Oxidation of Naphthalene by Cyanobacteria and Microalgae

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Eighteen different algal cultures were examined for their ability to metabolize naphthalene. The strains tested included nine cyanobacteria (blue–green algae), five green algae, one red alga and one brown alga; two diatoms were also examined. All these organisms oxidized naphthalene under photoautotrophic conditions. Experiments with [^{14}C]naphthalene showed that each organism oxidized naphthalene to at least six metabolites. One of the metabolites was identified as 1-naphthol. Under the experimental conditions used in this study the extent of naphthalene metabolism to organic-soluble derivatives ranged from 0·1 to 2·4%.

INTRODUCTION

In recent years there has been increasing concern over the fate of aromatic hydrocarbons in marine ecosystems since several of these compounds are known to exhibit toxic, mutagenic and carcinogenic properties (Daly *et al.*, 1972). Since naphthalene is a major component in the water-soluble fraction of crude and certain fuel oils (Winters *et al.*, 1976), we decided to investigate naphthalene oxidation by a variety of cyanobacteria and microalgae.

Recently, we have reported that the cyanobacteria *Agmenellum quadruplicatum*, strain PR-6 and *Oscillatoria* sp., strain JCM oxidize naphthalene to 1-naphthol as the major product (Cerniglia *et al.*, 1979, 1980). The results described in this paper suggest that the ability to oxidize naphthalene is widely distributed amongst the algae.

METHODS

Organism and growth conditions. Sources of algae, the growth media and growth temperatures are listed in Table 1. Metabolic studies were conducted in a 'closed flask' growth system as previously described (Cerniglia *et al.*, 1979, 1980).

Detection and quantification of naphthalene metabolites. After 24 h incubation, products formed by the metabolism of naphthalene were recovered as described previously (Cerniglia *et al.*, 1979, 1980). Each residue was redissolved in 0·1 ml acetone and samples (20 μl) were applied to a silica gel 60 F_{254} glass plate (E. Merck, Darmstadt, Germany). The chromatogram was developed three times in chloroform/acetone (8:2, v/v). Multiple development was necessary for the separation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) from its *trans*-isomer. Naphthalene metabolites were located on chromatograms by viewing under ultraviolet light (254 nm) and also by spraying with Gibb's reagent [2,6-dichloroquinone-4-chloroimide in methanol (2 %, w/v)].

Metabolism of [^{14}C]*naphthalene.* Incubations were carried out as described above except that [1-^{14}C]-naphthalene [35 μg, 1·0 μCi (37 kBq) in 0·1 ml 95 % ethanol] was used as the substrate. All culture volumes were 30 ml.

Detection of ^{14}*C-labelled metabolites.* Samples were prepared for chromatography as described above for experiments with unlabelled naphthalene. The radioactive extracts (20 μl) were applied to the base of a plastic thin-layer chromatography plate (Polygram Sil G/UV_{254}; Machery-Nagel & Co., Duren, Germany).

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Table 1. *Source and growth conditions of various cyanobacteria and microalgae examined for their ability to metabolize naphthalene*

Organism	Growth conditions*	Source†
Cyanobacteria		
Oscillatoria sp. strain JCM	2	C. Van Baalen
Oscillatoria sp. strain MEV	2	C. Van Baalen
Microcoleus chthonoplastes strain BA-1	2	C. Van Baalen
Anabaena sp. strain CA	1	C. Van Baalen
Anabaena sp. strain 1F	1	C. Van Baalen
Agmenellum quadruplicatum strain PR-6	2	C. Van Baalen
Coccochloris elabens strain 17a	2	C. Van Baalen
Nostoc sp. strain MAC	7	D. S. Hoare (available from C.V.B.)
Aphanocapsa sp. strain 6714	6	R. Rippka
Green algae		
Chlorella sorokiniana strain TX 71105	7	J. Myers
Chlorella autotrophica strain 580	4	R. R. Guillard
Dunaliella tertiolecta strain DUN	4	R. R. Guillard
Chlamydomonas angulosa	6	J. A. Hellebust
Ulva fasciata	8	Field collected, Port Aransas, Tex., U.S.A.
Diatoms		
Cylindrotheca sp., strain N-1	5	C. Van Baalen
Amphora sp., strain AMP-1	5	C. Van Baalen
Red alga		
Porphyridium cruentum	3	C. Van Baalen
Brown alga		
Petalonia fascia	8	Field collected, Port Aransas, Tex., U.S.A.

