Phytochemistry, Vol. 27, No. 3, pp. 731–735, 1988.
Printed in Great Britain.

0031–9422/88 $3.00+0.00

AN ALLELOPATHIC FATTY ACID FROM THE BROWN ALGA *CLADOSIPHON OKAMURANUS*

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(*Revised received* 3 *August* 1987)

Key Word Index—*Cladosiphon okamuranus*; Chordariaceae; brown alga; allelopathy; red tide; toxic phytoplankton; fatty acid; octadecatetraenoic acid.

Abstract—An allelopathic substance was isolated from the methanol extract of the brown alga, *Cladosiphon okamuranus*. Extraction and purification of the active substance was monitored by bioassays against the conchospores of *Porphyra yezoensis* and *Heterosigma akashiwo*. The compound was identified as 6*Z*,9*Z*,12*Z*,15*Z*-octadecatetraenoic acid and its activity was then tested against 36 species of microalgae. Many of eukaryotes tested were affected at 5 ppm, but prokaryotes were unaffected at 25 ppm. The tetraunsaturated fatty acid exterminated toxic red tide planktons such as *Chattonella antiqua* at 1 ppm concentration.

INTRODUCTION

Recent advances in plant biology have revealed that many kinds of plants keep or extend their territories by excreting allelopathy substances that inhibit the growth of other species of plants [1]. Although these allelopathic phenomena are well recognized in the terrestrial plant kingdom, very little is known about those for marine plants.

The brown alga *Cladosiphon okamuranus* is an edible seaweed, a cultivation method for which has recently been established [2]. In the course of exploiting the cultivation, one of us noticed [3] that although the sea-water in a tank where cut fragments of mature thalli of *C. okamuranus* were soaked became light brown, it remained clear and no other seaweeds grew in the water for several months. On the other hand, the sea-water without the seaweed fronds rapidly became turbid due to the growth of many kinds of seaweeds and microalgae. It seemed likely that *C. okamuranus* excreted some allelopathic substance that inhibited growth of other seaweeds. This finding prompted us to search for the inhibitory agent secreted by *C. okamuranus*. Herein, we describe the isolation and identification of the allelopathic substance.

RESULTS AND DISCUSSION

After several attempts, the following two bioassay systems were found to be effective, one using conchospores of the red alga *Porphyra yezoensis* (Assay I) and the other using the microalga *Heterosigma akashiwo* (Assay II). The algae were chosen because they were easily accessible and sensitive to the active substance.

Preliminary experiments revealed that the sea-water in which cut fragments of *C. okamuranus* were immersed inhibited attachment of the conchospores of *P. yezoensis* as shown in Table 1 (Expt. 1) (Assay I, see Experimental). However, attempted extraction of the active component from the sea-water was unsuccessful, because the concentration of the inhibitory substance was too low. Later, we found that a methanol extract of mature *C. okamuranus* exhibited a considerable inhibitory activity against the conchospores of *P. yezoensis* (Expt. 2) (Assay II, see Experimental), and that the extract destroyed the cells of *H. akashiwo*. Since *H. akashiwo* is readily accessible, the phytoplankton was mainly used for bioassay (Assay II).

The methanol extract of *C. okamuranus* was separated by silica gel chromatography monitoring the activity against *H. akashiwo*. Relatively nonpolar fractions were found to be active in the concentration range 20–100 ppm. Further purification was performed by repeated medium-pressure liquid chromatography. The active component was sensitive to air, and degassed solvents were used throughout the separation procedure.

The pure active substance thus obtained exhibited potent activity; at 2 ppm all cells of *H. akashiwo* were destroyed within 30 min. Also it inhibited the attachment

Table 1. Inhibitory activity of extracts of *Cladosiphon okamuranus* against conchospores of *Porphyra yezoensis* (Bioassay I)

Expt	Sample	N_0*	N_3*	A*
1	Active sea-water	89	23	74
	Control	252	142	44
2	MeOH extract (400 ppm)	6	0	100
	MeOH extract (200 ppm)	34	3	91
	MeOH extract (80 ppm)	190	100	47
	Control	179	97	46
3	ODTA (2 ppm)†	10	0	100
	Control	494	447	10

*These values were obtained from one experiment. N_0, initial number of conchospores on a piece of string, N_3, number of conchospores after three days, A, activity [$= 100\,(N_0-N_3)/N_0$].
†6*Z*,9*Z*,12*Z*,15*Z*-Octadecatetraenoic acid.

of conchospores of *P. yezoensis* completely at 5 ppm concentration [Table 1, (Expt. 3)].

