Jap. J. Phycol. (Sôrui) 29: 181-187. September 20, 1981

The influence of high molecular organic substances in sediment on the green alga *Chlorella pyrenoidosa* CHICK

Shuzi HINO and Kazuo ANDO

Hokkaido Research Institute for Environmental Pollution
Kita-ku, Sapporo, 060 Japan

HINO, S. and ANDO, K. 1981. The influence of high molecular organic substances in sediment on the green alga *Chlorella pyrenoidosa*. Jap. J. Phycol. **29**: 181-187.

High molecular organic compounds in the sediment of the Barato River (Hokkaido Ishikari-gun Ishikari-cho) were extracted with 1 N sodium hydroxide, and were found to stimulate the growth of *Chlorella pyrenoidosa*. Three extracts were obtained, an alkali, an acid, and a water. The alkali extract was separated into four fractions by DEAE-cellulose chromatography. The D-1 (0.1 M NaCl) fraction showed an observable stimulatory effect, whereas the D-4 (NaOH) fraction was inhibitory. After separating the D-1 fraction by Sephadex G-100, the growth stimulatory S-5 fraction was calculated as having a molecular weight of 24,000 by gel filteration. This fraction was composed of 32% sugars, 44% proteins, 0.31% iron, and 0.07% manganese. The growth inhibitory D-4 fraction contained gallic, protocatecuic, *p*-hydroxybenzoic, *p*-coumaric, and vanillic acids. All these phenolic compounds except for vanillic acid, which was not examined, were found to strongly inhibit the growth of *C. pyrenoidosa* at concentrations of 1 μM or over.

Key Index Words: Chlorella pyrenoidosa; *Chlorophyta*; *growth-inhibitory compounds*; *growth-stimulatory compounds*; *organic substances*; *phenolic compounds*; *sediment*.

The effects of organic and inorganic substances in the sediments of rivers, lakes and sea upon growth rates of algae have been extensively investigated (PRAKASH and RASHID 1968, HONJO and HANAOKA 1973, 1974, IIZUKA and NAKASHIMA 1975, COOKSEY and COOKSEY 1978, JACKSON and HECKY 1980, ISHIO and KONDO 1980). The effect of high molecular substances on algae have been found to be inhibitory (PRAKASH and RASHID 1968, COOKSEY and COOKSEY 1978) or stimulatory (GIESY 1976). It is well known that the growth of algae is stimulated by adding sediment to the growth medium (PRINGSHEIM 1946, STARR 1964).

The present study attempts to determine which constitutents of high molecular weight organic substances found in the sediment of a river in Northern Japan have a stimulatory effect on the growth of the green alga *Chlorella pyrenoidosa*. Both stimulatory and inhibitory fractions were analyzed by ion exchange chromatography, and molecular sieve gel chromatography to clarify which substances were active.

Meterials and Methods

Sediment; Samples of sediment were collected from the Barato River located in the center of Hokkaido, and kept in a cold room at 4°C in a wet state until use. This sediment was a fluffy precipitate and was black in color.

Alga; *Chlorella pyrenoidosa* (CHICK) was supplied by the Institute of Applied Microbiology, The University of Tokyo, and was used throughout the present experiments.

Extraction of organic substances; Organic substances were extracted from the sedi-

ment with distilled water, 1 N sodium hydroxide, or 1 N hydrochloric acid. Five hundred m*l* of each solvent was added to 100 g wet weight of the sediment and the mixture was incubated in a reciprocal shaker for 24 hr at room temperature. After shaking, the mixtures were centrifuged at 8,000 rpm for 20 min, and the pellets were discarded. After adjusting the supernatant to pH 7.0 with alkali or acid, solid ammonium sulfate was gradually added to 100% saturation. The precipitate was collected by centrifugation at 8,000 rpm for 20 min, and dissolved in 1 N sodium hydrooxide for water and alkali extracts and 1 N hydrochloric acid for acid extract. These solutions were dialyzed against distilled water for 3 hr and then against 50 mM Tris-HCl buffer (pH 7.2) overnight. The dialyzed solutions were designated as water, alkali, and acid extracts respectively. These samples were kept in a cold room at 4°C until use.