* Organisms were grown in Pyrex test tubes (175 × 22 mm) at 30 or 39 °C in 20 ml medium (see below). The tubes were bubbled with air enriched with $1 \pm 0{\cdot}1$ % (v/v) CO_2 and illuminated with two F20T12/D lamps placed 8·5 cm from the growth tubes on either side of the growth bath. The method is essentially a modification of that of Myers (1950). Media and growth temperatures: 1, ASP-2 (Provasoli *et al.*, 1957; Van Baalen, 1962) at 39 °C; 2, ASP-2 with vitamin B_{12} (8 μg l^{-1}) at 39 °C; 3, ASP-2 with vitamin B_{12} (8 μg l^{-1}) at 30 °C; 4, ASP-2 with vitamin B_{12} (8 μg l^{-1}) and vitamin B_1 (1 mg l^{-1}) at 30 °C; 5, ASP-2 with vitamin B_{12} (8 μg l^{-1}), vitamin B_1 (1 mg l^{-1}), NH_4Cl (1 mM) and $Na_2SiO_4.5H_2O$ (375 mg l^{-1}) at 30 °C; 6, Cg-10 (Van Baalen, 1967) at 30 °C, supplemented with 0·5 % (w/v) filter-sterilized glucose for heterotrophic (dark) growth; 7, Cg-10 at 39 °C, supplemented with 0·5 % (w/v) filter-sterilized glucose for heterotrophic (dark) growth (Bottomley & Van Baalen, 1978); 8, filtered seawater (2·5 ‰, v/v).

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Naphthalene, *cis*- and *trans*-naphthalene dihydrodiol, 4-hydroxy-1-tetralone and 1-naphthol were co-chromatographed with the radioactive metabolites. Each chromatogram was developed as described above. The standard compounds were visualized under ultraviolet light. The chromatogram was then cut into 1·0 cm strips which were placed in scintillation vials containing 10 ml Aquasol-2 (New England Nuclear). Radioactive metabolites were detected by scintillation counting in a Beckman model LS-250 spectrometer. Metabolites were also detected by autoradiography by exposing the developed chromatograms to Kodak X-ray film (blue-sensitive SB-54) for 2 to 4 weeks.

Analytical methods. Ultraviolet absorption spectra were obtained with a Beckman model 25 recording spectrophotometer. Radiochromatogram tracings were obtained with a Joyce-Loebl densitometer.

Chemicals. Naphthalene (99·95 %) was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. [1-^{14}C]Naphthalene [3·67 mCi $mmol^{-1}$ (136 MBq $mmol^{-1}$)] was from Amersham/Searle, Arlington Heights, Ill., U.S.A. *trans*-Naphthalene dihydrodiol was a generous gift from L. A. Kapicak, Union Carbide Corp., Charleston, W.V., U.S.A. *cis*-Naphthalene dihydrodiol was prepared as described previously (Jeffrey *et al.*, 1975). 4-Hydroxy-1-tetralone was also prepared as described previously (Cerniglia & Gibson, 1977). 1-Naphthol (J. T. Baker Co., Phillipsburg, N.J., U.S.A.) was purified by vacuum sublimation before use.