The IR spectrum of the active substance exhibited absorption bands at 3400–2400 (*br*) and 1720 cm^{-1} due to a carboxyl group. The ^1H NMR spectrum showed signals which are typical of unsaturated fatty acids. The presence of a terminal ethyl group was evident from the signals at δ0.99 (3H, *t*, $J = 3$ Hz) and 2.07 (2H, *quint*, $J = 7$ Hz). The nature of the methylene signal indicated that the methylene protons were further coupled with a proton, one of the olefinic protons appearing as an overlapped triplet ($J = 4$ Hz) at δ5.84 (8H), which was confirmed by double resonance experiments. The broad signal at δ2.84 (6H) is coupled with the olefin protons, and the signal was reminiscent of the doubly allylic methylene groups (=CH–CH_2–CH=) that are frequently encountered in the highly unsaturated fatty acids such as linolenic acid. Although the active component did not show a $[M]^+$ in the mass spectrum because of poor volatility, its methyl ester revealed the fragment at *m/z* 290 corresponding to the molecular formula $C_{19}H_{30}O_2$. From these features, the structure of the active substance was deduced to be 6,9,12,15-octadecatetraenoic acid. The IR spectrum was superimposable with that reported for 6*Z*,9*Z*,12*Z*,15*Z*-octadecatetraenoic acid (ODTA) [5]. The *Z*-configurations of the olefin groups at C-6, 9, 12, and 15 were confirmed by the absence of a band at 950 cm^{-1} in the IR spectrum of the methyl ester.

In order to determine if ODTA is active against a wide range of algae, 36 species of microalgae were tested under the same conditions as used in the assay for *H. akashiwo* (Table 2). The microalgae could be classified into two types; (i) organisms sensitive to ODTA at 5 ppm and (ii) organisms insensitive to ODTA even at 25 ppm. Consideration of the nature of the cell walls of the tested microalgae led to the conclusion that ODTA is active against phytoplanktons without cell coverings and inactive to ones with rigid cell walls such as *Tetraselmis chui*.

We were especially interested in *Chattonella antiqua*, *C. marina*, *Gymnodinium nagasakiense*, and *G. sanguineum*, because they are responsible for toxic red tides, which result in enormous damage to cultivated fishes and shellfish. These microalgae were tested under more dilute concentrations of ODTA. At 1 ppm, all these microorganisms were killed very rapidly (< 5 min). However, at 0.5 ppm, ODTA did not affect the planktons.

We next turned our attention toward the activity of other substances against *H. akashiwo*. 5*Z*,8*Z*,11*Z*,14*Z*-Eicosatetraenoic acid (arachidonic acid) and 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (EPA) have a similar activity to ODTA (Table 3). The sodium salt of ODTA is as effective as free ODTA. Less unsaturated fatty acids have weaker activities. Esters of unsaturated fatty acids also exhibited weaker activities than the corresponding free acids. Sodium laurylbenzenesulfonate and Tween 80 (typical surface-active agents) also showed activity, but to a lesser degree.

In parallel with the above assays, the allelopathic nature of ODTA against macroalgae was examined (see Experimental). Again, the same tendency as in the microalgae was observed; zoospores (naked) of *Undaria pinnatifida* were killed at 5 ppm although the eggs (with cell wall) of *Sargassum horneri* were unaffected even at 50 ppm. *P. yezoensis*, which was used in Assay I, is known to release conchospores with naked cell membranes. ODTA would have damaged them and prevented them from becoming attached to the string.

A large majority of seaweeds release spores or zoospores that have naked cell membranes at the reproduction period. Considering that these types of spores and zoospores are seriously damaged by ODTA, and other seaweeds are seldom found in the area where *C. okamuranus* is cultivated, we deduce that ODTA plays a role as an allelopathic substance in the marine plant kingdom.

There are a few reports on the toxicity of fatty acids isolated from microorganisms which are allelopathic substances against phytoplanktons [6–8]. It should be noted that ODTA was isolated from a macroalga.

