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GROWTH OF THE BRINE SHRIMP *ARTEMIA FRANCISCANA* KELLOGG (ANOSTRACODA) IN THE MATERIALS DISPERSION APPARATUS AS A SEALED MICROCOSM

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ABSTRACT

We report the hatching of brine shrimp nauplii and their growth to preadults, and occasionally adults, in vacuum-grease-sealed, 0.835–1.67 cc microalgal-based, xenic microcosms. Dormant brine shrimp cysts (1–5) were added to 0.75–1.50 ml *Chlorella* sp. cultures in Lexan™ plastic chambers (the microcosms) of the Materials Dispersion Apparatus (MDA); the chambers were then sealed. The assembled MDA was placed in a lighted incubator with continuous illumination at ca 26–30° C, on a shaker at 100 rpm. Occasionally the MDA was vibrated for 10 sec/day and sometimes mixed with 5–10 culture inversions at one time/day to insure particulate dispersion. Adult brine shrimp developed in as little as 9 days, with survival of preadults or adults (1 or 2/chamber maximum) for 12–56 days, at which times the experiments were terminated. Cultures with human fertilizer produced better brine shrimp growth and much longer survival compared to cultures without this fertilizer. That is, the nutrient wastes from the brine shrimp themselves were not sufficient without added human fertilizer to provide for best growth or survivorship of brine shrimp when compared to similar cultures that had this fertilizer. Some cultures (those that remained transparent and yellow to green) showed rather complete and sometimes obvious cyclic mineralization of their feces and shed chitinous exoskeletons, while other cultures (cloudy, and yellow to green) had much less particulate degradation. The concepts of bioregeneration and endogenous community culture regulation in a very small, xenic, microalgal-based metazoan closed-culture system are therefore demonstrated.

† † †

The Consort sounding rocket missions I, II and III carried the Materials Dispersion Apparatus (MDA, from Instrumentation Technology Associates), a device with 1–2 cc cylindrical Lexan™ plastic chambers for assessing materials processing in short episodes of microgravity. The microgravity intervals of seven minutes or less on the Consort missions have not been of sufficient duration to assess growth of even fast-growing eukaryotic organisms, although effects on swimming behavior of brine shrimp nauplii and metanauplii have been noted (DeBell et al., 1990, 1991). However, the small chambers of the MDA provide an opportunity to determine if a totally sealed micro-environment will support growth of communities at unit gravity providing that they can be physically accommodated and have room for growth.

In a previous study (Rosowski, 1989) it was shown that brine shrimp (Anostracoda: *Artemia franciscana* Kellogg) can rapidly grow to ca 7-mm adults in two weeks or less in xenic, microalgal-based community cultures. In that study, algae were provided with continuous illumination and the culture was left exposed to the atmosphere above its surface for gas exchange. The purpose of the present study was to determine if a similar culture system could support growth of brine shrimp when it was totally sealed, with a chamber volume similar to that of the MDA of the Consort rocket missions.

It is likely that the first opportunities for biological experiments in prolonged periods of microgravity (measured in days, weeks, or months) may be restricted to experimental chambers of small size. But regardless of chamber size, any demonstration of rapid growth of brine shrimp in a totally sealed system would provide support for the concept that endogenously regulated microcommunity controls can function properly with minimal exogenous inputs (e.g., appropriate light and temperature, and in these experiments at unit gravity, continuous culture movement to keep the particulate components dispersed). The reliability of the endogenous control concept, of obvious appeal because

Table I. Rationale for microalgal-based brine shrimp communities in extraterrestrial habitats.

1. Continuous closed-system productivity through light-driven biomass regeneration.
2. Soluble nutrient conversion into an insoluble biomass with removal through filtration by invertebrates or fish.
3. Oxygen production through photosynthesis eliminating need for electro-mechanical forms of oxygenation except as backup.
4. All community components can be useful in food chains of aquacultured species.
5. Provides a means for processing of astronauts' solid wastes as algal fertilizer.
6. Produces algal or algal-derived protein food supplements for astronauts.
7. High degree of endogenous culture regulation with a minimal of exogenous controls.

of its simplicity with respect to external controls, has been discussed and debated in the literature (Moore and MacElroy, 1982; Smernoff, 1986), but has yet to receive much support from prototype testing. Nevertheless, there remains considerable interest in closed systems because current spacecraft life support relies on nonrecycling of nutrients and wastes and these systems, although reliable, are not cost effective for long-term support of space stations or manned space missions of long duration (Schwartzkopf, 1992). Algal-based aquatic regenerative systems are intuitively more desirable than terrestrial systems since they produce a higher biomass on a square meter basis (Oswald, 1988) than terrestrial plant systems are capable of producing. Other arguments for microalgae in extraterrestial habitats in regenerative systems are summarized in Table I. This study reports preliminary evidence for successful endogenous regulation of brine shrimp growth in a small, closed, autotrophically-driven community from the dormant embryonic cyst to an adult in less than two weeks.