Assay of algal growth; Bold's Basal Medium (Brown and Bold 1964) was poured into test tubes (10m*l* per tube) and autoclaved at 120°C for 20 min. Sample extracts used in these experiments were dialyzed. After sterilization with an Amicon filter, the samples were adjusted to the same absorbance at 280 nm, and 1 m*l* was poured into each test tube. One drop of *C. pyrenoidosa* precultured in Bold Basal Medium for 5 days was inoculated into each of the test tubes with a micropipette. The inoculated test tubes were incubated at 20°C for one week under illumination at 2,000 lux. At the end of the experiment, algal growth was determined by measuring dry weight.

Determination of phenolic compounds; Phenolic compounds were extracted and purified following the method of KATASE and HANYA (1974), and determined with a Hitachi 635 A type high performance liquid chromatogram using a stainless steel column (0.4×15 cm) packed with Lichromosorb RP-18. The column temperature was ambient, the solvent was 5% acetonitrile and traced acetic acid, and the flow rate was 2.0 m*l*/min. A Hitachi UV monitor was used as a detector.

Assay of sugars, proteins, iron, and manganese; Sugars were assayed by the Anthron method (DREYWOOD 1964) with glucose as a standard. Proteins were assayed by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard. Iron and manganese were assayed colorimetrically by a JIS 0102 (1979).

Separation of the alkali extract by DEAE-cellulose column chromatography; The alkali extract was loaded on a DEAE-cellulose column (2×30 cm) equiliblated with 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl and the column was eluted with a gradient concentration of NaCl. After elution with NaCl, the column was washed with distilled water, and residual substances were further eluted with NaOH. One of the fractions of 5 m*l* were collected.

Reseparation of the DEAE-cellulose fraction by Sephadex G-100 column chromatography; One of the DEAE-cellulose fractions was subjected to gel filtration with a Sephadex G-100 column (2×90 cm). Equilibration and elution were carried out with 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl. One of the fractions of 5 m*l* were collected.

Determination of molecular weight; The molecular weight growth stimulatory fraction was determined by gel filtration. Catalase (58,000), chymotripsinogen (25,700), and cytochrome C (13,400) were added as marker proteins.

Degradation of the organic substances by C. pyrenoidosa; A closed dialysis tube containing the *C. pyrenoidosa* suspension, one of the Sephadex G-100 fractions, or both was immersed in 600 m*l* of the basal medium in 1 *l* flask with silicon cap. The medium was agitated with a magnetic stirrer at 20°C for 15 days. At intervals, a small amount of the medium was drawn out and the absorbance at 280 nm was measured.

Results

Effect of the extracts on C. pyrenoidosa; The three kinds of extracts were tested to ascertain their effects on the growth of *C. pyrenoidosa*. As shown in Table 1, the water and acid extracts stimulated growth 39% and 43%, respectively. The alkali extract stimulated growth 2.6 times the rate of the control.

Separation of the alkali extract by DEAE-cellulose column chromatography; The elution profile of the alkali extract is shown in Fig. 1. The extract was separated into at least four fractions (D-1 to D-4 fractions). The fraction were collected separately and their effects on algal growth were tested. The D-1 fraction stimulated the growth of *C. pyrenoidosa* (Table 2) two times that of the control. The D-2 fraction and D-3 fraction did not promote growth. Only the D-4 fraction showed inhibitory effects, repressing growth 20% below that of the control.

Table 1. Effects of the three extracts on the growth of *Chlorella pyrenoidosa*.

Substance	Algal growth	
	(mg/dry wt/*l*)	(%)
None (Control)	7.4	100
Water extract	10.3	139
Alkali extract	19.3	261
Acid extract	10.6	143

Control contained only Bold's Basal Medium.

Separation of the D-1 fraction by Sephadex G-100 column chromatography; This fraction was composed of at least six different molecular weights (Fig. 2). When these 6 fractions were tested for effects on algal growth rates, the S-5 fraction showed the

