on toxic test and measuring accurately the toxicity of organic matter in microbial test systems, due to the problems associated with the use of small volumes of test compounds, toxicant solubility, the nature of the solvent and other technical limitations (Stratton 1987). The final concentration used varied even up to 1% among the different authors (EI Jay 1996; Hughes and Vilkas 1983). It is possible that one level of solvent may affect the overall response elicited by a chemical, while another concentration of the same solvent would not (Stratton 1980). N, N-Dimethyl formamide (DMF) is an excellent solvent commonly used in production of chemical engineering and for aquatic toxicological studies (Hughes and Vilkas 1983).

This study investigated the effects of DMF on the growth and photosynthetic pigments of *Chlorella protothecoides* and *Chlorella pyrenoidosa*, in order to assess the toxicity of DMF used in pesticide or other lipophilic compound bioassays.

MATERIALS AND METHODS

Chlorella pyrenoidosa was axenic and was grown in liquid medium containing (g/liter) 0.04 Ca $(NO_3)_2$, 0.01 K_2HPO_4, 0.025 $MgSO_4$, 0.02 Na_2CO_3, 0.025 Na_2SiO_3, 0.03 citric acid and 0.03 ferric citrate. *Chlorella protothecoides* was maintained in

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a liquid sterilized medium previously described by Grant and Hommersand (1974).

The stock cultures were grown in 500mL flasks with 250mL of algal suspension media and incubated at 23±2°C under the fluorescent light, illumination of intensity nearly 80 μ $Em^{-2}s^{-1}$ for 14:10 light and dark period, respectively.

On the seventh day (exponential phase), DMF (99.9% analytical grade) was added to the culture medium to provide the desired test concentrations of 0.05%, 0.20%, 0.50%, 0.75% and 1.00% (volume/volume). Untreated samples of medium without DMF served as controls. In culture medium, initial algal cell concentrations were 10^5 cell/mL Each concentration of DMF to two species of algae was replicated three times. During the experimental period, samples were withdrawn after organic solvents treatment at 0, 1, 2, 3, 4 and 5 days for the determinations of algal cell number and at 1, 2, 3 and 5 days for photosynthetic pigment content.

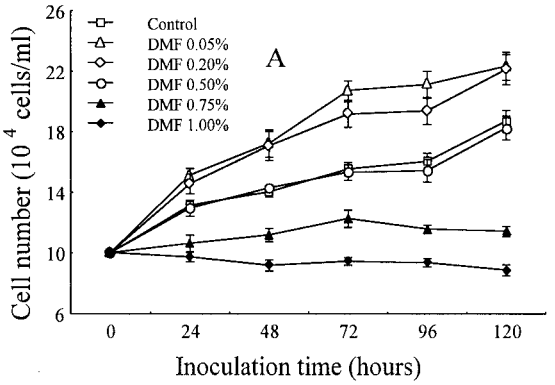
The growth rates of algae were measured by counting the cells density of the culture with a hemacytometer and microscope on test days. Inhibitory concentrations EC_{50} were determined by weighted non-linear regression analysis directly on the data using the Weibull equation to describe the concentration-response relationship (Nyholm et al. 1992). The absorption spectrum of the supernatant was measured spectrophotometrically in the wavelength from 350nm to 750nm by vis-UV spectrophotometric (Shimadzu, Japan. UV-1601). At the same time, the values of the absorption at 665, 649, 440nm were recorded for the quantification of chlorophyll (Chl) *a*, *b*, *a*+*b* and carotenoides (Caro)with the equations (Harris 1989).

Chl (*a*+*b*) =6.10 (A_{665})+20.04 (A_{649}) Chl *a* =13.70 (A_{665})–5.76 (A_{649})

Chl *b* =25.80 (A_{649})–7.60 (A_{665}) Caro =4.2 (A_{440})–0.27 [chl (*a*+*b*)]

RESULTS AND DISCUSSION

The cell numbers of both species of algae were plotted in Figure 1. Each curve represents the average value of three replicates. It was evident that lower concentrations of DMF induced significant increase in the growth rate of *C. pyrenoidosa.* Many previous studies indicated that some kinds of organic contaminants, which contain C, N, P and S elements, such as herbicides (Shabana and Abou-Waly 1995) and insecticides (Tian and Liu 1997) were always supplied as nutrition sources and stimulated the growth of the algae in low concentrations. DMF contains C, N atoms. It could be degraded into more simple substances by algae, which can further react to form CO_2 and other photolytic degradation by-products may serve as an additional carbon and nitrogen source for algal cells and could account for the stimulated growth of *C. pyrenoidosa* at low concentration.