MATERIALS AND METHODS

Chlorella growth and maintenance

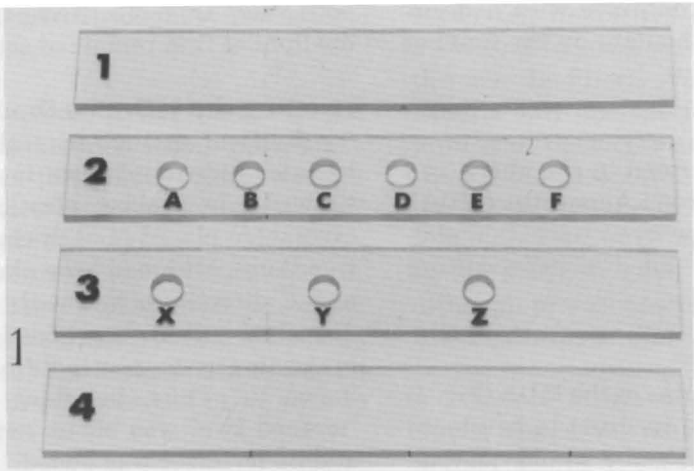
The basis of the culture system is a very small *Chlorella* sp., isolate MWB, which was maintained mostly in 1.5-l aliquots of saltwater medium as previously described (Rosowski, 1989). The culture was not unialgal (there was a filamentous blue-green algal contaminant), or was it free of other microorganisms, although *Chlorella* is clearly the dominant. For example, over time, protozoans occasionally become problem contaminants and we found it periodically necessary to reduce their numbers. Nitex™ monofilament cloth filtration to 5 μm did not work nearly as well in removing protozoans as did centrifugation at position 7 (top speed) on an International Clinical Centrifuge, Model CL, as follows. About 12 ml of the algal culture was centrifuged for 3–5 minutes; the supernatant (with protozoans) was discarded, while the pellet was saved and resuspended with fresh water (tapwater) which presumably had a lysing effect on pelleted protozoans that may have remained. This procedure was repeated 3–5 times until the resuspended pellet showed little evidence of protozoans upon microscopic examination. It was necessary to perform the freshwater rinsing procedures quickly in order to avoid lysing the algal cells. The final pellet was resuspended in the saltwater medium. This algal material then served as the inoculum for cultures of 170-ml size. Whole cultures of 170 ml were then used to start 1.5-l cultures after they were 4–5 days old.

Cultures of *Chlorella* were maintained on a mixture of Instant Ocean™ and NaCl (1:3–1:9 by weight or volume) adjusted with tap water to give a specific gravity of 1.025. Each 1.5-l culture received about 30 ml of dried activated sewage effluent (human fertilizer) to which was transferred 250 ml of the *Chlorella* suspension to initiate growth.

Source of the cysts

Cysts were obtained from the Sanders Brine Shrimp Company, Ogden, Utah, and are *Artemia franciscana* Kellogg, the warmwater, Great Salt Lake

Figures 1–4. **1**. The Materials Dispersion Apparatus (MDA). All four plates (1–4) shown disassembled and in surface view, in order of assembly from top to bottom, the individual chambers labeled (A–F and X–Z). **2**. Photograph of all four MDA plates assembled, in the 1 position. S = Spring clamps, and C = "C" clamps. **3**. A, B, and C are side-view drawings of the MDA: A is in the 1 position, B is in the 2 position, and C is in the 3 position. **A**. Two inner plates (2 and 3) of the MDA, position 1. Chambers A and B of plate 2 are centered above and between chambers X and Y of plate 3; chambers C and D are positioned similarly over chambers Y and Z; chambers E and F are positioned to the right of chamber Z. Each chamber has a volume of 0.835 cc. **B**. Two inner plates (2 and 3) of the MDA, position 2. Chambers A, C, and E are directly in line over chambers X, Y, and Z, creating a combined chamber volume of 1.67 cc. **C**. Two inner plates (2 and 3) of the MDA position 3. Chamber X of plate 3 is centered below and between chambers A and B. Y and Z are similarly centered between C and D, and E and F respectively. **4**. Photograph of MDA chamber with *Chlorella* sp. and brine shrimp. Actual size. A brine shrimp is barely visible between arrows.