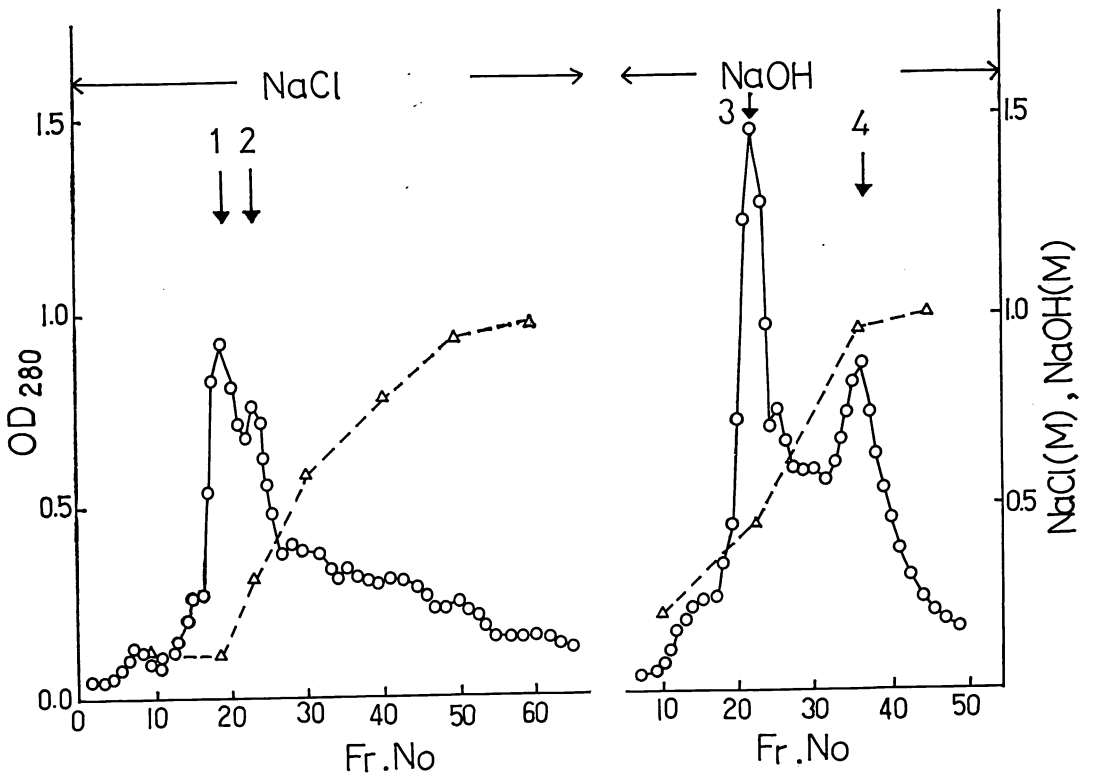

Fig. 1. DEAE-cellulose chromatography of the alkali extract. ○=OD280; △=concentration of sodium chloride or sodium hydroxide arrows: 1. D-1 2. D-2 3. D-3 4. D-4.

Table 2. Algal growth in the presence of each fraction separated by DEAE-cellulose column chromatography.

Fraction	Algal growth	
	(mg dry wt/*l*)	(%)
None (Control)	22.3	100
D-1 fraction (0.1 M NaCl)	43.1	193
D-2 fraction (0.3 M NaCl)	29.5	132
D-3 fraction (0.5 M NaOH)	24.2	109
D-4 fraction (1.0 M NaOH)	17.9	80.3

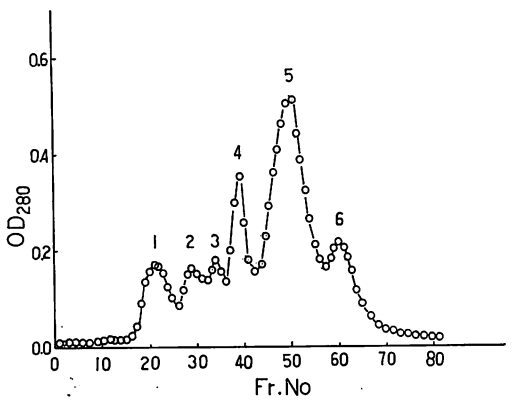

Fig. 2. Sephadex G-100 chromatography of the D-1 fraction (0.1 M NaCl).

Table 3. Algal growth in the presence of each fraction separated by Sephadex G-100 column chromatography.

Fraction	Algal growth	
	(mg dry wt/*l*)	(%)
Mixed	27.2	100
S-1 fraction	24.5	90.0
S-2 fraction	26.8	98.5
S-3 fraction	28.3	104
S-4 fraction	28.7	106
S-5 fraction	47.1	173
S-6 fraction	32.8	121

Fraction numbers are designated by the order of elution shown in Fig. 2. 'Mixed' was the sample before gel filtration.

maximum stimulatory effect (Table 3). The molecular weight of the fraction was determined to be about 24,000 by gel filtration.

Table 4. Composition of growth-stimulatory S-5 fraction.

