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Content and Uptake of Trace Metals in Benthic Algae, *Enteromorpha* and *Porphyra*. II. Studies on the Algae Cultured in Sea Water Supplemented with Various Metals.

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(Figs 7; Tables 7)

In order to investigate how the metal content of *Enteromorpha* and *Porphyra* grown in natural environment is influenced by the metal concentration in sea water, the metal contents of algae cultured in sea water supplemented with metals were examined. Also to trace the metals taken into fronds, these were divided into three component layers, so that the metal content of each layer was investigated. The culture of fronds in a large quantity presents a number of difficult problems so as the control pH, the chlorinity, the temperature, and the amount of light. Thus, the culturing was carried out as in the shortest period possible in this present investigation.

Materials and Methods

Sea water used in this experiment was collected approximately 15 km off the Fukuyama shore and stored in tanks. The sea water was filtered before use on a Toyo No. 2 filter paper, and the metals (Fe, Mn, Cd) were added to it. Iron was added as $FeCl_3$ in 0.1 N HCl for *Enteromorpha* and as Fe-EDTA (Imamura[1]) for *Porphyra*. Manganese and Cadmium were supplemented as $MnCl_2$ in 0.1 N HCl and $CdCl_2$ in 1 N HCl, respectively. PH of the water was adjusted to 8.1 with a sodium hydroxide solution. The metal concentrations of water adjusted to 10, 250, and 1000 times the analytical values (Fe 0.1, Mn 0.006, and Cd 0.002 mg/ℓ) given by Inoue[2] for the coast water of Fukuyama.

(1) **Culture of *Enteromorpha* and *Porphyra*.**

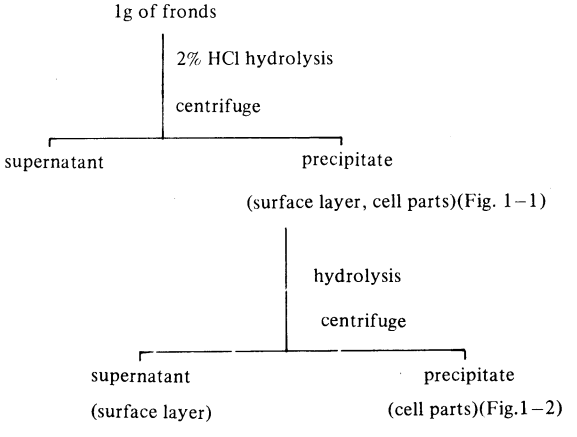
Fronds of *Enteromorpha* were cultured in a 5 ℓ aquarium under the conditions of temperature 20°C, chlorinity 17.6‰, pH 8.1, and exposing to a light of 3000–6000 lux for 12 hrs. a day. The culture periods were 72, 71, and 92 hrs. for metal additions of Fe, Mn, or Cd, respectively. Aeration was continued for days and nights. Cultivations were made in media containing 5 mg/ℓ of Fe, 2 mg/ℓ of Mn, and 0.4 mg/ℓ of Cd. After each culture period the algae were removed from the medium, rinsed successively with tap, deionized, and distilled waters, and dried at 105°C. Ten, 8 and 10 samples (each sample 0.3g) of dry material were taken for the measurements of Fe, Mn, and Cd, respectively.

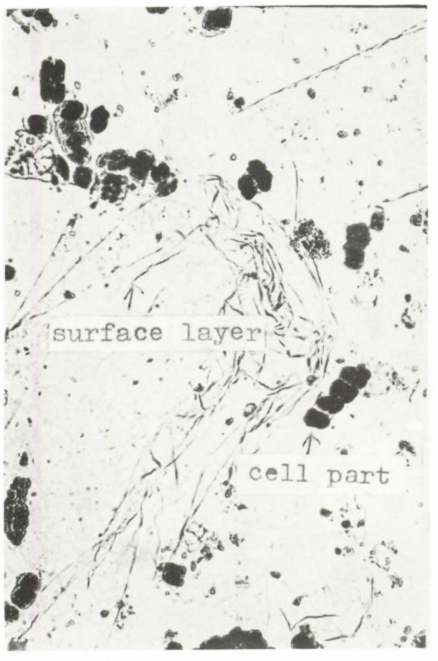
Each sample was treated by the method described in the preceding paper and analyzed by an atomic absorpiton spectrophotometer. Cd was measured without solvent extraction.