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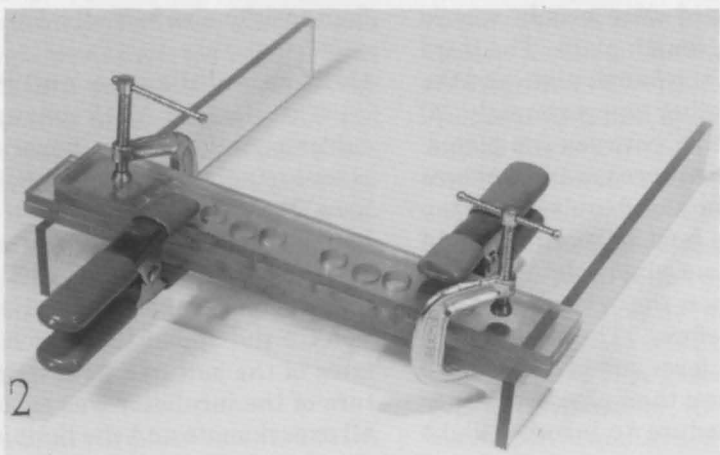

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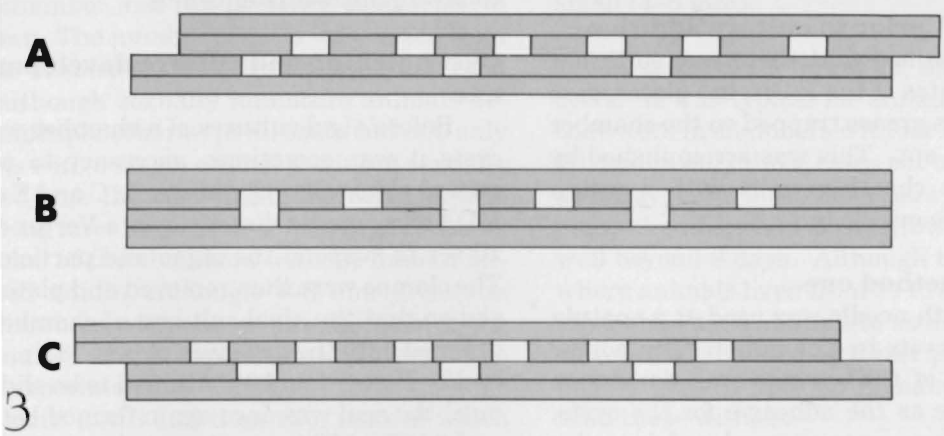

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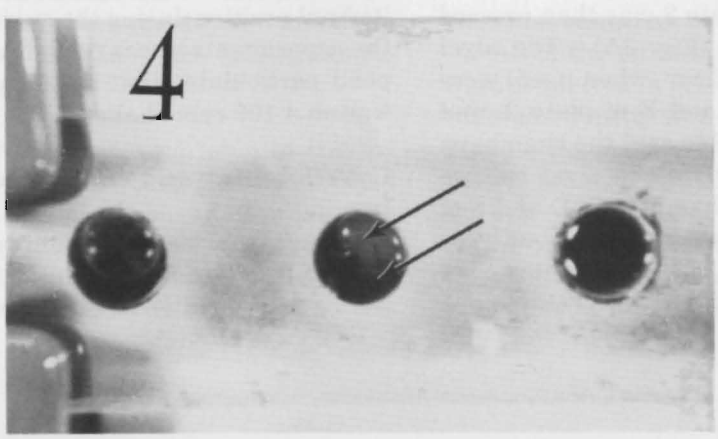

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biotype. They were stored covered until needed in a refrigerator in their original container with a desiccant. Tables II and III show the data on the number of cysts added to each chamber.

Experiments

All experiments were performed in chambers (cylinders) of the Materials Dispersion Apparatus (MDA). Each chamber became an experiment upon the addition of brine shrimp cysts to the *Chlorella* culture within the MDA.