Component	(%)
Sugars	32
Proteins	44
Iron	0.31
Manganese	0.07
Others	23.6

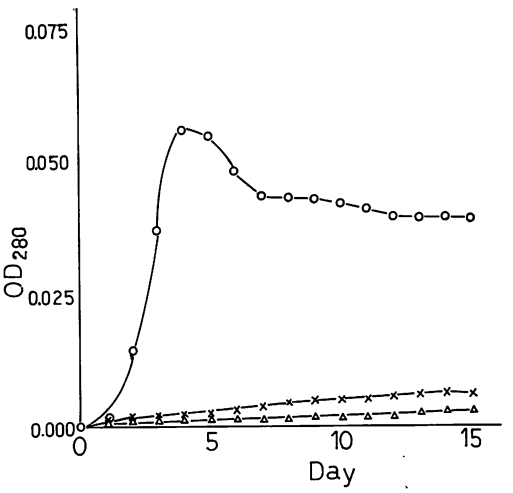

Fig. 3. Degradation of growth-stimulatory the S-5 fraction by *Chlorella pyrenoidosa*. OD280=amount passed through dialysis tube ○= growth stimulatory S-5 fraction and *C. pyrenoidosa* suspension: △=growth-stimulatory S-5 fraction; ×=*C. pyrenoidosa*.

Components of S-5 fraction; The S-5 fraction was analyzed for sugars, proteins, iron, and manganese. As shown in Table 4, proteins were the most dominant component.

Degradation of the S-5 fraction by C. pyrenoidosa; We attempted to examine how the S-5 fraction interacted with the alga. If the fraction is degraded by the alga, smaller molecules should accumulate and be measurable. The results of our experiment are shown in Fig. 3. In the case of both the algal suspension and the S-5 fraction alone, absorbance did not change. After the S-5 fraction was added to the algal suspension, absorbance at 280 nm increased rapidly, indicating degradation of organic substances

in the S-5 fraction.

Growth inhibitory substances; As shown in Table 2, the D-4 fraction inhibited growth. The D-4 fraction showed two absorption peaks at 250 and 300 nm. After the fraction was hydrolyzed with 6 N NaOH and dialyzed, the dialysate was analyzed by high performance liquid chromatography. The results are shown in Fig. 4. Absorption spectra were recorded and compared with those of established standards and consequently the organic substances in the D-4 fraction were identified as gallic, protocatecuic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids.

Effects of phenolic compounds on alga; Standard samples of gallic, protocatecuic, *p*-hydroxybenzoic, and *p*-coumaric acids were examined in respect to their effect on algal

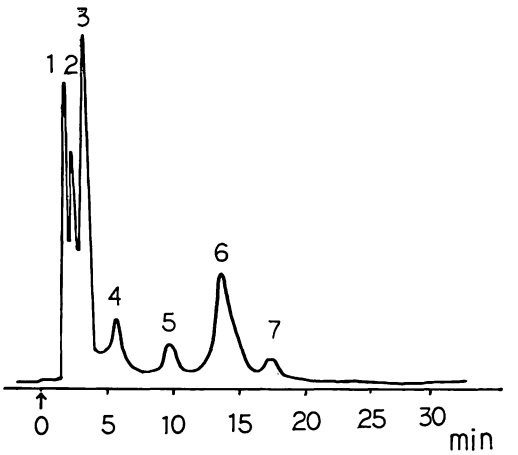

Fig. 4. High performance liquid chromatography of phenolic compounds. 1. gallic acid, 2. protocatecuic acid, 3. *p*-hydroxybenzoic acid, 4. unknown, 5. vanillic acid, 6. *p*-coumaric acid, 7. unknown.

Table 5. Effects of phenolic compounds on the growth of *Chlorella pyrenoidosa*.

Phenolic compounds	*p*-Coumaric acid	*p*-Hydroxybenzoic acid	Protocatecuic acid	Gallic acid
(μM)	Algal growth (%) relative growth	Algal growth (%) relative growth	Algal growth (%) relative growth	Algal growth (%) relative growth
0	100			
1	84.3	57.5	86.4	92.3
10	29.9	15.0	27.0	38.4
100	8.5	0.6	4.3	15.0
1000	0.4	0.0	0.0	1.2

growth. As shown in Table 5, *p*-hydroxybenzoic acid inhibited growth about 40% at only 1 μM. The other phenolic compounds inhibited growth about 60-70% at 10 μM, and all compounds inhibited the algal growth almost completely at 1 mM.