EXPERIMENTAL

Plant material. C. okamuranus was purchased from the Onna Fishery Organization. *H. akashiwo* was donated by Prof. M. Chihara. Other microalgae were supplied from the Microbial Culture Collection at the National Institute for Environmental Studies.

Media. (i) *Modified Suto ASP-6 medium.* Dist H_2O 1 l, NaCl 25 g, $MgSO_4$ 4 g, KCl 0.7 g, $CaCl_2$ 0.37 g, $NaNO_3$ 0.2 g, K_2HPO_4 25 mg, $NaHCO_3$ 0.168 g, PL solution* 2 ml: *PL soln; dist H_2O 1 l, $FeSO_4$ 8 mg, $MnCl_2$ 0.1 mg, $ZnCl_2$ 15 mg, $CoCl_2$ 3 mg, $CuSO_4$ 1.2 mg, boric acid 0.6 g, EDTA diNa salt 3 g. (ii) *PES medium.* Sea-water 1.5 l, dist H_2O 1 l., PES conc soln† 50 ml: †PES conc soln; dist H_2O 1 l, Na_2NO_3 3.5 g. sodium β-glycerophosphate 0.5 g, vitamin B_{12} 100 μg, thiamine hydrochloride 5 mg, biotin 50 μg, tris(hydroxymethyl)aminomethane 5 g, EDTA diNa salt 25 mg, PII metals soln‡ 250 ml: ‡ PII metals soln; dist H_2O 1 l; boric acid 1.145 g, $FeCl_3 6H_2O$ 48 mg, $MnCl_2$ $4H_2O$ 0.144 g, $ZnCl_2$ 10 mg, $CoCl_2$ $6H_2O$ 4 mg, EDTA diNa salt 1 g.

General methods. ^1H and ^{13}C NMR spectra were recorded at 90 MHz. GC/MS were obtained using a 1.5 m × 3 mm column packed with 10% OV-1 at 150°. *Bioassay.* For Assay I an appropriate amount (5–100 mg) of a test sample was dissolved in dist H_2O (10 ml) with the aid of 7 drops of Tween 80 (polyoxyethylene sorbitan monolaurate). The soln was added to 990 ml of modified Suto's ASP-6 medium. A control soln was prepd by adding 7 drops of Tween 80 to 1 l of the modified ASP-6 medium. This soln did not affect the conchospores of *P. yezoensis*. Oyster shells into which conchocelis of *P. yezoensis* penetrated [4] were stored in the modified ASP-6 medium under long-day conditions (14 hr light and 10 hr dark; 18°). Prior to bioassay, the shells were kept under short-day conditions (10 hr light and 14 hr dark) for *ca* 2 weeks. As soon as the conchocelis began to release conchospores, the shell was transferred to a test soln. The oyster shells used in the assay contain 75–100 colonies of the conchocelis per 3.5 cm^2 and released 10^3 conchospores per shell on average during 24 hr. The oyster shell was then placed in a 1 l beaker and 500 ml of the test soln added. A wood frame with nylon string was floated on the surface of the soln and a constant stream of air was bubbled through the system. The system was allowed to stand under the short-day conditions for 24 hr, during which time the released conchospores attached to the string. The central four pieces of the string were taken off, and the number of conchospores attaching on each string was counted under a microscope. The average number of conchospores on a piece of the string was defined as an initial number of conchospores (N_0). The index N_0 is not necessarily parallel with the activity of the sample, because (i) it depends on the number of spores that are released from the conchocelis in the oyster shells, this number considerably varying in each assay and (ii) it includes the number of such spores that are killed or seriously damaged by the active substance and are attached to the strings only by physical means. Therefore, the four pieces of the string were further cultivated in 300 ml of the test soln (12 hr light and 12 hr dark) for 3 days and the number of