In the case of *Porphyra* the fronds were taken from a commercial culture ground and cultured in media containing 0.76, 19.0, and 76.0 mg/ℓ of Fe (Fe-EDTA); 0.06, 1.6 and 6.6 mg/ℓ of Mn; and 0.02, 0.5, 2.0 mg/ℓ of Cd in 5 ℓ aquaria under the conditions of temperature 10°C, chlorinity 19.2‰, pH 8.1 and by using the same light conditions as those of *Enteromorpha*. Aeration was made only for day times. Approximately 10g (by wet weight) of *Porphyra* was removed from the culture medium every 24 hrs, rinsed with water, and dried. The dry material (1g) was decomposed, and the metal content was determined. Cadmium was determined after extracting with solvent.

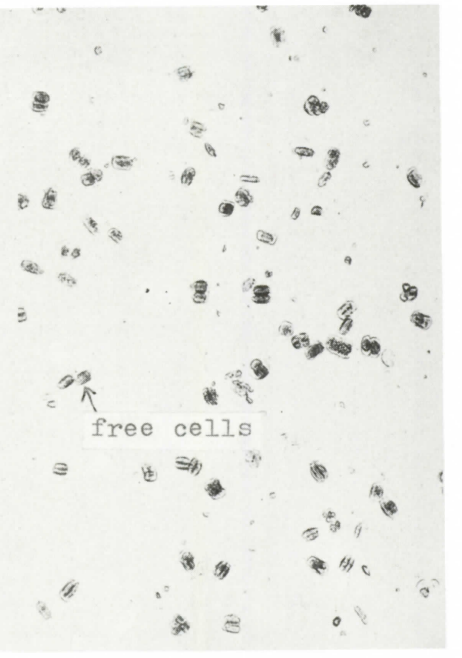
(2) The part of *Porphyra* Fronds for Metal Uptake.

As a method to examine in what quantity how much the metals were present and in which part of the fronds, dry *Porphyra* (1 g) was placed in a 300 mℓ Kjeldahl flask equipped with a condenser, and 100 mℓ of 2% HCl were added. The content was heated at 100°C for 20 min., immediately cooled, and centrifuged at 3000 rpm for 15 min. The portion dissolved by this first hydrolysis is assumed to be the intercellular substance (major component is mannan) and is called the middel layer. The precipitate consists of the surface layer (cuticle layer, crystalline mannan with little, crystalline xylan) and of the cell part (cell wall consists of xylan) which can be recognized by a microscope (Frei and Preston[4~6]). Water (50 mℓ) was added to this precipitate and the mixture was boiled for 30 min. to dissolve the surface layer and centrifuged. Finally, the cell part remained as precipitate. Figure 1 shows these procedures. Each layer separated by this method was treated in the same manner of the algal decomposition and then analyzed. Cadmium was determined after solvent extraction.





1.



2.

Fig. 1 Separation of the fronds of *Porphyra* by hydrolysis

(3) Difference in Metal Uptake between Light and Dark conditions.

Porphyra was cultured in the sea water supplemented with Fe, Mn, and Cd, and examined whether there were differences in metal uptake between light and dark conditions. The culture was done under the same conditions as before, but only in sea water containing the fold of the natural metal concentrations. The *Porphura* culture was started from dark period. After 12 hrs under dark and light conditions, they were collected, rinsed with water, and the metal contents were determined by the decomposition method and also by separating each layer (Miwa[3] , Frei and Preston[4~6], Cronshaw[7]).

(4) Metal Concentration of Sea Water.

The metal concentrations in the sea water used for the culture media were determined by a modified method of Biecher[8]). Thus, Chelex 100 (a chelate resin) was rinsed with 4 N NHO_3 and loaded on a column (1 cm inside diameter) with distilled water to a height of 10 cm. The sea water was filtered on a Milipore filter HA (0.45μ), and was added an ammonium acetate buffer solution in the proportion of 2 mℓ to 100 mℓ sea water. The treated sea water (500 mℓ) was passed through the column at a rate of approximately 11 mℓ per minute. In order to elute the ions adsorbed, the column was washed with 4 N HNO_3 (25 mℓ) at a rate slower than 1.5 mℓ/min. The eluted solution was recovered in a 50 ml volumetric flask and made up to 50 mℓ with 4 N HNO_3. This solution was used for the analyses by the atomic absorption spectrophotometry. The calibration curve were made by diluting 1000 mg/ℓ solution of Fe, Mn, and Cd with 4 N HNO_3.