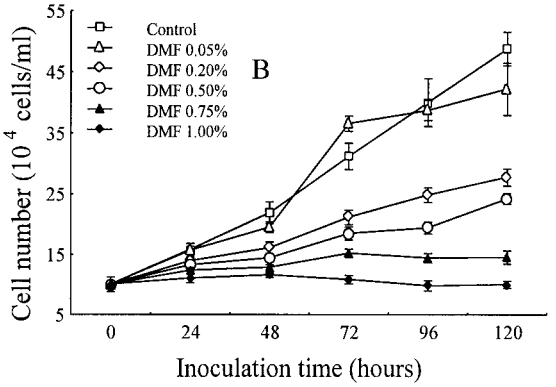


Figure 1. Growth response of *Chlorella pyrenoidosa* (A) and *Chlorella protothecoides* (B) expressed as the cell number after treatment with DMF

This hypothesis was similar to Rousch and Sommerfeld (1998) who supposed that low concentration of methyl *tert*-butyl ether (MTBE) enhanced *Selenastrum capricornutum* growth. In this case MTBE may be attributed to as an additional carbon source. In similar experiments, EI Jay (1996) found that the stimulating effect appeared at 0.1% DMF concentration for *C. vulgaris* and *S. capricornutum*, whereas the significant inhibiting effects appeared at 0.5%. The degree of stimulation was about 29% for *C. vulgaris* and 14% for *S. capricornutum*, which was less than that of the present study.

Chlorella pyrenoidosa was so heavily affected by 0.75% DMF, the inhibition ratio of the growth rate ranged from 54% to 82%. With 1% DMF, the growth of *C. pyrenoidosa* ceased and the number of algal cells decreased ($\mu<0$) (Figure 1). But low concentrations of DMF didn't show stimulation effect on the growth of *C. protothecoides*. Merely at concentration of 0.05% DMF, the growth rate of *C. protothecoides* had no significant alteration. The other *C. protothecoides* samples were inhibited by DMF above 0.05%. As DMF concentration increased, the effect of DMF became more pronounced.

The EC_{50} values calculated for DMF on *C. pyrenoidosa* and *C. protothecoides* ranged from 0.60% to 0.93% and from 0.45% to 0.62% during the experiment and

generally decreased with time (Table 1).

Table 1. Variation in sensitivity of *C. protothecoides* and *C. pyrenoidosa* to DMF (%) expressed as EC_{50} at different duration of exposure (mean of three replicates). Figures in brackets are 95% confidence limits.

Species	Test duration (hr)					Mean
	24	48	72	96	120	
C. pyrenoidosa	0. 93 (0. 13)	0. 74 (0. 11)	0. 71 (0. 07)	0. 68 (0.08)	0. 60 (0.05)	0. 73 (0.12)
C. protothecoides	0. 62 (0. 09)	0. 59 (0.08)	0. 53 (0.09)	0. 48 (0.06)	0. 45 (0.05)	0. 54 (0.10)

The mean EC_{50} values were 0.73% and 0.54%, respectively. *C. protothecoides* was more susceptible to DMF than was *C. pyrenoidosa*. Stratton and Smith (1988) reported that the EC_{50} of DMF towards growth in the *C. pyrenoidosa* was 0.94%.

After 48 and 120 hr inoculation in different concentrations of DMF, the peaks of the photosynthetic pigments in the both species of algae could be detected at blue (440nm) and red (670nm) areas in their absorption spectrum except for that of *C. pyrenoidosa* in 1% DMF after 120 hr. The absorption value of *C. pyrenoidosa* pigments in 0.2% DMF was stronger than those in control and in 1% DMF at 72hr and 120hr (Figure 2). It also showed clearly that the growth of *C. pyrenoidosa* wasstimulated by low concentration of DMF and inhibited by higher concentration. For *C. protothecoides*, the strength of absorption decreased as DMF concentrations increased (Figure 3). Though EC_{50} value of DMF to *C. protothecoides* was much smaller than that to *C. pyrenoidosa*, the absorption peaks of pigments in *C. protothecoides* were always clearly detected, even exposed to 1% DMF after 120 hr inoculation. Additionally, there existed an obvious difference at 460 nm in pigment absorption spectrum between *C. pyrenoidosa* and *C. protothecoides*. From this result, we considered that the cell division of *C. protothcoides* might be more sensitive to DMF than that of *C. pyrenoidosa*. However the resistance of the former mature cell was stronger than the latter under the toxic press of DMF. The idea that the algal species in this study was differentially sensitive to DMF is supported by many other studies, which showed that algae under similar environmental conditions are uniquely affected by toxins (Rousch and Sommerfeld 1998).