Table 2. Activity† of 6Z,9Z,12Z,15Z-octadecatetraenoic acid against microalgae

Plant family	Genus and species	Concentration of ODTA (ppm) 25	5	1*
Cyanophyceae	*Microcyctis wesenbergii*	—	—	
	Oscillatoria raciborskii	—	—	
Rhodophyceae	*Cyanidium caldarium*	—	—	
Bacillariophyceae	*Aulacosira ambiqua*	—	—	
	Chaetoceros debile	+	+	
	Coscinodiscus granii	—	—	
	Skeletonema costatum	+	+	
	Tabellaria flocculosa	—	—	
Dinophyceae	*Gymnodinium nagasakiense*	+	+	+
	Gymnodinium sanguineum	+	+	+
	Heterocapsa triquetra	+	+	
	Prorocentrum micans	+	+	
Raphidophyceae	*Chattonella antiqua*	+	+	+
	Chattonella marina	+	+	+
	Olisthodiscuc luteus	+	+	
	Heterosigma akashiwo	+	+	
Haptophyceae	*Cricosphaera roscoffensis*	±	±	
Cryptophyceae	*Cryptomonas* sp.	+	+	
Prasinophyceae	*Hafniomonas reticulata*	+	+	
	Nephroselmis sp.	+	+	
	Pterosperma cristatum	+	+	
	Plymnesium parvum	+	+	
	Pyramimonas sp.	+	+	
	Tetraselmis cordiformis	±	±	
	Tetraselmis chui	—	—	
Euglenophyceae	*Euglena gracilis*	—	—	
	Eutreptia sp.	+	+	
Chlorophyceae	*Chlamydomonas augustae* var. *ellipsoidea*	—	—	
	Chlamydomonas sp. (marine)	+	+	
	Chlorella pyrenoidosa	—	—	
	Chlorosarcinopsis caeca	—	—	
	Chlorosarcinopsis delicata	—	—	
	Haematococcus lacustris	—	—	
	Oltmannsiella sp.	+	+	
	Scenedesmus quadricauda	—	—	
	Volvox aureus	—	—	
Conjugatophyceae	*Closterium acerosum*	—	—	

*Only four species of algae, *G. nagasakiense*, *G. sanguineum*, *C. antiqua*, and *C. marina*, were tested at this concentration.

† +: All the cells of the alga are destroyed during 30 min.

—: The sample does not affect the alga.

±: The sample seems to affect the alga, but the effect is not so clear.

conchospores on each string recounted. The average number of conchospores on each string was defined as number of conchospores after 3 days (N_3). In the case of an active test soln, most of the conchospores detached from the string after 3 days. The conchospores still remaining attached to the string after 3 days germinated normally even in the active soln and no difference was observed between the resulting thallis and those of control after 2 weeks cultivation. It seems that the conchospores are affected by the active substance before they attach to the string, and, once they attach without being damaged by the active substance, they grow normally. Activity (A) was defined by the following equation.

$$A = \frac{N_0 - N_3}{N_0} \times 100$$

For Assay II, the samples were dissolved in C_6H_6 (each 10 mg/ml) in order to prevent oxidation and polymerization and the solns stored at $-20°$ until used. A portion of the soln was transferred to a 10 ml vol. flask. The C_6H_6 was evapd and the residue dissolved in 0.25 ml of EtOH. PES medium was added gradually to make a 10 ml soln. A control soln was prepd by evapn of a portion of C_6H_6, followed by addition of 0.25 ml of EtOH and 10 ml of PES medium. *H. akashiwo* was constantly cultivated in the medium, which was made by addition of Na_2NO_3 (200 mg) and Na_2HPO_4 (40 mg) to PES medium (1 l), at 20° under fluorescent lamps (3000 lm/m^2; 12 hr light/12 hr dark). When the density of the cells reached 10^5 (cells/ml), a 100 ml portion of the medium was added to 1 l of the fresh medium, and this was further cultivated to maintain the continuous supply of the alga. Portions of the medium (1 ml, 7×10^4 cells/ml) were transferred to the wells of a multidish (24 wells, Nunc), and an