Discussion

As shown in Fig. 1 and Table 2, the D-1 and D-2 fractions stimulated algal growth, whereas the D-4 fraction was inhibitory. The D-4 fraction is therefore assumed to have more groups such as phenols and carboxyls than the D-1 and D-2 fractions. The D-4 fraction had two definite absorption peaks at 250 and 300 nm, consistant with the results obtained for phenolic compounds by GOLDSCHMIDT (1953). This fraction was analyzed and the presence of *p*-coumaric, vanillic, *p*-hydroxybenzoic, protocatecuic and gallic acids was determined. COOKSEY and COOKSEY (1978) suggested that phenolic compounds (such as tannin) were present in sediment and inhibited algal growth in case of eluting with rain and sea water. We found that pure phenolic compounds inhibited the growth of *Chlorella pyrenoidosa* at or above 10 μM (Table 5). However, although the D-4 fraction contained large quantities

of phenolic compounds, it inhibited the algal growth to a lesser extent, only about 20% (Table 2). It is likely that in the D-4 fraction, the phenolic compounds may combine with some other substances such as protein, sugars, and lignin, so consequently the inhibitory effect may be decreased.

Some phenolic compounds have been reported to stimulate sporeling in red algae (BONEY 1967) as well as respiration of *Chlorella vulgaris* (DEDONDER *et al.* 1971), and to enhance the growth of *Goniotrichum elegans* when presented together or coupled to peptides (FRIES 1970, 1972, and 1973). Our research has found an inhibitory effect on algal growth rates, but we have not yet determined the role of these compounds in the river. This problem has to be solved in the near future.

The alkali extract showed a highly stimulatory effect on algal growth in our research. On the other hand, IWASAKI (1969), and IWASAKI *et al.* (1969) reported that the boiled extract of sediment had a remarkable stimulatory effect. HIRAYAMA and NUMAGUCHI (1972) reported that an acid extract had maximum stimulatory effect among the alkali, acid, and boiling water extracts. HONJO and HANAOKA (1973, 1974) reported that the acid extract had a stimulatory effect on algal growth. However, they did not investigate the effects of high molecular alkali extracts upon algal growth.

WARIS (1953), PRAKASH and RASHID (1968) suggest that low molecular humic acid extracted with alkali had a maximum growth stimulatory effect upon algae. GISEY (1976) suggested that humic acid extracted with alkali (M. W 30,000) had growth stimulatory effects, this due to chelating and other factors. Sediment and soil extract with alkali involve humic acid (GOLDSCHMIDT 1953). We separated the S-5 fraction from the alkali extract by several technique. The humic acid was included in the D-3 and D-4 fractions. The S-5 fraction was different from humic acid. The fraction composed of proteins and sugars was high stimulatory to algal growth. The humic acid dose not affect algal growth on our experiments.

Our research showed that the S-5 fraction was utilized by *C. pyrenoidosa* (Fig. 3). We suggest that the growth stimulatory effect of the fraction might be due to the supply of carbon, nitrogen, and/or energy sources avaiable for the alga after the degradation of high molecular weight fractions such as the S-5 fraction.

However, since we have not examined which component or combination is most effective in promoting growth, we cannot exclude the possibility that some component which was not determined in this study may be an essential stimulatory factor.

Acknowlegements

The authors are thankful to Miss. K. OSANAI for her technical assistance.

References

BONEY, A. D. 1967. The effects of coumarin on the growth and viability of sporeling of red algae. Planta (Berl.) 74: 114-123.

BROWN, R. M. and BOLD, H. C. 1964. Phycological studies. V. Comparative studies of the algal genera *Tetracystis* and *Chlorococcum*. Univ. Texas Pub. 6417.

COOKSEY, K. E. and COOKSEY, B. C. 1978. Growth-influencing substances in sediment extracts from a subtropical wetland: Investigation using a diatom bioassay. J. Phycol. 14: 347-352.

DEDONDOR, A. and VAN SUMERE, C. F. 1971. The effect of phenolics and related compounds on the growth and respiration of *Chlorella vulgaris*. Z. Pflanzenphysiol. 65: 70-80.

DREYWOOD, R. 1946. Sugar measurement. Anal. Chem. 18: 499-504.

FRIES, L. 1970. The influence of microamounts of organic substaces other than vitamins on the growth of some red algae in the axenic culture. Br. phycol. J. 5: 39-46.