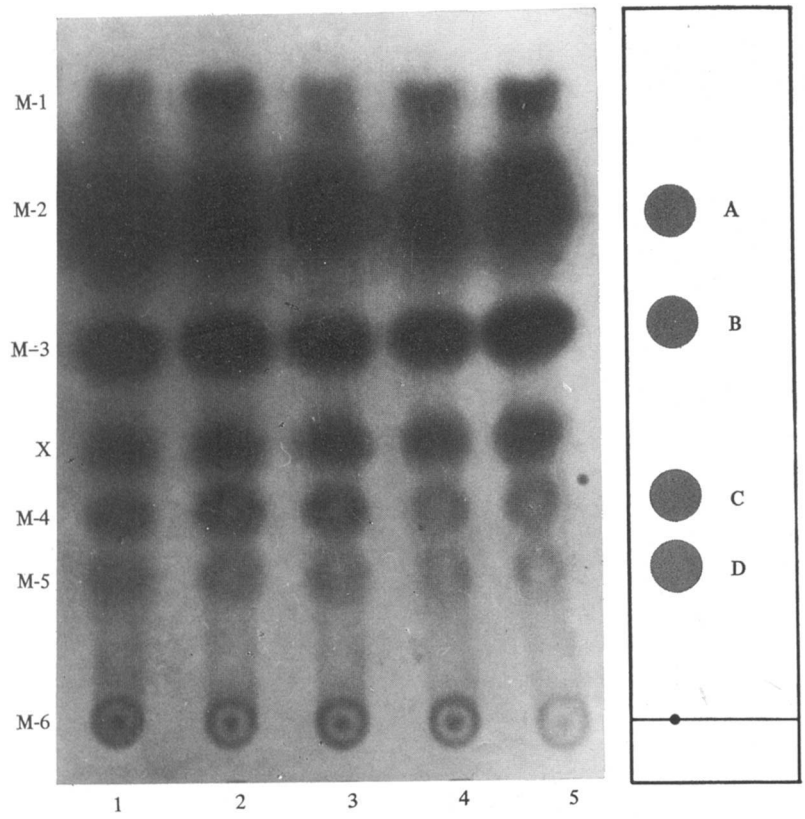

Fig. 1. Autoradiogram of metabolites formed from [1-^{14}C]naphthalene by different algae: 1, *Chlorella sorokiniana* (green alga); 2, *Chlorella autotrophica* (green alga); 3, *Porphyridium cruentum* (red alga); 4, *Cylindrotheca* sp., strain N-1 (diatom); 5, *Amphora* sp., strain AMP-1 (diatom). A, B, C and D show the mobilities of 1-naphthol, 4-hydroxy-1-tetralone, *cis*-naphthalene dihydrodiol and *trans*-naphthalene dihydrodiol, respectively. The solvent was chloroform/acetone (8:2, v/v).

RESULTS AND DISCUSSION

Eighteen organisms representing five major algal groups were screened for their ability to oxidize naphthalene (Table 1). After 24 h growth in the presence of naphthalene each culture was analysed for metabolites. All the organisms produced a compound whose chromatographic properties were identical to those of 1-naphthol. This compound gives a characteristic blue–purple colour with Gibb's reagent after exposure to ammonia vapour. Although no attempt was made to identify 1-naphthol by more rigorous criteria, we have reported its conclusive identification as a product formed from naphthalene by *Agmenellum quadruplicatum*, strain PR-6 (Cerniglia *et al.*, 1979) and *Oscillatoria* sp., strain JCM (Cerniglia *et al.*, 1980).

In order to quantify the naphthalene oxidation products formed by each organism the experiments were repeated with [^{14}C]naphthalene. Autoradiograms were prepared from each thin-layer chromatogram. The results for five organisms representing three of the major groups are shown in Fig. 1. All of the organisms listed in Table 1 gave a similar profile of metabolites. Naphthalene oxidation was not observed in control experiments with heat-killed organisms or in the absence of organisms. In the preceding paper (Cerniglia *et al.*, 1980), we reported the identification of 1-naphthol, 4-hydroxy-1-tetralone and *cis*-naphthalene dihydrodiol as products formed from naphthalene by *Oscillatoria* sp., strain JCM; these compounds correspond to the metabolites designated M-2, M-3 and M-4 in Fig. 1. Thus, it appears that both prokaryotic and eukaryotic algae may form the same metabolic

Table 2. *Quantitative analysis of metabolites formed from* [1-^{14}C]*naphthalene by different species of cyanobacteria and microalgae*

Each metabolite from the [1-^{14}C]naphthalene incubation was cut from the thin-layer chromatography (t.l.c.) plate and its radioactivity was measured as described in Methods. Metabolite numbers refer to spot numbers in Fig. 1.

