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Microbial production of auxin indole-3-acetic acid in marine sediments

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ABSTRACT: Auxin indole-3-acetic acid (IAA) was detected in marine sediments for the first time using HPLC and immunological identification methods. The IAA content of the interstitial water in sediments varied, in the order of 10^{-10} to 10^{-6} mol l^{-1}, with sampling season and location. The content in seawater was much lower than that of sediments, ca 10^{-11} to 10^{-10} mol l^{-1} Considerable amounts of IAA were produced by various marine bacteria in vitro, especially in the presence of tryptophan. IAA production and accumulation was also enhanced when glucose, tryptophan and natural seaweed substrates were added to sediments and incubated under both aerobic and anaerobic conditions in the dark, which suggests that the natural microbial community contributes to IAA production in sediments. The amount of IAA that accumulated in the water surrounding sediments reached 10^{-7} mol l^{-1} in the presence of seaweed substrate, and corresponded to the level in glucose plus tryptophan. Auxin-active compounds produced during microbial decay of natural organic materials may affect algal growth in natural environments.

INTRODUCTION

Auxin- and cytokinin-like plant hormonal compounds have been detected in natural seawater (Bentley 1960, Pedersén & Fridborg 1972, Kentzer et al. 1980). Using gas chromatography-mass spectrometric analysis, Pedersén (1973) identified a major cytokinin-active compound in seawater filtrates as 6-(3-methyl(-2-butenylamine) purine (isopentenyladenine). Since plant hormones are present in various marine algae, multicellular (Abe et al. 1972, Tay et al. 1985, 1987, Hofman et al. 1986, Mooney & Van Staden 1987), unicellular (Grotbeck & Vance 1972) and natural planktonic organisms (Bentley 1960), it is possible that plant hormones in seawater originate from algal plants (Pedersén 1973, Kentzer et al. 1980).

Recently we found that many bacterial strains isolated from seawater and sediments were able to produce cytokinin-active compounds under culture condition (Maruyama et al. 1986). Furthermore, we identified one of the cytokinin-active compounds from marine bacteria as isopentenyladenine (Maruyama et al. 1988), the same compound reported as a major cytokinin in seawater (Pedersén 1973). These findings allowed us to postulate on the microbial origin of cytokinins in marine environments. In addition, Mishra & Kefford (1969) previously reported auxin-like effects of coral-sand (rich in microorganisms) on the growth of the multicellular green alga *Caulerpa sertularioides*; suggesting that marine sediments probably contain certain auxin compounds or auxin-producing microorganisms. However, there has been no report on the presence of auxins or their producers in marine sediments.

In this paper, we try to demonstrate that microbial production of auxin indole-3-acetic acid (IAA) occurs in marine sediments. HPLC and enzyme-linked immunosolvent assay are used to identify and quantify IAA in seawater, sediment and bacterial culture samples. Tryptophan, which is known as a common precursor in IAA synthetic pathways among terrestrial plants and bacteria, was used to evaluate its effect on IAA production in both marine bacterial monoculture and fresh sediment culture. In addition, IAA production and accumulation in marine sediments is confirmed using a seaweed (predominant in the surrounding environment) as a possible substrate for sedimentary microorganisms.

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MATERIAL AND METHODS

Seawater and sediment samples. Locations of sampling areas are illustrated in Fig. 1. Samples of seawater and sediments were collected in Aburatsubo Inlet, Kanagawa Prefecture, Japan, from 1984 to 1985 and in Tokyo and Sagami Bays during the KT-87-2 cruise on board the R V 'Tansei-maru' (Ocean Research Institute, University of Tokyo) in March 1987. Sediments from the center of Tokyo Bay were black, muddy, and smelled of hydrogen sulfide, indicative of highly anaerobic conditions. Seawater samples were taken using a Van-Dorn sampler, and sediment with an Ekman-Birge sampler in Aburatsubo Inlet and with a box-corer in Tokyo and Sagami Bays. The seawater thus collected was immediately filtered through a glass fiber filter (Whatman GF/F) and the filtrate stored at −20 °C. The upper 0 to 5 cm layer of sediment samples was also kept at −20 °C prior to use. In the laboratory, the thawed sediment sample was centrifuged at 10 000 × *g* for 30 min at 4 °C and the supernatant used in IAA analysis.