Results

(1) Culture of *Enteromorpha* and *Porphyra*.

In the case of *Enteromorpha*, the Fe contents were 478μg/g on the average of 10 samples cultured in the control sea water, and 1340μg/g in the samples culture in water that had 5 mg/ℓ of Fe added. The contents of Mn were 116μg/g on the average of 8 samples from the control sea water, and 698 μg/g in the samples from the water containing 2 mg/ℓ of Mn. The contents of Cd were traced in the control, but 58.5μg/g in the samples from the water supplemented with 0.4mg/ℓ of Cd.

In the case of *Porphyra*, the Fe content before culture was 322 μg/g. The average length of 20 fronds of this alga was 9.0 cm. The content of Fe decreased with cultivation time in the control water and in the water which had 0.76 and 19.0 mg/ℓ of Fe added. However, it somewhat increased in the samples cultured in the water supplied with 76.0 mg/ℓ of the metal, although there was no difference of uptake in the course of culture time (see Fig. 2 and Table 5). The average length of *Prophyra* used for the experiement of Mn uptake was 10.5 cm. There was no variation in the Mn content of the alga cultured in the control and in the water supplied with 0.06 mg/ℓ of Mn. However, in the waters with 1.6 and 6.6 mg/ℓ of the metal added the content of Mn increased in proportion to the culture duration (see Fig. 3 and Table 5).

The average length of *Porphyra* fronds used for the experiment of Cd uptake was 17.4 cm. There was no time variation in the Cd content of *Porphyra* when cultured in the control water. However, the concentration of Cd increased as the metal addition increased from 0.02 to 0.5 and to 2.0 mg/ℓ. The Cd content of alga increased in proportion with the culture time, but did not do so with Cd concentration of media (see Fig. 4 and

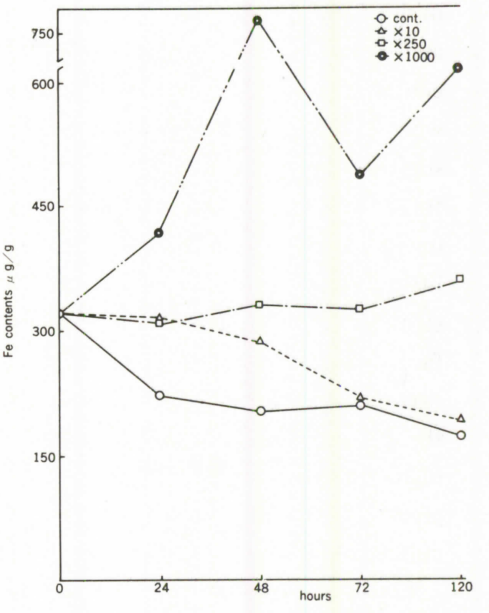


Fig.2 Variations of Fe contents of *Porphyra* cultured in the waters containing various amounts of Fe

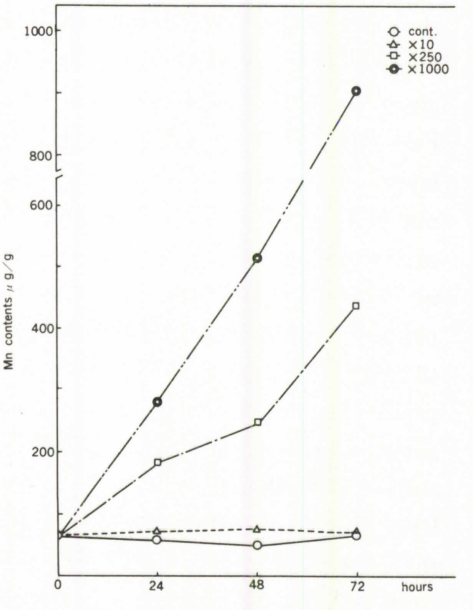


Fig.3 Variations of Mn contents of *Porphyra* cultured in the waters containing various amounts of the metal

Table 5).

