Microorganisms and Plant of Autonomous Biological Systems (ABS) Samples

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Abstract Distribution of microorganisms and cellular structure of an Autonomous Biological Systems (ABS) were studied with a special attention to the effect of space environments. Viable cell densities measured by the direct fluorescence microscopic method were in the order of 10^5 cells/ml for fractions 1 (upper suspension) and 2 (lower suspension), and 10^6 cells/ml for fraction 3 (sediments). These values were 10 to 100 times larger than the values obtained by the classical colony forming unit method. No difference between flight and ground samples was observed in the vertical distribution of viable microorganisms when fractionation and analysis were carried out after recovery.

Intracellular distribution of chloroplasts in higher green plants, *Ceratophyllum demersum*, of flight samples was disturbed after 10 days of flight (24hrs/day light on). After 4 months of flight (Mir /STS-79/81) with 24 hrs light on, *Ceratophyllum demersum* was completely disintegrated. On the other hand, in the second 4-months-flight experiment with 16 hrs/day light on, *Ceratophyllum demersum* was only slightly deteriorated.

Introduction

All organism on Earth is under severe control of genetic information and environmental factors. Among the environmental factors, gravity is very unique since organisms have never experienced it's change. It is often said that organisms accumulate their ancestor's properties/memories as courses of mutation and natural selection imposed by environmental factors in their genetic information. If this is true, we have no information on gravity other than 1g and have no preparation for its change.

Many studies have been carried out with special attention to the effect of gravity on organisms. Every organism is always involved in ecological systems and for the comprehensive understanding of life, we have to investigate not only individual organisms but also ecological systems. Compared to individual organisms, ecological systems are much sensitive to environmental changes. It is a familiar sight that habitats are localized vertically due to gravity, with typical examples of birds and fishes. Even the distribution of organic materials including detritus and subsequently over-all material flow

are dependent on gravity. Therefore, it seems that gravity plays an essential part for the creation and maintenance of ecological systems.

Under these situations it is very important what kind of ecological structures can be formed if gravity changes. Niche of organisms and material flow are expected to change dramatically under microgravity. For the preliminary study on the change described above, simple but stable model is required. We have investigated the structure and its stability of miniature materially closed ecological systems in space environment. Here we report the results of the study on the distribution of microorganisms, and the structure of chloroplasts in plant cells in closed ecological systems.

Materials and Methods

Material processing including fractionation is described elsewhere (MacCallum *et al.* 1998, Ishikawa *et al.* 1997). Aliquots of the fractioned sample solution were filtered with membrane filters (millipore GTBP2500, pore size:0.2 μ m). Trapped cells were washed with 3 ml of sodium phosphate buffer (pH 7.0, 20mM). The washed filters were placed on slide glasses, and 0.1 ml of staining solution {20mM sodium phosphate buffer pH 5.9 supplemented with 10 μ M of CFDA -AM (5-carboxyfluorescein diacetate acetoxymethyl ester) and 0.05% pluronic F127} was poured. Measurements were carried out from 20 min

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Fig.1 Microscopic images of microorganisms contained in the closed ecological system. A: DIC(differential contrast) image, B: fluorescence image of 5-CF (hydrolytic fluorogenic product of CFDA-AM), C: fluorescence image of chlorophyll. Suspension (liquid) fraction of the ground control for 10 days mission by Space Shuttle was stained with CFDA-AM to detect viable cells (B). Plants and algae were detected by chlorophyll fluorescence (C).

through 50 min after staining. Distribution of microorganisms and fine structure of a water plant, *Ceratophyllum demersum*, were measured by fluorescence method (Tsuji *et al.* 1995, Kawasaki *et al.* 1996, Kawasaki and Tsuji 1996, Kawasaki 1998) using a fluorescence microscope (Zeiss Axiovert 135M) equipped with a cooled CCD camera (Photometrics CH250). Viable cells were detected by 5-CF(5-carboxyfluorescein : cleavage product of CFDA-AM) and algae cells were detected by chlorophyll fluorescence. Fine structure of *Ceratophyllum demersum*

was measured by differential contrast (dic) method. The images recorded with the cooled CCD were stored in magneto-optical disks. Cell density was determined by an image analysis software, IPLab (Signal Analytics).

Colony forming unit of bacteria was measured by spreading a fraction of specimens to agar plates (90 mm diameter) supplemented with Nutrient Broth and cultured for 3 weeks at 37°C, and the number of colonies were counted by naked eyes. Colony forming unit of fungi were measured by almost same way except that standard potato dextrose plates supplemented with Chloramphenicol (15mg/plate), Streptomycin (2mg/plate) and Penicillin (50,000 units/plate) were used and temperature was 27°C.

Results

1. Vertical distribution of microorganisms

Microorganisms contained in the present ecological systems are shown in Figure 1. It is evident that this system is composed of many kind of microorganisms judging from the morphology shown in A. The fibrous objects are algae since they contain chlorophyll (C) and they are the primary producers together with green plants such as *Ceratophyllum demersum*. The objects in B are viable cells. The small dots with diameters less than 5 μm are specifically localized around the algae. Judging from the size, they are mainly viable bacteria. Most bacteria are supposed to be consumers (in other word, decomposers) and depend on primary producer such as algae. Fig.1 clearly pictured this currently accepted relationship between primary producers and

Fig.2 Vertical distribution of microorganisms (3rd mission). A: viable cell density, B:chlorophyll-containing-cell density. Sample terminology: Take G2-1cf for example, G2 stands for sample number 2 of ground control, and 1cf stands for fraction 1 and 5-CF (dye used for fluorescence measurements). chlo means chlorophyll (used for fluorescence measurements). As for fraction 3, samples were diluted 50 times. Therefore, true cell density values of fraction 3 are 50 times of the values shown in these figures. Significant point is cell density of fraction 3 is several tenth times higher compared to those of fraction 1 and 2 both in ground and flight samples. However, no difference was obtained between ground and flight samples.