Bacterial culture. Ten marine bacterial strains and 4 type-strains were used (Table 3). Three types of basal media were prepared, 1 defined medium (A) and 2 undefined media (B and C). Medium A contained glucose (5 g), NH_4Cl (0.2 g), K_2HPO_4 (7 mg), and Fe-EDTA (iron-ethylenediaminetetraacetate, 4 g) in 1 l of artificial seawater (ASW): NaCl (17.6 g), $MgCl_2 \cdot 6H_2O$ (8.0 g), Na_2SO_4 (2.9 g), $CaCl_2 \cdot 2H_2O$ (1.1 g), KCl (0.5 g), tris(hydroxymethyl)-aminomethan (9.1 g), at pH 7.8. Medium B contained glucose (2 g), trypticase peptone (BBL; 2 g) and Bacto-Yeast extract (Difco; 1 g) in 1 l ASW; and Medium C had a composition of trypticase peptone (4 g) and Bacto-Yeast extract (1 g) in 1 l ASW. Tryptophan was added at a concentration of 100 mg l^{-1}. Bacteria were cultured in 50 ml L-shape tubes containing 10 ml of medium at 20 °C on a reciprocal shaker and harvested at late-log phase by centrifugation at 11 000 × *g* for 20 min at 4 °C – the supernatant was then used.

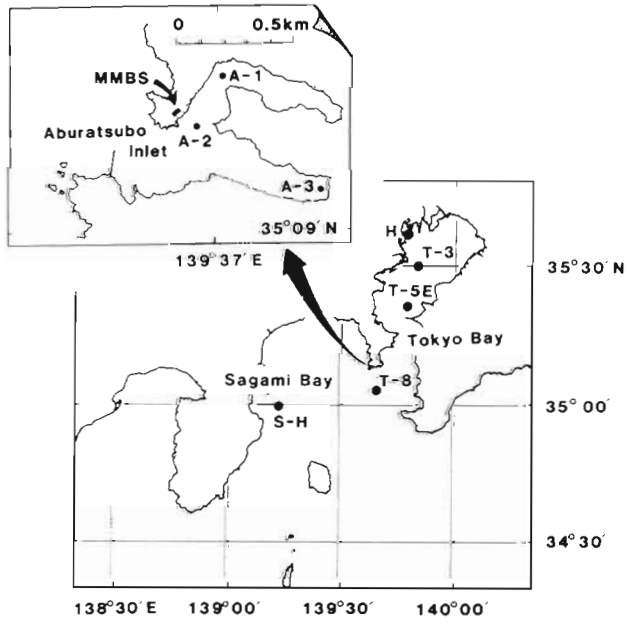

Fig. 1. Location of sampling stations. MMBS: Misaki Marine Biological Station, University of Tokyo

Incubation of sediment samples. Two sediment samples were collected in August, 1987 from Stns A-2 and A-3 in Aburatsubo Inlet (Fig. 1). Immediately after collection, each fresh sediment sample was divided into a total of 8 subsamples of 300 cm^3 and stored in four 3 l flasks and in four 500 ml flasks. Sterilized ASW (300 ml) was added to each and the flasks mixed. Among the 4 flasks prepared from 1 sediment sample, 3 were supplemented with either glucose (0.6 g), glucose (0.6 g) plus tryptophan (6 mg) or a green alga *Ulva* sp. (10 g wet wt); the remaining flask acting as control. These were then incubated for 7 d on a rotary shaker (at 200 rpm, eccentricity = 7 cm). The other 4 (smaller) flasks were incubated for the same period, but without aeration. All subsamples were incubated in the dark at 20 °C. After incubation, the sediment-water mixture of shaken samples was centrifuged at 10 000 × *g* for 30 min and the supernatant used in IAA analysis. For non-shaken flasks (i.e., unaerated), the water fraction above sediments was collected by decantation and filtered by Millipore GS-type filters (pore size 0.22 µm) and the filtrate used in IAA analysis. The remaining sediment was then centrifuged at 10 000 × *g* for 30 min and the supernatant also used for analysis. The green alga (used as a culture substrate) was collected from the surrounding littoral zone of the Inlet near the Misaki Marine Biological Station, University of Tokyo (Fig. 1). To estimate its original IAA content, a sample was extracted 3 times with 80 % methanol at below 4 °C. This extract was then filtered using a Toyo No. 3 paper filter and the IAA content of the filtrate determined.