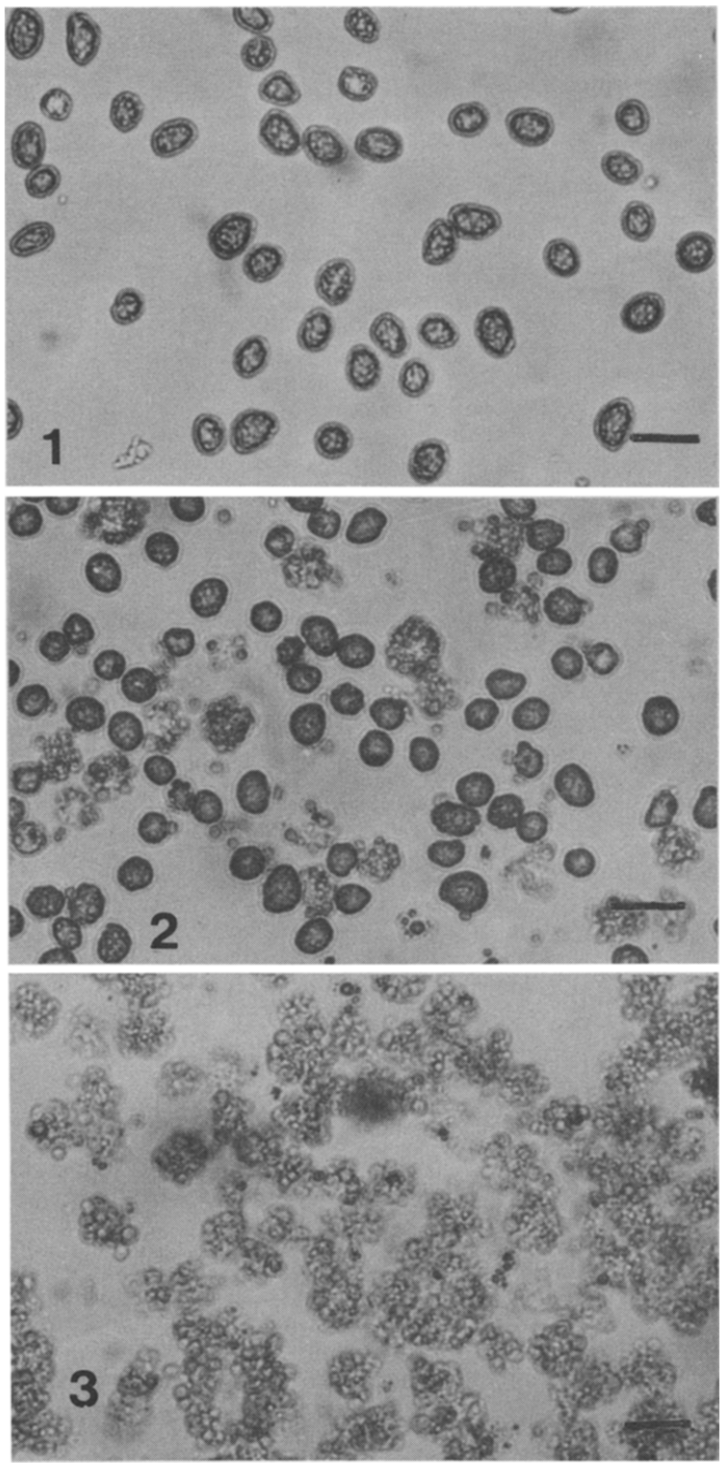

Fig. 1. Time course of Bioassay II using *Heterosigma akashiwo* (1; 0.5 min, 2; 5 min, 3; 30 min, after an active sample was added.). The active sample is a crude MeOH extract of *Cladosiphon okamuranus*, and the concentration is 200 ppm.

Table 3. Activity of unsaturated fatty acids and surface-active agents against *Heterosigma akashiwo*

Acid/compound	Concentration† (ppm)
ODTA (6*Z*,9*Z*,12*Z*,15*Z*-octadecateraenoic)	2
Arachidonic*	2
Eicosapentaenoic*	2
γ-Linolenic*	25
Linoleic*	25
Oleic*	50
ODTA methyl ester	25
Methyl linolenate*	50
ODTA Na salt‡	2
Sodium linolenate‡	25
Trilinolenine*	75
Sodium laurylbenzenesulfonate*	100
Tween 80*	100

†The compounds were tested at 100, 75, 50, 25, 15, 5, 2, 1 and 0.5 ppm under the same conditions used in Assay II. The minimum concentrations necessary to kill the microorganism during 30 min are listed.

*Purchased from Tokyo Kasei Co. Ltd.

‡Prepared by adding equimolar amount of NaOH to the fatty acid in ethanol and then evaporating the solvent.

appropriate amount (0.25–1 ml) of the test soln added to each well. If the sample is active, the motility of the planktons decreases (Fig. 1.1), the cells swell (Fig. 1.2), and finally burst (Fig. 1.3). Activity was defined as the minimum concn which resulted in the rupture of all cells during 30 min.