Fig.3 Structure of *Ceratophyllum demersum*. A: 1st mission (10 days mission on Space Shuttle). B: 2nd mission (4 months mission on Mir, 24 hrs/day light on). G and F mean ground control and flight samples, respectively.

consumers.

Vertical distribution of viable microorganisms and cells containing chlorophyll fractionated after recovery of the third mission (4 months flight on Mir with 16 hrs/day light on) are summarized in Fig. 2. Significant is that fraction 3 showed 10 to 50 times higher viable and algae cell densities compared to fractions 1 and 2 in ground as well as in the flight samples. However, no significant difference was obtained between the flight and ground samples. In the first flight on Mir (4 months but always light on), the similar results were obtained.

Cell densities shown in Fig.2 are several hundreds times larger than those obtained by the classical colony counting

Fig.4 Microscopic structure of *Ceratophyllum demersum*. A: 1st mission (10 days mission on Space Shuttle). B: 3rd mission (4 months mission on Mir, 16 hrs/day light on). G and F mean ground control and flight sample, respectively.

method (data not shown). Similar discrepancies were often reported (Tsuji *et al.*, 1995). No fungi were detected in the samples of Mir missions (data not shown).

2. Fine structure of *Ceratophyllum demersum*

Macroscopic and microscopic structures of *Ceratophyllum demersum* examined by the dic microscope are shown in Figs.3 and 4. As shown in Fig.3A, after 10 days mission, *Ceratophyllum demersum* in both flight and ground samples looked healthy. Different from the apparent morphological similarity between the flight and ground samples, changes in fine structure was observed as shown in Fig.4A. Significant decrease in the number and disordering of chloroplasts were observed in the flight samples.

This finding opens the idea that this change will be promoted after longer flight. We, therefore, tried the 2nd experiment of 4 months using the Russian space station Mir. Results are shown in Fig.3B. Dramatic difference was observed in this mission. In the flight samples, *Ceratophyllum demersum* was completely decomposed. We, therefore, could not record the microscopic images this time. As explained in Materials and Methods, light for photosynthesis was always on, and the light illumination stress may be added besides space-specific stresses.

In the 3rd experiment, we modified the light irradiation cycle time to 16 hrs on and 8 hrs off. Results are shown in Fig.4B and their macroscopic morphology is given in this volume (Ijiri, *et al.*, 1998). Although the plants reduced their volume and looked partially deteriorated (see Ijiri *et al.* in this volume), over-all decomposition was not observed even in the flight samples. Within plant cells, decrease in the number and disordering of chloroplasts were observed both in the flight and ground samples with no clear difference between them.

In summary, in the first (10 days with 24 hrs/day light on) and 2nd (4 months flight with 24 hrs/day light on) experiments, the difference in the structure of *Ceratophyllum demersum* between the flight and ground samples was obtained. On the other hand, no difference was obtained in the 3rd experiment (4 months on Mir with 16 hrs/day light on).

Discussions

Distributions of microorganisms examined after recovery showed no difference between flight and ground samples. This does not mean that distribution is identical under both space and ground environment since we could fractionated only after recovery. Rather, as easily speculated, under space environment, microorganisms may homogeneously distribute, and redistribute quickly when they are subjected to 1g. Difference in the distribution of microorganisms can be detected as a difference in turbidity. By improving the resolving power and sensitivity of the monitoring system, the dynamics of the distribution of microorganisms can be detected. It can be concluded,

therefore, that in our ABS, the distribution of microorganisms formed under space environment transforms into a similar type to that of ground within few hours after recovery. Microgravity of 4 month and light on cycle seem not to alter the sedimentation velocity of the organisms significantly .

Through the 2nd and the 3rd missions, the number of algae cells and the chlorophyll content per cells were not different between flight and ground, and in ground samples some animals such as shrimps were actively moving even after 7 month when *Ceratophyllum demersum* were completely deteriorated. Photosynthesis of algae may have supplied the oxygen to animals.

We obtained some puzzling results on the morphology of *Ceratophyllum demersum*. In the first and the 2nd missions (10 days and 4 months, respectively, with whole-day light illumination) , the morphology and distribution of chloroplasts were distorted in the flight samples. However, when light irradiation was reduced to 16 hrs/day, these differences were not observed. We have no clear explanation at present. One possibility is that synergetic effect of light on cycle time and microgravity may induce the change observed in the present experiments. It is probable that continuous light illumination gives stress to some plants. Dark duration is required for the growth of short-day plants (Larkin *et al.*, 1990). Even in some long-day plants, gene expressions are sensitive to light/dark duration (Wu *et al.*, 1996, Xu *et al.*, 1997).

If other space-specific stresses is applied to these plants, the synergetic stimuli may enlarge the changes and induce different effects and finally result in the dramatic change. Synergetic effect of radioactive particle radiation and microgravity was suggested for early development. (Neubart *et al.* 1986). Further flight experiments changing light on cycles will give the answer whether this assumption is true or not. As described in the first report of the present series (MacCallum *et al.*, 1998) , 0.5°C lowering of the average temperature and the introduction of heat-resistant strain of *Ceratophyllum demersum* may be alternative explanations.

Primary energy producers in the present system, green plant and algae, are the most important members of the closed ecological system. As described above, under the light-dark cycles, they were alive at least for 4 months under space environment, which enabled the survival of the decomposers, consumers and subsequently the whole system. This guarantees that the present system can be a model for the study on long-term effects of space environments to closed ecological systems or life support systems.

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