Extraction and identification of IAA All samples were subjected to the following series of extractions for IAA. Each subsample was partitioned twice against diethyl ether at pH 8.5 to 9.0. The aqueous phase was then adjusted to pH 2.5 to 3.0 and partitioned twice against diethyl ether. The acid-ether phases were combined and evaporated under vacuum at 40 °C, and the dried sample was then dissolved in distilled water. This extract was immediately applied to an HPLC using a Senshu Pak ODS-1251-N column (4.6 × 250 mm) packed with 5 µm octadesylsilica gel (Nargel, FRG) in a Jasco Tri Rotar-VI HPLC system equiped with a Hitachi F-1000 fluorescent spectrophotometer (with an excitation at 280 nm and an emission at 350 nm). IAA peak was determined by comparison to a retention time of authentic IAA (Wako), and the content calculated from the peak area using a chromatogram integrator (DPL-225, Jasco). Samples

were eluted (in duplicate) at a flow rate of 1 ml min^{-1} with an isometrical condition of 25 % CH_3CN in 50 mM CH_3COOH. For accurate determination of IAA from marine sediments and bacterial cultures, every 0.5 ml fraction eluted from HPLC was methylated by treatment with excess amounts of ethereal diazomethan (Schlenk & Gellerman 1960) and then subjected to ELISA (enzyme-linked immunosolvent assay) using the monoclonal antibody for IAA methylester as described by Mertens et al. (1985). Polystyrene flat-bottom microtitration plates (Nunc), precoated with rabbit anti-mouse immunoglobulin (Miles, Israel), were used. Monoclonal antibody for IAA methylester and alkaline phosphatase-labeled IAA methylester (Idetek, USA) were applied to the immunoassay according to the instructions. Each sample was assayed in duplicate and the authentic IAA methylester (Fluka) subjected to calibration at every assay.

RESULTS

IAA in seawater and sediments

The presence of IAA in sediment samples was comfirmed by HPLC-ELISA analysis. A fluorescent compound with a retention time identical to authentic IAA appeared in the sample chromatogram (Fig. 2A). A methyl ester derivative of this compound also exhibited specific cross-reactivity to the antibody for an authentic IAA methyl ester (m-IAA).

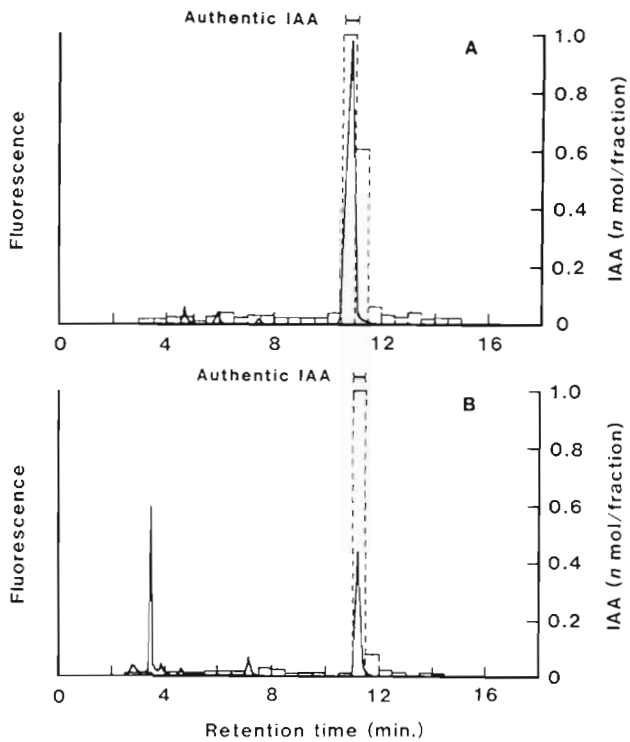

Fig. 2. HPLC separation of the diethylether extract of: (A) centrifuged supernatant of marine sediment collected at Stn T-3 in Tokyo Bay March 1987; (B) Strain 8E-13 culture fluid after incubation for 3 d in artificial Medium A enriched with tryptophan. Determination of IAA in fractions made by enzyme immunoassay

The IAA content of sediment samples tended to fluctuate with both season and location (Table 1). In samples from Aburatsubo Inlet, the IAA content was higher during the summer than in the other seasons: 3.8 to 5.0×10^{-9} mol l^{-1} in sediments collected in summer, 0.7 to 2.1×10^{-9} mol l^{-1} in sediments of other seasons. Significantly more IAA was found in Tokyo Bay than in Aburatsubo sediment samples. In the central region of Tokyo Bay, IAA content was 0.6 to 1.4×10^{-6} mol l^{-1} sediment which was over 10-fold larger than those in the outlet of the same bay and in Sagami Bay. The fine particle content (under ∅ 250 μm) in the sediment was measured to clarify possible correlation with the amounts of IAA in sediments. Sediments collected from Tokyo and Sagami Bays were rich in fine sand and mud (over 70 %) compared with that from Aburatsubo Inlet (30 to 70 %), which corresponded well with the difference in IAA content between the inlet and bay samples (Fig. 3).