(2) The Part of *Porphyra* Fronds for Metal Uptake.

The weight percentages of surface layer, middle layer, and cell part was 24.5, 44.0 and 31.5%, respectively. The metal concentrations of each part of *Porphyra* fronds were listed in Table 1 to Table 4. The results suggest that Fe uniformly distributes in the three parts. The distribution of Mn is high in the surface and middle layer, but quite little in the cell part. The uptake of Mn is stronger in the middle layer than in the surface layer. In the case of Cd, the concentration in the cell part changes little even when the Cd concentration of the water becomes high, but the uptake by the middle layer is greatly influenced by the concentration of the culture medium.

(3) Differences in Metal Uptake between Light and Dark conditions.

An average length of *Porphyra* used in this experiment was 17.4 cm. The results are shown in Figure 5 to Figure 7. The metal concentration in each part of the fronds are given in Table 6. The results reveal that the Fe concentration decreases in the surface layer during dark periods, although it can not be regarded as an unambiguous tendency of Fe uptake. Yet, there is a clear difference in the Mn uptake between light and dark periods, that is, the concentration of Mn in the surface layer becomes high during light periods and low during dark peiods. The amout of decrease in the surface layer was equal to the amount of increase in the middle layer at dark periods. This fact suggests that the metal taken in the surface layer during light periods moves into the middle layer during dark periods. In the case of Cd uptake there was no difference between light and

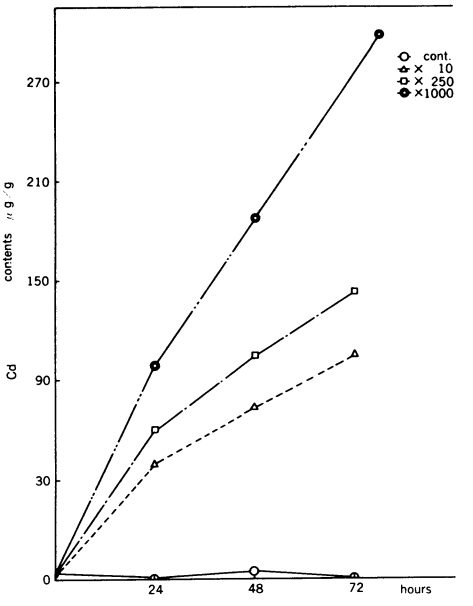


Fig.4 Variations of Cd contents of *Porphyra* cultured in the waters containing various amounts of the metal

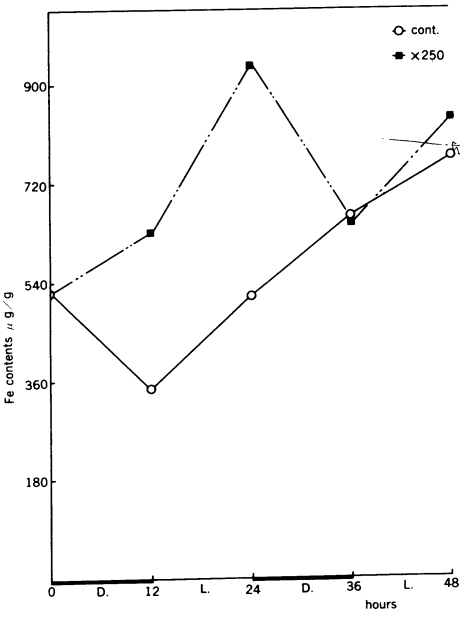


Fig.5 Changes of the Fe uptake of *Porphyra* between light and dark period

Table 1. Fe contents of each part of *Porphyra* fronds cultured in the waters of various Fe concentration (μg/g)

Time (hr)	0	24	48	72	120
control sea water					
Total	322.0	222.0	–	210.0	174.0
surface layer	285.0	142.0	190.0		
middle layer	446.0	330.0	250.0		
cell parts	269.0	133.0	148.0		
0.76 mg Fe/ℓ					
Total		207.0	–	220.0	192.0
surface layer		359.0	301.0		
middle layer		696.0	343.0		
cell parts		459.0	201.0		
19.0 mg Fe/ℓ					
Total		241.0	–	325.0	359.0
surface layer		321.0	291.0		
middle layer		615.0	508.0		
cell parts		306.0	120.0		
76.0 mg Fe/ℓ					
Total		408.0	–	487.0	616.0
surface layer		284.0	509.0		
middle layer		609.0	1320.0		
cell parts		333.0	182.0		