Assembly of the MDA

The four Lexan™ plastic plates of the MDA (Fig. 1) were greased so that the liquid medium to be placed within the chambers of plates 2 and 3 would not leak between, or from above or below, these plates. Three spots of Apiezon vacuum grease were evenly spaced along the surface of the bottom fourth plate. The third plate was then placed on top of the fourth plate and the plates were then rubbed together approximately 20 times to spread the grease evenly between the plates. To insure that there was sufficient grease and uniform adhesion to prevent leaking from the chambers, the top plate of the pair (plate 3) was held to see if it would support the weight of the bottom plate (plate 4). The first plate was then placed upon the second, and the procedure was repeated as before. The two sets of plates were then assembled to form one MDA (Fig. 2). That is, plates 1+2 and 3+4 were then placed together using the same greasing procedure to insure a tight seal.

Cleaning of the MDA prior to culture addition

Once it was determined that there was sufficient grease between the plates of the MDA, the plates were slid apart so that excess grease trapped on the chamber walls could be cleaned out. This was accomplished by repeatedly wiping the chamber walls with a cotton swab until a swab came out clean.

Loading the MDA, method one

A 10-ml syringe with needle was used as a spatula to transfer individual cysts to a chamber. The hollow metal tapered needle of the syringe was dampened with tapwater to serve as the adhesive for the cysts. Usually from one to four cysts were placed in a dry chamber (x, y, z) of plate 3. Plate 2 was then pressed onto plate 3 to provide a seal (Fig. 3A). The algal culture and granular solid fertilizer (when used) were then placed in chambers A, C and E of plate 2, and plate 1 was pressed onto plate 2 to seal the chambers; there was a small air space above the algal culture when the plates were sealed. Chambers B, D and F of plate 2 were envisioned to contain a fixative but were left empty in these experiments. The four plates were then clamped on both ends with one-inch "C" clamps and flat spring clamps were then placed, staggered, on both sides (Fig. 2). Thus the cysts and the culture medium at first remained separate in this procedure.

Loading the MDA, method two

A 10-ml syringe was also used as a spatula to transfer individual cysts to a chamber; however, the tip was a dry, hollow, plastic tube. From one to three cysts were placed in each chamber in plate 3. Plate 2 was then positioned over plate 3 with chambers A, C, and E directly in line with the chambers of plate 3. The algal culture was added directly to the chambers containing cysts, nearly filling chambers of both plates 2 and 3. Thus, the final MDA culture volume of "method two" was about twice that of "method one," and no fertilizer was added. Plate 1 was then pressed onto plate 2 and flat spring clamps were placed in all four positions as in method one (Fig. 2).

Culture stabilization and growth

The clamped MDA apparatus with its microalgal cultures and cysts in separate chambers was then placed in a lighted incubator (Percival Mfg. Co., Boone, Iowa) on a shaker (Gyrotory shaker, Model G2, New Brunswick Scientific) operating at 100 rpm for 24 hr. The curved bottom of the "C" clamps in contact with the moving shaker table allowed for a "rocking motion" of the apparatus, which helped to keep particulates of the cultures in suspension. The air temperature of the incubator was ca 26–30° C in the course of all experiments and the light intensity from cool white fluorescent lamps (20-watt) at the MDA surface was ca 50–70 microeinsteins/m^2/s.

Cyst hatching and culture development, method one

Before algal cultures of a chamber were exposed to cysts it was sometimes necessary to resuspend any settled algal cells. Chambers A, C and E of the clamped MDA were placed directly over a Vortex-Genie™ for 5–10 sec to suspend the algae and particles of fertilizer. The clamps were then removed and plates 1 and 2 were slid so that the algal cultures of chambers A, C and E dropped onto the dry cysts of plate 3, in chambers X, Y and Z. Plates 1 and 2 continued to be slid and squeezed until the seal was once again formed between plates 2 and 3. The MDA was then reclamped in this position, its final position during the growth period. Throughout the experiments it was periodically necessary to resuspend particulates that settled even though the MDA was on a 100 rpm shaker.

Cyst hatching and culture development, method two

There was no prior stabilization and growth of the algal cultures in this method. That is, algal cultures were added directly to the chambers containing cysts,

the plates were clamped, and the resulting position of the chambers was maintained throughout the experiments.

