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Growth and Aggregation Behavior of Representative Phytoplankton as Affected by the Environmental Contaminant Di-n-butyl Phthalate

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Dialkylphthalates (phthalate acid esters, PAEs) are now well-recognized as environmental contaminants. Due to their use as industrial plasticizers, di-n-butyl phthalate (DNBP) and di-ethylhexyl phthalate are the predominant molecular forms present in the environment (Meyer et al. 1973). The contamination of the environment by these substances is widespread. The surface waters of the Gulf of Mexico approach 1 ug/L (ca. 5 nM DNBP) (Giam et al. 1978). This level is still three orders of magnitude below the concentration which is acutely toxic to Artemia (brine shrimp) larvae (Hudson et al. 1981) and other aquatic species (Mayer and Sanders 1973), but with annual production rates of phthalates in the range of 5×10^8 kg/yr, the elevation of current levels of phthalates is certain to occur in coastal regions. Still, the rate of oceanic contamination cannot be easily estimated from existing data (Giam et al. 1978; Giam and Atlas 1980). Meyer et al. (1972) reported much higher concentrations in the Missouri River and as much as 0.3 ppm (ca. 1.5 uM DNBP) in Lake Superior. Such relatively high concentrations are a hazard to fresh water aquatic life, especially in regard to the reproductive capacities and the known sensivities of embryonic aquatic organisms to phthalate esters (Autian 1973). In spite of the fact that phthaltes exhibit low chronic and acute toxicities in adult organisms, biomagnification of phthalate esters (Hudson et al. 1981), their mutagenic and teratogenic potential, and the sensitivities of embryonic and early developing organisms to phthalate ester intoxication indicate the need for much further study of their envirnomental distribution, fate and mechanisms of action.

Our continuing efforts to characterize the molecular basis for the developmental-stage-dependent phthalate ester toxicity in the brine shrimp, Artemia, (Hudson et al. 1981) led us to consider a number of microorganisms as foraging species for Artemia. The sensitivity of these microorganisms to phthalate esters was

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surprising and suggests that the nature and distribution of fresh water phytoplankton may already have been significantly altered by phthalates. The purpose of our experiments was to demonstrate the extent to which present levels of oceanic contamination by phthalates may be approaching those necessary to affect the distribution and survival of phytoplankton in the biosphere.

MATERIALS AND METHODS

Algae or diatoms were supplied from stock cultures maintained by the Marine Biology Laboratory at California State University Long Beach or were obtained from the Carolinia Biological Supply Co. (Burlington, NC). All microorganisms were grown under constant illumination with fluorescent lights at 26°C in sterile F/2 (modified artifical sea water) media (Guillard and Ryther, 1962) in 250-mL Erlenmeyer flasks capped with cotton plugs. Growth rates were determined using a Reichart-Neubauer hemocytometer. Experiments were conducted with the green algae Dunaliella parva , the diatom, Thalassioria pseudomona, and the blue-green algae, Synechococcus lividus. Species which exhibited growth of irregularly shaped or filamentous aggregrates were abandoned. Synechococcus lividus, however, at nearly all concentrations and growth conditions exhibited formation of roughly spherical aggregating colonies. Numbers of individual organisms present in these aggegrates were estimated reproducibly using a modified hemocytometry procedure. The radius of each aggegrate appearing in the microscopic field was determined and divided by the diameter of a single organism. The radius of each aggegrate is thus given as a function of the number of organisms which fit it and was used to calculate the numbers of organisms present in each spherical aggegrate. All hemocytometric determinations of concentrations of monodispersed organisms or spherical aggegrates were carried out in triplicate. In general, growth rates were sufficiently slow that these microscopic procedures could be carried out sequentially during the same day and gave acceptable reproducibility.

Solutions of DNBP were prepared from analytical reagent grade DNBP (Sigma, St. Louis, MO). Analysis by high performance liquid chromatography (HPLC) (Hudson et al. 1981) showed a single peak with no detectable trace of the monohydrolysis product, mono-n-butyl phthalate. A primary standard of this monoester was prepared by methods previously published (Hudson et al. 1981) and shown to be distinctly separated from DNBP under the HPLC conditions employed. Analyses were conducted by reverse-phase (ODS-18 stationary phase) HPLC using an 80:20 methanol:water (v/v) isocratic solvent system. DNBP was added to the modified artifical sea water culture medium at concentrations well below its solubility limit (ca. 100 uM). Rates of algae or diatom growth were then determined using the same diluted culture microorganisms. Control rates of growth were determined over

several days by hemocytometry and compared with rates in the presence of various concenrations of DNBP. These were 10, 25, 50 and 100 uM for Thalassoiria pseudomona , 1 and 10 uM for Dunaliella parva , 10, 100 and 500 nM, 1 and 10 uM for Synecchococcus lividus . Triplicate assays were determined for each time point and error determined as an average deviation from the mean. Several sets of experiments were conducted at beginning concentrations of organisms in the range of 200-700/uL. While the results were seen to be similar, these data were not treated statistically as the experiments were primarily designed to demonstrate the approximate DNBP concentrations at which either the growth rates or aggregation behaviors of these representative organisms were altered.