Organism	Percentage of total radioactivity on t.l.c. plate in each metabolite						Total radioactivity (d.p.m.)	Percentage metabolism of naphthalene
	M-1	M-2	M-3	M-4	M-5	M-6		
Cyanobacteria								
Oscillatoria sp., strain JCM	20	37	20	6	6	11	38210	2·4
Oscillatoria sp., strain MEV	64	9	6	4	3	14	4965	0·3
Microcoleus chthonoplastes strain BA-1	55	16	5	5	3	16	2945	0·2
Nostoc sp., strain MAC (light)	2	44	28	0	4	22	2315	0·1
Nostoc sp., strain MAC (dark)	5	23	23	0	40	9	2795	0·2
Anabaena sp., strain CA	15	52	18	5	5	6	31315	2·0
Anabaena sp., strain 1F	17	46	26	4	4	3	24300	1·5
Agmenellum quadruplicatum strain PR-6	13	45	25	5	4	10	22620	1·4
Coccochloris elabens strain 17a	0	12	43	28	14	2	32881	2·0
Aphanocapsa sp., strain 6714 (light)	6	7	51	21	10	4	8505	0·5
Aphanocapsa sp., strain 6714 (dark)	21	16	45	12	4	3	11530	0·7
Green algae								
Chlorella sorokiniana strain TX 71105	8	31	45	8	6	1	18835	1·2
Chlorella autotrophica strain 580	19	36	31	7	5	3	24785	1·5
Dunaliella tertiolecta strain DUN	9	31	42	11	4	2	15340	1·0
Chlamydomonas angulosa	29	37	21	4	4	4	20045	1·3
Ulva fasciata	13	26	33	8	12	8	18910	1·2
Diatoms								
Cylindrotheca sp., strain N-1	8	38	40	7	5	2	23165	1·4
Amphora sp., strain AMP-1	6	27	44	13	6	3	18655	1·2
Red alga								
Porphyridium cruentum	9	26	44	8	10	2	18875	1·2
Brown alga								
Petalonia fascia	13	44	26	4	7	7	29975	1·9

products. However, we are aware that chromatographic properties alone are insufficient for firm identification purposes and the exact structures of the metabolites formed by most of the organisms in the present study remain tentative. Nevertheless, it is clear that both prokaryotic and eukaryotic microalgae oxidize naphthalene to several metabolites. The formation of 4-hydroxy-1-tetralone by bacteria (Bollag *et al.*, 1975) and fungi (Cerniglia & Gibson, 1977, 1978; Cerniglia *et al.*, 1978) has been reported and this product is formed by the further metabolism of 1-naphthol. However, *cis*-naphthalene dihydrodiol has only been observed as a bacterial metabolite (Jerina *et al.*, 1971; Catterall *et al.*, 1971; Jeffrey *et al.*, 1975). If *cis*-naphthalene dihydrodiol is formed by eukaryotic algae it will represent the first demonstration of the *cis*-hydroxylation of naphthalene by higher organisms.

Other metabolites produced by all of the organisms are designated M-1, X, M-5 and M-6 (Fig. 1). The identity of these products remains to be determined. M-1 may be a non-enzymic oxidation product of 1-naphthol, as previously seen with *Oscillatoria* (Cerniglia *et al.*, 1980). The product M-5 has chromatographic properties similar to those of *trans*-naphthalene dihydrodiol; however, we have shown for *Oscillatoria* sp., strain JCM (Cerniglia *et al.*, 1980) that M-5 does not resemble *trans*-naphthalene dihydrodiol when analysed by high-pressure liquid chromatography.