Pigment content may be a satisfactory method of toxicity assessment. In our study, the contents of chlorophyll (*a*+*b*), *a*, *b* and the carotenoid pigment in all cultures during five days had been determined (Table 2,3). Chlorophyll (*a*+*b*) contents in *C. pyrenoidosa* exposed to 0.2% DMF were higher than those in the control or in 1% DMF. In accordance with decreasing of alga growth rates, *C. protothecoides* exhibited an important reduction on chlorophyll (*a*+*b*) contents as DMF concentration increased.

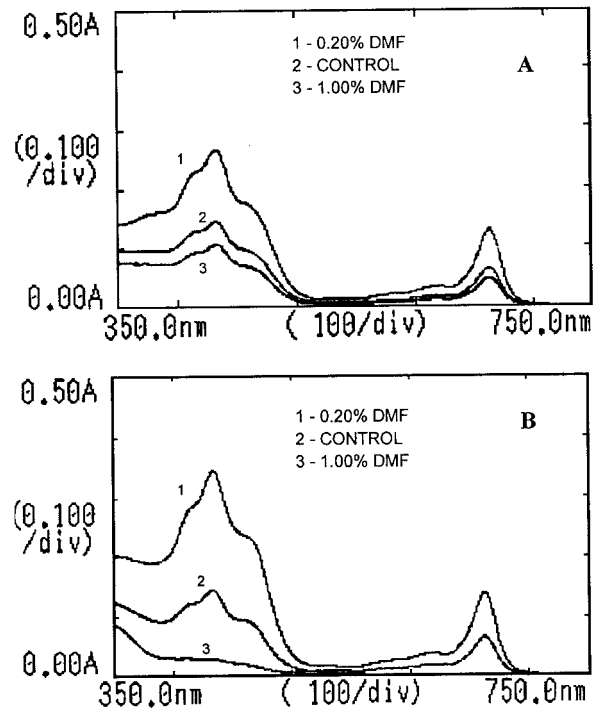


Figure 2.
Absorption spectrum of photosynthetic pigments in *C. pyrenoidosa* after 72 (A) and 120 hr (B)

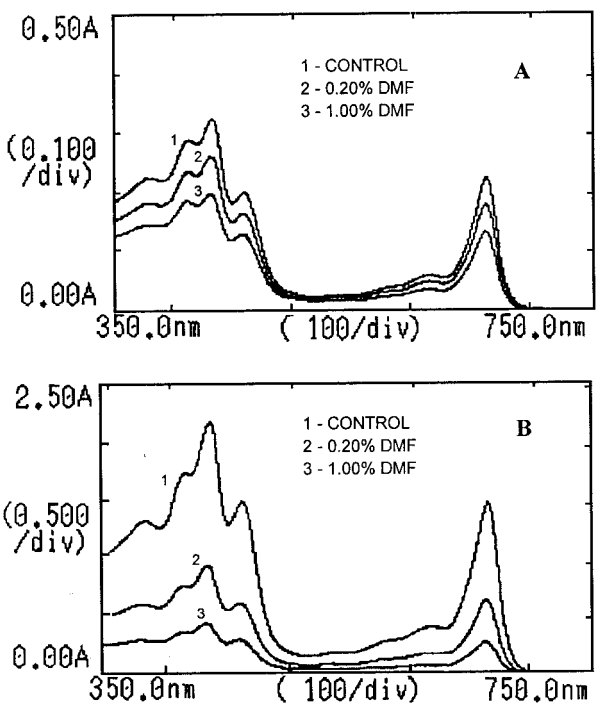


Figure 3.
Absorption spectrum of photosynthetic pigments in *C. protothecoides* after 72 (A) and 120 hr (B)

No significant difference was found in value of chlorophyll (*a*+*b*) and carotenoid among controls, 0.2% and 1% DMF to both algae during inoculation time except that the lowest ratio occurred in the 0.2% DMF to *C. pyrenoidosa* and 1% DMF to *C. protothecoides* on the fifth day. On comparing the chlorophyll (*a*+*b*)/caro values in two species of algae, the values in *C. protothecoides* were always 1.7-fold as much as those in *C. pyrenoidosa.* This showed that the chlorophyll synthesis in *C. protothecoides* was more efficient than that in *C. pyrenoidosa.*

Table 2. Algal pigment contents, values of chl *a*/*b*, chl (*a*+*b*)/carotenoides in *C. pyrenoidosa* in the control and the cultures treated by 0.2% and 1% DMF.