RESULTS AND DISCUSSION

The growth rates at various concentrations of DNBP for the green algae, Dunaliella parva, the diatom, Thalassiosira pseudomona, and the blue-green algae, Synechococcus lividus are shown in plots in Figure 1. In all cases only the unaggregated form of the organism was counted as a function of time. Only the blue-green algae species showed sizable and DNBP-dependent variability in the amount of the organism present in culture in aggregates of variable size. However, as aggregates were in roughly spherical shape, the number of organisms present within could be directly and reproducibly estimated by a modified hemocytometry procedure. Systematic examination of Synechococcus lividus growth rates showed a complex interrelationship between the growth of the organism in its aggregrate form cf. unaggregated forms as a function of the concentration of DNBP. The data show an apparent maximum for the rate of growth of the unaggregated organism at 500 nM DNBP; however, as shown in Table 1, it is only the relative percentage of the dispersed organism which is increased at 500 nM DNBP. When both dispersed and aggegrated forms are counted, the overall rates of formation of this cyanobacterium grown in media containing between 10 nM and 1 uM DNBP, are essentially identical. Indeed, the growth rate of the organism in the absence of DNBP was significantly lower.

The alteration in the growth pattern of the Synechoccus species employed is interesting. The tendency for this cyanobacterium to aggegrate may derive from a common slime layer (Rippka et al., 1979). Relatively low concentrations of DNBP clearly stimulate the growth of the aggegrate forms or rapid aggregation of new monodispersed organisms (Table 1). Dilution of cultures containing DNBP to picomolar concentrations of DNBP with greater than 90% of the organisms in the aggegrate form allows regrowth of the cultures at rates not significantly different from naive cultures (organisms not exposed to DNBP). However, growth in the aggregate form predominates in the DNBP exposed cultures and organisms are not significantly disaggregated in the short term. At the present time is is not clear if DNBP provides a sensitive