Table 2. Mn contents of each parts of *Porphyra* fronds cultured in the waters of various Mn concentrations (μg/g)

Time (hr)	0	24	48	72
control sea water				
surface layer	4.2	53.2	44.6	60.8
middle layer	146.0	111.0	96.0	119.0
cell parts	–	–	–	–
0.067 mg Mn/ℓ				
surface layer		22.1	98.9	39.6
middle layer		154.0	117.0	141.0
cell parts		–	–	–
1.67 mg Mn/ℓ				
surface layer		115.0	106.0	124.0
middle layer		356.0	508.0	941.0
cell parts		–	–	1.8
6.67 mg Mn/ℓ				
surface layer		112.0	290.0	470.0
middle layer		579.0	1010.0	1800.0
cell parts		–	3.3	14.7

Table 3. Fe contents of each part of *Porphyra* fronds cultured in the waters of various concentrations of Mn (μg/g)

Time (hr)	0	24	48	72
control sea water				
surface layer	169.0	133.0	299.0	167.0
middle layer	557.0	443.0	373.0	388.0
cell parts	190.0	153.0	105.0	130.0
0.067 mg Mn/ℓ				
surface layer		146.0	201.0	128.0
middle layer		450.0	330.0	351.0
cell parts		113.0	135.0	120.0
1.67 mg Mn/ℓ				
surface layer		169.0	119.0	132.0
middle layer		394.0	358.0	390.0
cell parts		131.0	78.7	102.0
6.67 mg Mn/ℓ				
surface layer		184.0	153.0	118.0
middle layer		414.0	431.0	329.0
cell parts		122.0	92.8	70.9

Table 4. Cd contents of each part of *Porphyra* fronds cultured in the sea waters of various concentrations of Cd (μg/g)

Time (hr)	0	24	48	72
control sea water				
surface layer	–	–	–	0.55
middle layer	2.79	1.37	4.27	0.85
cell parts	0.88	0.55	–	0.14
0.02 mg Cd/ℓ				
surface layer		18.00	44.10	70.70
middle layer		77.70	144.00	197.00
cell parts		0.16	0.23	4.15
0.50 mg Cd/ℓ				
surface layer		38.40	43.60	109.00
middle layer		112.00	212.00	266.00
cell parts		2.71	4.23	2.23
2.00 mg Cd/ℓ				
surface layer		29.70	47.70	69.00
middle layer		206.00	398.00	629.00
cell parts		1.70	3.64	12.20

Table 5. Concentration factor in *Porphyra* in various metal concentrations.
(F : μg/g dry tissue / μg/ml sea water)

Fe : F

concentration \ hours	0	24	48	72	120
0.076 μg Fe/mℓ	4,239	2,922	2,676	2,769	2,293
0.76		415	379	290	253
19.00		16	17	17	19
76.00		6	10	6	8

Mn : F

concentration \ hours	0	24	48	72
0.006 μg Mn/mℓ	10,392	9,803	8,870	10,663
0.066		1,104	1,135	1,071
1,66		111	149	266
6.66		42	77	135

Cd : F

concentration \ hours	0	24	48	72
0.002 μg Cd/mℓ	753	379	939	275
0.02		1,933	3,707	5,266
0,5		119	211	289
2.0		49	94	149