Once the MDA was loaded, it was placed in the lighted incubator on the shaker at 100 rpm. Sometimes it was necessary to invert the MDA cultures several consecutive times in order to resuspend material that had settled out. That is, the shaker action alone was not able to keep sediment from collecting on the bottom of the MDA culture chambers. Furthermore, use of the Vortex-Genie™ was often necessary to free sediments that appeared to be stuck to the bottom. There was no obvious short or long-term effect on the brine shrimp from these procedures.

Proof of chamber seal

The chambers with cultures that were greased and sealed tightly with clamps were inverted regularly as previously mentioned. On a very few occasions the chambers leaked fluid but no farther than a cm, and the medium did not leak totally out of the MDA. Since the chambers mostly retained their liquid at their original volume, and did so when inverted (the bottom becoming the top and vice versa), we assume that the seals were tight and that no gas exchange could occur through the vacuum grease.

RESULTS

There were two principal variables in these MDA sealed-microcosm experiments, the number of brine shrimp cysts/chamber and the presence or absence of human fertilizer. The number of cysts was varied from 1 to 5/chamber (Tables II, III). However, it was soon evident that although sexually immature animals developed with multiple cohorts, in all cases but one only those chambers with a single animal produced sexually mature adults (from the very beginning of an experiment or occurring later as a result of deaths of cohorts). In addition, none of the chambers without human fertilizer produced adults, although 4–6 mm preadults occurred in chambers with one or two animals at the time of termination of the experiments. It should be noted that in experiment 36, Table III, three animals were present until day 8 and then two, none of which become adults in about a month. However, adult brine shrimp did occur sometime between days 30 and 37 in experiment 36, the only case in which two adults occurred in a single chamber.

The protozoan density was a variable in the experiments that was difficult to control and that had the potential to become an algal consumer problem as the MDA cultures aged. Nitex™ monofilament cloth in a pore range of 5–20 μm was used to filter the algal suspensions to reduce protozoan density prior to culture addition to the MDA. However, this procedure was often ineffective as protozoans larger than the average filter pore size nevertheless managed to pass through the filters. When protozoans bloomed, it was usually only one species. Most effective for reducing protozoan density was centrifugation, but again, although protozoans were greatly reduced by this procedure, they were never eliminated. Nevertheless, community regulation of protozoan density usually occurred in the MDA, keeping them at a manageable density although protozoans were sometimes abundant in the MDA cultures at their termination.

Several MDAs were placed on a single shaker so that it was easy to make direct comparisons of the color of the cultures as they aged in the presence of growing brine shrimp. After only a few days, differences in color of the cultures were often noted, with those containing human fertilizer becoming greener than those without this fertilizer. The decision to terminate an experiment was usually based upon the length of the brine shrimp and on the culture color in those chambers with at least one live animal. Those MDA cultures which had growing animals and/or were dark green were allowed to continue their development whereas those which had begun to turn yellow-green were usually terminated even if live animals were present. Experience suggested that once a culture had a major shift in color from a dark, rich green to an off-green or yellowish-green transparency, the animals usually failed to grow much. In chambers with two or three animals that lacked fertilizer, the animals mostly remained very small (4–5 mm).

In seven cases out of 60, no nauplii emerged from cysts. It was typical for animals to die in 3–9 days if they were in chambers without human fertilizer (Table II), and in only three cases out of 24 were animals alive at 9 days. In those chambers with human fertilizer, many chambers had active growth of their brine shrimp well beyond 9 days. Although there were experiments where animals lived from 20 to over 50 days (Table III), others had dead animals in as few as 2–4 days. Clearly, cultures with added fertilizer provided for more satisfactory growth and maintenance of the brine shrimp than those without.

General remarks about the experiments

All of the following experimental sets of the MDA were connected in groups of three and the medium and *Chlorella* density were the same in each chamber for a group. Some data for the following experiments is patchy because initially we were concerned with only the end result, that of obtaining an adult brine shrimp within the shortest time possible. After accomplishing this within 9 days, a precedent was set, and many experiments were ignored after two weeks if an adult

Table II. MDA microcosms *without* human fertilizer.