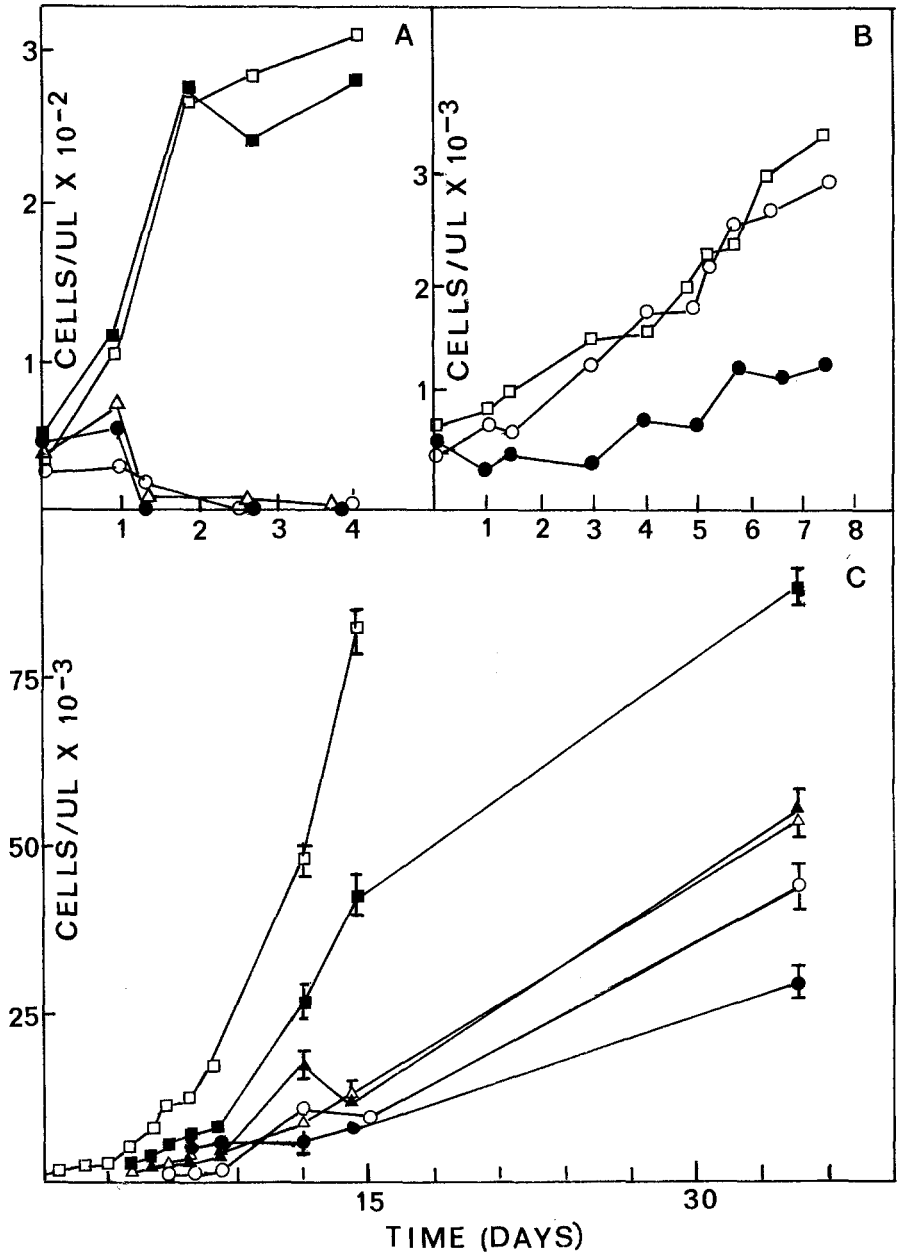

Figure 1. The growth rates of the monodispersed microorganisms Thalassioria pseudomona, panel A: control (□), [DNBP] at 10 (■), 25 (△), 50 (●) and 100 (○) uM; Dunaliella parva, panel B: control (□), [DNBP] at 1 (○) and 10 (●)uM; Synecchococcus lividus, panel C: control (□), [DNBP] 10 (○), 100 (△) and 500 (■)uM and 1 (▲) and 10 (●)uM.

signal such as the simple, though difficult to reverse, physical association with the slime layer. Alternatively, DNBP may initiate more complex changes in the organism causing it, in

Table 1. Comparison of growth of monodispersed and aggegrates of Synechococcus lividis on day 14-15 for identical starting cultures grown in the absence of and at various concentrations of DNBP

Monodispersed Mean[a]	Percent Dispersed	Aggegrate Mean[a]	Percent Aggregrate	Total Organisms	
82,500 ± 1,750	78	23,250 ± 3,000	22	105,750 ± 4,750	[b]
8,750 ± 250	5	184,500 ± 7,750	95	193,250 ± 8,000	[c]
14,750 ± 750	8	175,250 ± 14,250	92	190,000 ± 15,000	[d]
43,250 ± 2,500	21	161,250 ± 9,250	79	204,500 ± 11,750	[e]
13,500 ± 750	7	175,250 ± 32,750	94	188,750 ± 33,500	[f]

[a] average deviation from the mean of three determinations
[b] control
[c] 10 nM
[d] 100 nM
[e] 500 nM
[f] 1 uM

either event, to continue to grow in the aggregate form even after nominal removal of DNBP. Thus, DNBP was found to markedly affect the growth and/or aggegration behavior of the representative algae and diatom species employed. DNBP caused aggregation and altered growth characteristics of the blue green algae Synechococcus lividus. The diatom, Thalassisoria pseudomona, was also relatively less sensitive to the effects of DNBP.

If the sensitivity of fresh water phytoplankton rivals that observed here, then the disappearance of various aquatic species from the lakes and streams may inevitably be linked to the level of phthalates now present. As early as 1972 Meyer at al. reported micromolar level concentrations of DNBP in the Missouri River and in Lake Superior.

While other fresh water pollutants abound, phthalates will, in time, disappear from the environment, provided sources of entry are blocked. Some microorganisms can deal successfully with phthalates by metabolizing them. In bacterial systems (Englehardt and Wallnofer, 1978; Kurane et al. 1980a and 1980b) both mono- and di-alkyl phthalate hydrolyzing enzymes occur. In Norcardia these enzymes are inducible. Phthalic acid produced was subsequently oxidized to 3,4-dihydroxyphthalic acid and then to procatechuic acid. Such benzene ring opening oxidation does not appear to be a significant feature of phthalic acid ester metabolism in higher species.

Given the rate of oceanic contamination by phthalates, these substances could alter the distribution of the salt water phytoplankton such as those which have been the focus of the

studies reported here. Rates of reproduction may be subject to direct toxic effects, the mechanisms underlying which remain to be determined. Also differential effects on the rates of reproduction of the various subspecies which make up this important element of the oceanic food chain could contribute to a changing distribution of the phytoplankton populating the coastal regions of the oceans over the next several years.

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