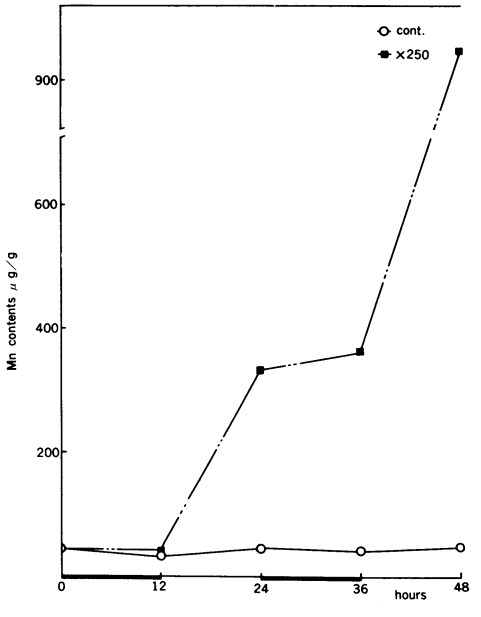


Fig.6 Changes of the Mn uptake of *Porphyra* between light and dark period

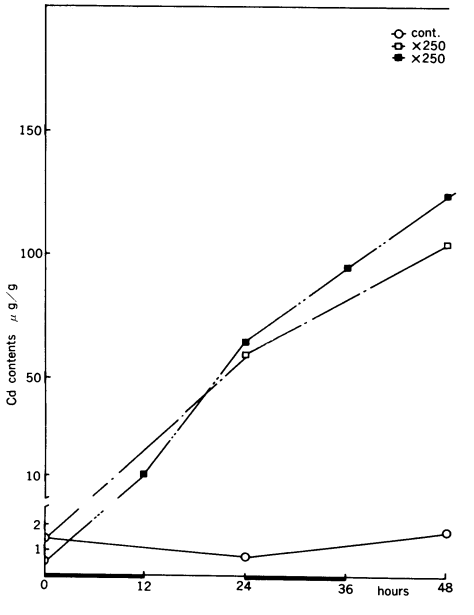


Fig.7 Changes of Cd uptake of *Porphyra* between light and dark period

Table 6. Concentrations of metals in each part of the fronds of *Porphyra* during light and dark period (12-12 h) (μg/g)

hours / metal conc. part	0	0 to 12 dark	12 to 24 light	24 to 36 dark	36 to 48 light
control sea water					
Fe: surface layer	344.0	288.0	488.0	432.0	594.0
middle layer	601.0	555.0	636.0	732.0	730.0
cell parts	561.0	934.0	379.0	672.0	925.0
19.0 mg Fe/ℓ s.w.					
surface layer		292.0	675.0	488.0	527.0
middle layer		749.0	1090.0	933.0	1210.0
cell parts		733.0	922.0	379.0	533.0
control sea water					
Mn: surface layer	17.20	3.02	17.40	5.39	7.92
middle layer	86.10	63.50	87.30	90.20	99.30
cell parts	2.67	–	5.40	4.19	3.78
1.67 mg Mn/ℓ s.w.					
surface layer		10.0	518.0	90.0	702.0
middle layer		91.0	445.0	752.0	1720.0
cell parts		–	38.5	35.0	58.8
control sea water					
Cd: surface layer	–				
middle layer	0.875				
cell parts	0.873				
0.5 mg Cd/ℓ s.w.					
surface layer		5.15	17.70	29.60	37.90
middle layer		21.10	136.00	197.00	261.00
cell parts		0.40	2.64	3.77	0.90

dark periods, but the uptake steadily increased in proportion to the culture time.

(4) Metal Concentration of Sea Water.

The concentrations of Fe, Mn, and Cd in control sea water were 0.076, 0.006, and 0.002 mg/ℓ, respectively. Also the percentages of dissolved metals to the amounts of the metals added to the control water were checked (Table 7). The result showed that 10-80% of Fe and Mn added were in dissolved state, while Cd completely dissolved. There were no changes in the concentrations of dissolved metals when they were measured by changing pH of the solution from 8.1 to 8.7.

Table 7. Concentrations of metals in the sea water added witht he metals at PH 8.1 and PH 8.7

Amount of metals added	Concentration PH 8.1	PH 8.7
76.0 mg Fe/ℓ sea water	34.4 mg/ℓ	34.4 mg/ℓ
19.0	4.7	4.6
0.76	0 .17	0.16
6.76 mg Mn/ℓ sea water	5.56	5.25
1.67	0.39	0.26
0.06	0.05	0.05
2.00 mg Cd/ℓ sea water	2.10	2.00
0.50	0.50	0.50
0.02	0.023	0.025

Discussion

Since the culture of *Enteromorpha* was done only as a preliminary experiment, whether the metals added in the sea water were dissolved or not was not taken into account. Since aeration continued day and night, the pH of culture water increased to about 9.0 after the period of culture.