A peak identical to IAA was also detected in the chromatogram of seawater extracts. The IAA content in the seawater was estimated at 1.2 to 22×10^{-11} mol l^{-1}, which was 1 to 5 orders of magnitude less than that of sediment samples. Although the seasonal and the locational variations of IAA in seawater were not as great as that in sediments, IAA levels tended to be slightly higher in Tokyo Bay than in Aburatsubo Inlet (Table 2).

IAA production of marine bacteria

IAA production of Strain 8E-13 was examined using the defined medium (A) with and without tryptophan. The IAA produced was clearly identified by HPLC-ELISA (Fig. 2B). Growth occured to nearly the same extent in both tryptophan-containing and control medium. However, IAA was only produced in the presence of tryptophan. This inducing effect of tryptophan on IAA production was found in all the other marine bacteria isolated previously and in 3 of 4 type-strains (Table 3) tested. Amounts of IAA produced in tryptophan-containing medium were of the order 10^{-7} to 10^{-6} mol l^{-1}, almost 1 to 2 orders greater than in the control medium.

Evaluation of IAA production in marine sediments

Fresh sediment samples shaken during incubation yielded a higher IAA content. Similarly, the addition of organic substrates greatly promoted IAA production in

Table 1. Indole-3-acetic acid (IAA) content (calculated as a unit of total sediment volume, $\times 10^{-9}$ mol l^{-1}) in interstitial water of marine sediments

Sampling area	Station no.	Date	IAA content
Aburatsubo Inlet	A-1	Jan 1985	0.7
		Aug 1985	3.8
		Nov 1985	2.1
	A-2	Nov 1984	1.3
		Jan 1985	1.2
		Aug 1985	5.0
		Nov 1985	1.7
Tokyo Bay	T-3	Mar 1987	1400
	T-5E	Mar 1987	610
	T-8	Mar 1987	88
Sagami Bay	S-H	Mar 1987	20

both the samples (Table 4). The effect of glucose plus tryptophan on IAA production was much greater than that of glucose alone, suggesting that the stimulative effect was mostly induced by tryptophan. The addition of seaweed as a sole substrate also enhanced IAA production (similar to glucose plus tryptophan). In cultures without aeration, IAA accumulation tended to be higher in the water above the sediment than in the interstitial water of the sediment (Table 5). As observed in shaken flasks, organic substrates stimulated IAA production in both sediment samples. The greatest stimulative effect was found in glucose plus tryptophan

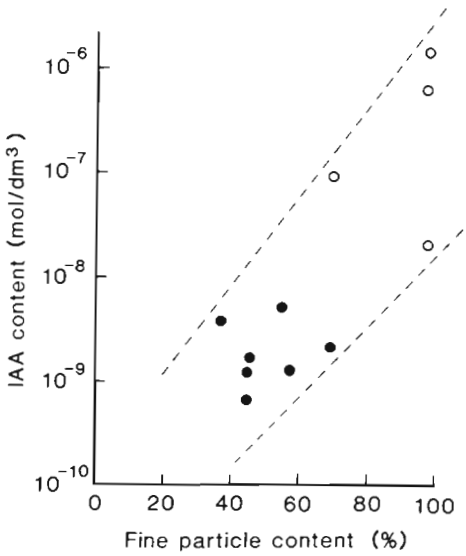

Fig. 3. Relation between IAA content of the interstitial water and fine (< ⌀ 250 μm) particle content of marine sediments obtained from Aburatsubo Inlet (●) and from Tokyo and Sagami Bays (○)

cultures, the IAA content reaching a maximum of 7.5×10^{-6} mol l^{-1} in the water overlying the A-3 sediment. Seaweed substrate enhanced IAA production to the same extent as glucose plus tryptophan even in this condition.

As a whole, greater IAA production (stimulated by enrichment) was found in A-2 sediment samples than in A-3 samples under shaken conditions, while non-shaken A-2 samples were more susceptible to enrichment than A-3 samples. This shows that the extent of IAA production and accumulation in the sediments was influenced not only by the kinds of substrates introduced, but also by the natural activity of the sediments.