Time		Pigments contents (mg/g dry weight)				Ratio	
Simple		chl (*a*+*b*)	chl *a*	chl *b*	caro	chl *a*/*b*	chl(*a*+*b*)/ caro
24hr	Control	2. 4	2. 0	0. 5	0. 7	4. 19	3. 30
	0.2%	3. 1	1. 5	0. 6	0. 9	3. 97	3. 28
	1%	2. 0	1. 6	0. 4	0. 6	3. 96	3. 48
48 hr	Control	9. 6	7. 5	2. 0	2. 9	3. 69	3. 32
	0.2%	19. 1	14. 5	4. 6	5. 8	3. 11	3. 31
	1%	7. 9	6. 1	1. 9	2. 3	3. 25	3. 48
72 hr	Control	7. 3	5. 5	1. 8	2. 5	3. 01	2. 92
	0.2%	18. 9	14. 5	4. 4	6. 1	3. 30	3. 11
	1%	6. 1	4. 2	2. 0	2. 0	2. 13[b]	3. 12
120 hr	Control	9. 8	7. 4	2. 5	3. 5	2. 98	2. 83
	0.2%	20. 6	14. 2	6. 4	8. 9	2. 22[a]	2. 31[a]
	1%	N.D	N.D	N.D	N.D	—	—

The values are arithmetic means (n=3) in each column; N. D: no detected;
[a] significant differences $p<0.05$; [b] high significant differences $p<0.01$

Results of this study showed that DMF led to decrease not only chlorophyll *a*, but also chlorophyll *b* and both species of algae had a different behavior in the values of chlorophyll *a*/*b* among the treated samples. DMF had marked different effects on *C. pyrenoidosa* only in the latter part of the experiment period, while *C. protothecoides* present significantly lower values of chlorophyll *a*/*b* from the 1st day to the end of the experiment under 1% DMF and from the 3rd day to 5th day under 0.2% DMF than that under controls. It was similar to reports by EL-DIB et al. (1991) who induced that chlorophyll *a* appeared to be inhibited by phenylureas more than chlorophyll *b* yielding a decrease in the chlorophyll *a*/*b* ratio. The drop in chlorophyll *a* content at higher concentrations of DMF might be attributed to the inhibition of photosynthesis and cell division. Similar effects were observed by E1 Jay (1996) who indicated that DMF could cause chlorophyll destruction as well as the cell wall damage of either *C. vulgaris* or *S. capricornutum* after a few

days contact (5 to 6 days). Conversely, the content changing of chlorophyll *a*, the most important pigment in algal cells for collecting solar energy for photosynthesis, also influenced algal growth. Most reports demonstrated that the reduction in the growth rate of algae under organic contamination might be due to a decrease in algal photosynthesis caused by inhibition of synthesis of the chlorophyll *a*. (Yan et al. 1997). It was also found that the value of chlorophyll *a/b* of two species of algae showed different time-response models. With exposing time increasing, the values of chlorophyll a/b decreased in *C. pyrenoidosa*. However, exposing time seemed unrelevant to *C. protothecoides*.

Table 3. Algal pigment contents, values of chl *a/b*, chl (*a+b*)/carotenoides in *C. protothecoides* in the control and the cultures treated by 0.2% and 1% DMF.

Time		Pigments contents (mg/g dry weight)				Ratio	
Simple		chl(*a+b*)	chl *a*	chl *b*	caro	chl *a/b*	chl(*a+b*)/ caro
	Control	10.0	7.6	2.3	1.8	3.27	5.55
24hr	0.2%	8.4	6.2	2.2	1.6	2.83	5.33
	1%	7.7	5.5	2.1	1.4	2.60[b]	5.44
	Control	33.4	25.3	8.1	6.2	3.12	5.36
48 hr	0.2%	27.3	20.1	7.1	4.9	2.82	5.58
	1%	20.6	14.8	5.8	3.7	2.55[a]	5.60
	Control	41.8	32.0	9.8	7.4	3.25	5.63
72 hr	0.2%	25.3	18.3	7.0	5.1	2.60[a]	5.01
	1%	18.5	13.1	5.4	3.3	2.44[b]	5.54
	Control	53.3	40.1	13.1	9.0	3.06	5.91
120 hr	0.2%	22.7	16.1	6.5	4.4	2.47[a]	5.21
	1%	9.8	7.0	2.7	2.3	2.56[a]	5.22[a]

Results mentioned above indicated that a suitable concentration should be chosen necessarily when DMF was used in toxicity bioassays. Low concentrations of DMF could make different effects on test organisms. Therefore, the ecotoxicological effects induced by DMF are possible a change in dominant species in ecosystem due to the species-dependent sensitivity. For instance, *C. pyrenoidosa* and *C. protothecoides*, which exist in most freshwater bodies, should have different consequences under DMF toxic pressure. A reduction of primary production related to green algae is due to partial inhibition of photosynthesis. chlorophyll *a* of both species of algae appears inhibited by DMF more than chlorophyll *b*.

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