The relative amounts of each metabolite varied according to the organism under investigation (Table 2). This is also clearly demonstrated in densitometer tracings of the autoradiograms obtained after incubating naphthalene with *Oscillatoria* sp., strain JCM,

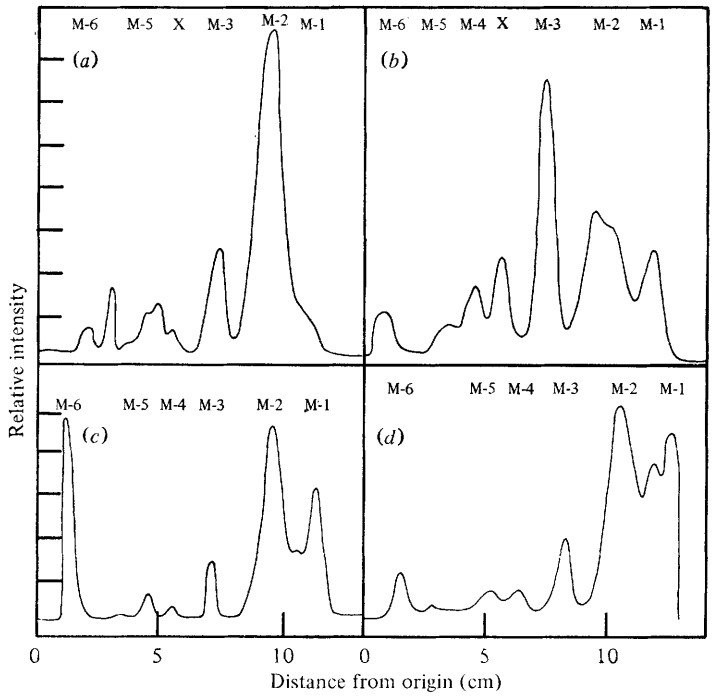

Fig. 2. Radiochromatogram tracings showing metabolites formed from naphthalene by different algae: (*a*) *Oscillatoria* sp., strain JCM (cyanobacterium); (*b*) *Porphyridium cruentum* (red alga); (*c*) *Petalonia fascia* (brown alga); (*d*) *Chlamydomonas angulosa* (green alga). M-1 to M-6 represent metabolites shown in Fig. 1.

Porphyridium cruentum, *Chlamydomonas angulosa* and *Petalonia fascia* (Fig. 2). The extent of naphthalene metabolism to organic-soluble metabolites ranged from 0·1 to 2·4% of the total added [^{14}C]naphthalene. No attempt was made to quantify the water-soluble products formed by the different organisms; in the case of *Oscillatoria* sp., strain JCM this amounts to 2·8% (Cerniglia *et al.*, 1980).

It is interesting to note that *Chlamydomonas angulosa* oxidized naphthalene to several metabolites including 1-naphthol. Soto *et al.* (1975*a*, *b*) found naphthalene to be extremely toxic and inhibitory to growth of this strain. In addition no metabolic products were detected. The concentrations of naphthalene (78 μM) used in the present investigation were not toxic for any of the organisms except the two heterotrophic cyanobacteria *Nostoc* sp., strain MAC and *Aphanocapsa* sp. (Hoare *et al.*, 1971; Beauclerk & Smith, 1978; Bottomley & Van Baalen, 1978). These were extremely sensitive to naphthalene concentrations above 10 μM as shown by obvious bleaching of photosynthetic pigments after a few hours. Both organisms oxidized naphthalene to a slight extent under both dark and light conditions. The significance of these results remains to be investigated. *Anabaena* sp., strain CA, a marine filamentous cyanobacterium capable of rapid growth under nitrogen-fixing conditions (Stacey *et al.*, 1977), and *Anabaena* sp., strain 1F, also a nitrogen-fixing cyanobacterium, oxidized naphthalene (Table 2).

The ability of algae to metabolize aromatic compounds has been reported previously. Thus, Ellis (1977) showed that $^{14}CO_2$ was formed from [^{14}C]catechol by six algal strains, and four of these organisms also metabolized phenol to CO_2. However, we believe that the results presented in this paper represent the first demonstration that the ability to oxidize aromatic hydrocarbons is widespread in the algal kingdom. These observations could have profound implications for the fate and effects of this class of compounds in the environment.

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