DISCUSSION

The plant hormone auxin, IAA, was detected in marine sediments for the first time. The IAA content of interstitial water from sediments tended to be higher in eutrophicated environments, 0.7 to 5.0×10^{-9} mol l^{-1} in the Aburatsubo Inlet and 0.9 to 14×10^{-7} mol l^{-1} in the Tokyo Bay. Since the interstitial water sample for extraction was centrifugally separated from the sediment sample, we assume that the present value represents the amount of free IAA, not tightly absorbed to sediment particles. The amount of IAA in seawater filtrates was almost constant with location at the low level of 1.2 to 22×10^{-11} mol l^{-1}, compared with that in the sediments. The IAA content of seawater filtrates corresponds well with previous estimates of the total auxin content of 1.9×10^{-11} mol l^{-1} (Bentley 1960).

One possible origin of auxins in natural seawater is algal plants. Previous studies have shown that auxins are present in seaweeds: 5.7×10^{-11} mol g^{-1} fresh wt (Abe et al. 1972), below 5.7×10^{-10} mol g^{-1} fresh wt (Buggeln & Craigie 1971). Almost the same level of auxin was detected in green alga used here as a bacterial substrate: 1.0×10^{-11} mol IAA g^{-1} fresh wt. In

Table 2. IAA content ($\times 10^{-11}$ mol l^{-1}) in seawater filtrates

Sampling area	Station no.	Water layer	Date	IAA content
Aburatsubo Inlet	A-1	Surface	Jan 1985	1.2
	A-2	Surface	Nov 1984	2.3
		Surface	Jan 1985	2.0
		Surface	Sep 1985	2.8
		Bottom	Sep 1985	3.8
Tokyo Bay	H	Surface	May 1985	22
	T-3	Surface	Mar 1987	5.0
		Bottom	Mar 1987	2.8

addition, auxin-like compounds have been detected in unicellular algae (Hussain & Boney 1971, Grotbeck & Vance 1972) and in marine phytoplankton samples (Bentley 1958, 1960). Grotbeck & Vance (1972) estimated the auxin content of *Chlorella pyrenoidosa* as 3.0 to 8.3 $\times 10^{-9}$ mol g^{-1} dry wt. Although it is not clear whether auxins are released from decaying organisms or from living ones, our results which indicate a much lower IAA concentration in seawater than in sediment suggest that living algae contribute a minor part to the total IAA production of the sea.

Another possible origin of auxin in seawater is marine bacteria. IAA production by marine bacteria was clearly demonstrated in the present study. Amounts of IAA detected in cultures in the late logarithmic growth phase ranged from 10^{-8} to 10^{-5} mol l^{-1}. Tryptophan remarkably enhanced IAA production in almost all samples where it was applied, thus indicating its ability to stimulate IAA production by marine bacteria. Since the inducing effect of tryptophan on IAA-production is known among various terrestrial bacteria – e.g. *Agrobacterium tumefaciens* (Liu & Kado 1979), *Pseudomonas syringae* pv. *savastanoi* (Hutcheson & Kosuge 1985), *Azotobacter vinelandii* (Lee et al. 1970), *Azospirillum brasilense* (Tien et al. 1979), *Rhizobium leguminosarum* (Wang et al. 1982), several pseudomonads and xanthomonads (Fett et al. 1987) – it seems that the conversion of tryptophan to IAA is a general function of bacterial communities in the natural environment.

In fresh sediment cultures, tryptophan appeared to stimulate IAA production as well as in the bacterial culture. Since sediment was incubated under dark conditions, our results suggest that there is a great contribution by heterotrophic microorganisms in sediments, rather than by any photosynthetic organisms. While IAA was produced under both aerobic and anaerobic conditions, it is remarkable that when sediment was supplemented with fresh seaweed substrate and incubated anaerobically, greater amounts of IAA accumulated in the water just above sediments. Since the amount of IAA produced and accumulated in both the overlying water and the interstitial water of sediments was far beyond the original IAA content of seaweed, it is reasonable to suppose that most of the IAA was produced during microbial decomposition of

Table 3. Effect of tryptophan on growth (at OD_{600}) and IAA production ($\times 10^{-7}$ mol l^{-1}) of marine bacteria. Medium A: free of IAA; B and C: originally contained IAA, 0.2 to 0.3 $\times 10^{-7}$ mol l^{-1}