FRIES, L. 1972. The influence of phenolic compounds on the growth of *Goniotrichum elegans* (CHAUV.). Proc. 7 th Intl. Seaweed Symp. p. 575-579.

FRIES, L. 1973. Requirements for organic substances in seaweed. Bot. Mar. 16: 19-31.

GOLDSCHMIDT, D. 1953. The effect of alkali and strong acid on the ultra violet absorption

spectrum of lignin and related compounds. J. Am. Chem. Soc. **75**: 3780-3786.

GIESY, J. P. 1976. Stimulation of growth in *Scenedesmus obliquus* (Chlorophyceae) by humic acids under iron limited conditions. J. Phycol. **12**: 172-179.

HIRAYAMA, K. and NUMAGUCHI, K. 1972. Growth of *Gymnodinium* Type-65 causative organism of red tide in Omura Bay, in medium supplied with bottom mud extract. Bull. Plankton Soc. Japan **19**: 13-21.

HONJO, T. and HANAOKA, T. 1973. Studies on the mechnisms of red tide occurrence in Hakata Bay (II). General features of red tide flagellate, *Heterosigma* sp. Bull. Plankton Soc. Japan **19**: 75-81.

HONJO, T. and HANAOKA, T. 1974. Studies on the mechanisms of red tide occurrence in Hakata Bay (III). The chracteristics of effective bottom mud and its geographycal distribution pattern. Bull. Plankton Soc. Japan **20**: 126-130.

IIZUKA, S. and NAKASHIMA, T. 1975. Response of red tide organisms to sulfide. Bull. Plankton Soc. Japan **22**: 27-32.

ISHIO, S. and KONDO, K. 1980. Studies on the scarcity of red tide in the eutrophycated waters of Ariake Bay (1). Dissolution of phosphate ion from bottom mud by hydrogen sulfide. Bull. Jap. Soc. Scient. Fishers. **46**: 977-989.

IWASAKI, H. 1969. Studies on the red tide dinoflagellates (III). On *Peridinium hangoei* SCHILLER appeared in Gokasho Bay, Shima Peninsula. Bull. Plankton Soc. Japan **16**: 132-139.

IWASAKI, H., OKADA, Y. and TANABE, S. 1969. Studies on the red tide dinoflagellates (IV). On *Rhodomonas ovalis* NYGAARD appeared in coastal area of Fukuyama. Bull. Plankton Soc. Japan **16**: 140-144.

Japanese Standards Association. 1979. Testing method for Industrial Water (JIS 0102). Japanese Standards Association, Tokyo.

JACKSON, T. A. and HECKY, R. E. 1980. Depression of primary productivity by humic matter in lake and reservoir waters of the boreal forest zone. Can. J. Fish. Aquat. Sci. **37**: 2300-2317.

KATASE, T. and HANYA, T. 1974. Microdetermination of *p*-coumaric acid in water by gas chromatography. Jap. Analyst. **23**: 1211-1217.

LOWRY, O. H., ROSEBROGH, N. J., FARR, A. L. and RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**: 265-275.

PRAKASH, A. and RASHID, M. A. 1968. Influence of humic substances on the growth of marine phytoplankton dinoflagellates (*Gonyaulax*). Limnol. Oceanogr. **13**: 598-606.

PRINGSHEIM, E. G. 1946. Pure cultures of algae. Cambridge Univ. Press, London.

STARR, R. C. 1969. Structure, reproduction and differentiation in *Volvox carteri* f. *nagarienses* IYENGAR, strains HK 9 and 10. Arch. Protistenk. **111**: 204-222.

WARIS, H. 1953. The significance for algae of chelating substances in the nutrient solution. Physiol. Plant. **6**: 538-543.

日野修次・安藤和夫：河川堆積物に含まれる高分子有機物の緑藻 *Chlorella pyrenoidosa* CHICK に与える影響

北海道中央部に位置する茨戸川の堆積物に含まれる高分子有機物が，*Chlorella pyrenoidosa* に与える影響，およびその組成，性状を調べた。DEAE-Cellulose, Sephadex G-100 カラムクロマトグラフィー，および，高速液体クロマトグラフィーにより，糖質，蛋白質を主成分とする分子量約24,000の生長促進物質群と，フエノール化合物を主とする生長阻害物質群の存在が明らかにされた。また，促進物質群は *Chlorella pyrenoidosa* によって低分子化されることが判明した。(060 札幌市北区北19条西12丁目北海道公害防止研究所)