Isolation and identification of active substance. Activities of the chromatographic fractions were monitored by Assay II and specified with act. in parentheses after each fraction. Fresh *C. okamuranus* (15 kg) was treated with NaCl (10 kg) immediately after collection at the Onna beach in Okinawa Island. It was sent to Tsukuba and soaked in MeOH (15. l). After 3 weeks extn at room temp, the ext. was concd on a rotary evaporator (35°). The residue (act. 200 ppm) was treated with cold (0°) EtOH and the mixt. filtered to remove salt. The filtrate was concd and H_2O (500 ml) added to the residue (act. 150 ppm). The mixt. was extd with 3 × 500 ml portions of Et_2O and the Et_2O layer evapd. The green material (act. 25 ppm) was chromatographed (Wakogel C-300) using CH_2Cl_2–MeOH [100:0 (act. 20 ppm), 49:1 (act. 8 ppm), 19:1 (act. 20 ppm), 1:1 (act. 100 ppm), and then 0:100 (act. 100 ppm)]. The fraction eluted by CH_2Cl_2–MeOH (49:1) was further sepd by medium-pressure LC [Merck, Lobar Column; LiChroprep RP-8; dioxane–H_2O (4:1)]. The oily substances obtained by concentrating the fractions rapidly polymerized on standing, and therefore, all the solvents used in chromatography were degassed and Ar was applied when the vacuum of the evaporator was released. The most active fraction [the second one (act 4 ppm) of the four fractions] was re-chromatographed using dioxane–H_2O (2:1) to give four fractions. The second fraction (act 2 ppm) was most active and showed one spot on TLC (Merck, Kieselgel GF_{254}) with various solvent systems. The active component was identified as 6*Z*,9*Z*,12*Z*,15*Z*-octadecatetraenoic acid [5]. The substance was methylated with CH_2N_2–Et_2O. The product was purified by silica gel chromatography (hexane–EtOAc) to give a pure Me ester; GC/MS 20 eV, *m/z* (rel. int.): 290 $[M]^+$ (100), 261 $[M-C_2H_5]^+$ (80), 259 $[M-OMe]^+$ (16); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3040 (=CH), 2975 (CH_n), 2857 (CH_n), 1730 (COOMe), 1410 (CH_n), 1155 (C–O); ^1H NMR (90 MHz, $CDCl_3$) δ0.98 (3H, *t*, *J* = 7 Hz), 1.10–1.94 (4H, *m*), 2.08 (4H, *br s*), 2.32 (2H, *br t*, *J* = 7 Hz), 2.84 (6H, *br s*), 3.64 (3H, *s*), 5.84 (8H, *br t*, *J* = 4 Hz).

Effect of ODTA upon macroalgae. Undaria pinnatifida. A piece of sporophyll (*ca* 8 cm diam.) was soaked in sea-water (50 ml) for 1 hr, during which time the sporophyll released zoospores. Portions (1 ml) of sea-water were transferred to the wells of a multi-dish. A sample soln was prepd by dissolving ODTA (5 mg) in EtOH (0.3 ml) and dil. the soln to 10 ml with sea-water. An aliquot (1 ml) of the soln was added to the zoospores-containing sea-water in the well (the resulting soln contained 25 ppm ODTA) and the motion of the zoospores was observed under a microscope. After 5 min, most of the zoospores were stationary and the cells began to swell; after 10 min all the cells had burst. At 5 ppm rupture of all zoospores was complete after 30 min. At 2.5 ppm, ODTA had no effect on the spores. As control, zoospores containing sea-water (2 ml) were treated with EtOH (30 μl): this amount of EtOH did not influence the motility of the zoospores. *Sargassum horneri.* Sample solns were prepd in the same manner as described above. The solns (0, 5, 25, 50 and 100 ppm) were put in the wells of a multidish. Three fertilized eggs of *S. horneri* were added to each soln and the system allowed to stand for 48 hr at 24–26°. The eggs germinated during this period. However, there were no recognizable differences in morphology and growth of the rhizoids between the eggs in the control and the ones in the ODTA solns.

Acknowledgement—We are grateful to Dr Makoto Watanabe, National Institute for Environmental Studies, for supplying microalgae and for his helpful suggestions.

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