Strain	Source	Medium	Growth		IAA content	
			Tryp. added	Control	Tryp. added	Control
8E-13	Seawater	A	0.32	0.33	26.3	<0.1
8H-9		A	0.55	0.60	2.2	<0.1
8I-6		A	0.19	0.18	1.6	<0.1
AA-9	Sediments	C	1.69	1.68	2.4	0.9
S1-1		C	1.22	1.25	3.4	1.3
S1-18		C	1.22	1.24	1.6	0.8
KI-13	Seaweeds	A	0.51	0.43	75.4	0.1
KJ-3		B	0.47	0.52	15.4	1.4
HO-13		C	1.63	1.47	13.7	2.2
CC-16	Unicellular algal culture	B	0.78	0.77	2.1	0.3
Aeromonas hydrophila NCMB 86		C	1.12	1.08	15.7	3.8
Pseudomonas aeruginosa NCMB 10		C	1.23	1.23	4.3	1.1
Vibrio parahaemolyticus ATCC 17802		C	1.65	1.68	24.0	3.3
Corynebacterium sp.[a]		C	1.54	1.42	1.4	1.8

[a] Strain isolated from seawater and identified by Dr U. Simidu

Table 4. IAA content in centrifuged supernatant of the sediment-ASW medium mixture after incubation in aerating condition in the dark. Values are IAA content ($\times 10^{-9}$ mol l^{-1}). Seaweed: per 10 g fresh wt *Ulva* sp. originally contained IAA at 0.1 $\times 10^{-9}$ mol

Sediment source	Pre-incubation	Substrate enriched to the ASW medium			
		None	Glucose	Glucose+Tryptophan	Seaweed
A-2	<0.1	26.0	44.5	271.0	278.0
A-3	<0.1	21.5	56.0	148.0	118.0

Table 5. IAA content in the interstitial water of the sediment (sediment) and in the water overlying the sediment (water) after incubating without aeration under the dark. Values are IAA content ($\times 10^{-9}$ mol l^{-1})

Sediment source	Sample	Pre-incubation	Substrate enriched to the ASW medium			
			None	Glucose	Glucose+Tryptophan	Seaweed
A-2	Water	<0.1	13.0	39.0	525.0	134.0
	Sediment	<0.1	1.5	2.5	83.5	3.5
A-3	Water	<0.1	10.5	73.5	7540.0	255.0
	Sediment	0.1	2.5	6.0	566.0	682.0

seaweed. Therefore, it seems that the settlement of a large amount of organic debris, as a result of active primary production, induces IAA production and accumulation in stagnant near-bottom water. Although there is little data on the vertical distribution of IAA in seawater, we found higher IAA concentrations in the bottom-water than in the surface-water of Aburatsubo Inlet. In addition, higher cytokinin activity has been detected (another plant hormone group of bacterial origin in the sea; Maruyama et al. 1986) in estuary near-bottom water than in surface water over 1 yr (Kentzer et al. 1980).

In general, algal growth is enhanced by supplements of extracts of terrestrial soil, suggesting the presence of certain biologically active compounds in the soil (Provasoli et al. 1957). Marine sediments also contain growth-promoting compounds for unicellular algae, especially for red-tide flagellates (Iwasaki 1979, Takahashi & Fukazawa 1982). These compounds may be released from the bottom sediments under low-oxygen conditions, together with some nutrients, organic chelators and vitamins (Iwasaki 1979, Fukazawa et al. 1980). Although the exact chemical nature of the compounds which stimulate growth of red-tide flagellates is still obscure, our results suggests that auxin, IAA, may be one such active compound in marine sediments.

A previous study on the multicellular green alga *Caulerpa sertularioides* indicated that its long-term thallus growth could be attained, not only in the presence of an auxin IAA, but also in the presence of marine-sand rich in microorganisms (Mishra & Kefford 1969). This finding is comparable with our results, that marine sediments contain IAA and IAA producing microorganisms. Dawes (1971) also observed the stimulative effects of IAA on thallus proliferation of the green alga *C. prolifera*, at a concentrations of 1 to 5×10^{-6} mol l^{-1}, whereas at concentrations beyond 10^{-5} mol l^{-1} growth was inhibited. Although the level of IAA reported for green algae was slightly higher than the range of IAA concentration in marine sediments examined in this study, it is possible that further IAA production by sediment microorganisms affects growth. In addition, Harrison (1978) showed that microbial decay of eelgrass promoted growth of the green alga *Ulva fenestrata*, suggesting that certain compounds, released by bacterial activity, affect growth. Further studies on algal growth regulators in marine environments are necessary to elucidate, not only the growth of multicellular algae but also the formation of red tides in the sea.

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