In the case of *Porphyra* the culture water was aerated only during day time. There the pH of the water rose only to a maximum 8.7, and it was not able to maintain a constant value. According to Oohusa[9], there are no differences in the amout of growth of *Porphra* when cultured in a pH range between 7.5 and 8.4 for 5 to 10 days, but the growth rapidly declines at a pH above 8.5. Hence, it seems that pH is an important factor affecting the uptake of Fe. Seshadri Kannan[10,11] reported that the maximum uptake of Fe by rice was at pH 5.0-5.5. Further detailed experiments are necessary for stricter determination of the influence of the pH factor.

In the separation of fronds by hydrolysis with hydrochloric acid, the weight ratios were 24.5, 44.0, and 31.5 for the surface layer, middle layer, and cell part, respectively. This separation method is not fully satisfactory, because contamination with other layers is always possible, especially of the middle one with the other two. However, we had to proceed in this manner because there is not yet a better method available for measuring the metal concentrations of each layer of fronds. If it were only for the presence, and not for the concentration of metals in each layer different methods are availbale. In this respect, J. R. Walton[12] confirmed by means of an electron probe X-ray analyzer that lead taken from the mitochondria of rat liver had accumulated in granules. Other investigators (Richard[13], Albert[14]) have reported that intramitochondrial metals were closely linked to the electron and energy transfer reactions. The distribution of insouble or structurebound minerals was studied by an electron micrscope coupled with high resolution microincineration techniques. The present study wants to direct itself to the elucidation of the physiological function and the intracellular and intercellular accumulation of metals by employing such techniques as X-ray fluorescence analysis, electron microscope, electron diffraction, and ultramicro radioautography.

Summary

1. In the culture of *Porphyra* in the sea water supplemented with metals, the uptakes of Mn and Cd were relatively high and increased in proportion to culture time when the metal concentration in water was high.

2. Fe distributed evenly in all the three parts of fronds. Mn was concentrated in surface and middle layer, while Cd was accumluated mainly in the middle layer and a little in the surface layer. In general the uptake was high in the middle layer.

3. In the uptake of Mn there was a clear distinction between light and dark conditions, that is, Mn was absorbed only during light period. While, Cd was aborbed regardless of light and dark periods.

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アオノリ，アマノリの金属添加海水での培養に関する研究

前田正智・藤山虎也

自然状態で生育するアオノリ，アマノリの金属含有量が海水の金属含有量により異なってくるかを調べるため，海水に金属を添加し，葉体中の金属含有濃度を調べた。アサクサノリ等の細胞層一層のParenchyma組織では，細胞部を中心にその両側に細胞充間物質とクチクル層の五層からなり，成分的には三層よりなっている。したがってとりかこまれた金属が葉体のどの部位に存在するか葉体を三層に分離し層別の金属含有濃度を調査した。添加金属はFe，Mn，Cd の3種で海水中の各金属濃度は対照海水の金属濃度10，250，1000 倍とした。葉体の金属添加海水での培養時間は24×3時間，明期・暗期12×4時間とした。

アオノリの対照海水中の培養では各金属含有濃度はFe：478μg/g，Mn：116μg/g，Cd：Trace，24時間後の金属添加海水ではFe：1340μg/g，Mn：698μg/g，Cd：58.5μg/gであった。

アマノリでは対照海水中での各金属の含有濃度はFe：200～300μg/g，Mn：50～60，Cd：0.5～1.5であった。金属添加海水では葉体中のFe濃度はあまり大きな増加はみとめられなかったが，Mn，Cdについては高濃度海水において葉体中の金属含有濃度は培養時間と比例的関係が認められた。アマノリ葉体の部位別金属含有濃度について，Feはどの部位にも平均的に分布し，Mnでは細胞部に少なく表層と中層に高

い含有濃度を示した。また，とりこみについても中層部が強く，表層部でもとりこまれた。Cdの場合は細胞部の濃度は海水中のCd濃度が高くなっても変化は少なく，中層部へのとりこみが非常に多かった。明期暗期別の金属のとりこみについては，Feでは明白な傾向が認められなかったが，Mn，Cdについては興味ある結果が得られた。すなわち，Mn，は明期にとりこまれ，暗期にはとりこまれない。Cdは明・暗期に関係なく培養時間と比例的にとりこみが行なわれることが明白になった。