Chamber #	# Cysts/ chamber	# Cysts hatched	Brine shrimp age at death in days	Temp. range °C (mean)	*Chlorella* sp. cells/ml	Nitex™ size μm
1.	1	1	8	28–29	—	10
2.	2	2	8, 9	(28.6)		
3.	3	3	9, 9+, 9+			
4.	1	1	4	26–29.5	5.5×10^7	—
5.	2	2	4, 4	(28.9)		
6.	3	3	5, 6, 6			
7.	4	3	9+, 9+, 9+	29–32	—	20
8.	4	3	6, 6, 8	(30.5)		
9.	5	5	9, 9, 9, 9+, 9+			
10.	4	0	0	—	4.4×10^7	5
11.	4	2	3, 6			
12.	4	2	3, 4			
13.	3	0	0	20–29	4.4×10^7	5
14.	3	2	3, 6	(28.2)		
15.	3	2	3, 4			
16.	3	3	2, 2+, 2+	28–29	3.1×10^7	5
17.	3	3	2, 2+, 2+	(28.7)		
18.	2	2	2+, 2+			
19.	3	2	3, 3	10–29	4.4×10^7	5
20.	3	2	7, 7	(22.3)		
21.	3	0	0			
22.	2	2	5, 6	27–29.5	4.0×10^7	10
23.	3	2	5, 9	(28.7)		
24.	4	2	2, 5			

explanation of symbols: + = alive at termination of experiment; 0 = none; — = no data

did not develop by that time. This is why there are often lapses ranging from days to weeks where we are unsure of when an animal became an adult, or when, exactly, an animal died. Therefore, we can only report the range in which these events occurred. Also, experiments were often terminated when the animals were still alive, when more time had elapsed than was desired for the animals to become adults, or, if an animal's growth was so slow that it would unlikely become an adult. Later we became interested in the longevity of brine shrimp within the closed system. Still, because the chambers were sealed, it was possible to only assess qualitative aspects of brine shrimp growth and maturity.

Growth of the animals within the chambers was, on the average, faster towards the beginning of the experiments in the first week, with growth slowing and stopping accompanied by periods of small growth spurts. Some observations made during the time of the growth spurts include the death of another animal within the chamber, lowering their density, and the disappearance of detritus and other suspended particulates. There may have been other significant changes in the water quality in the MDA of which we are unaware that affected growth. Fast animal growth didn't seem to be particularly dependent upon a strictly grass-green algal suspension; culture color was highly variable, and the reasons for a fast versus a slower rate are unclear. Also, there was a flux of suspended materials during culture development and sedimentation did not appear to correlate with *Chlorella* density. We assume that these particulates were shed exuviae, feces, bacterial aggregates, or small broken

Table III. MDA microcosm experiments *with* human fertilizer.

Chamber#	# Cysts/ chamber	# Cysts hatched	Brine shrimp age at death in days	Age at maturity in days	Temp. range °C (mean)	*Chlorella* sp. cells/ml	Nitex™ size µm
1.	2	2	19+, 19+		28–29.5	2.8×10^7	20
2.	3	3	13, 19+, 19+		(28.7)		
3.	4	1	2				
4.	2	2	4+, 4+		28.5–29.5	2.3×10^7	20
5.	3	3	4+, 4+, 4+		(28.9)		
6.	4	4	4+, 4+, 4+, 4+				
7.	2	1	9		26–30	2.5×10^7	20
8.	3	2	3, 13+		(28.0)		
9.	4	3	11, 11, 13+				
10.	2	1	7		26–30	2.5×10^7	20
11.	3	1	13+	10	(27.9)		
12.	4	2	4, 13+	0, 9			
13.	2	2	17+, 17+		26–28	4.8×10^7	20
14.	2	2	2, 17+	0, 12–17	(27.3)		
15.	3	3	2, 2, 2				
16.	2	1	16+	9–16	26–28.5	4.7×10^7	20
17.	2	0	0		(27.2)		
18.	2	0	0				
19.	3	3	16, 16, 16+		26–28.5	4.7×10^7	20
20.	2	2	16+, 16+		(27.3)		
21.	2	0	0				
22.	2	2	4, 9		28.5–29	1.8×10^7	0
23.	2	2	11, 14+		(28.9)		
24.	2	2	4, 14+				
25.	2	1	11		28.5–29	5.0×10^7	0
26.	2	0	0		(28.9)		
27.	2	2	11+ ,11+				
28.	2	1	2		—	—	0
29.	3	3	9, 11, 11				
30.	2	2	11+, 11+				
31.	1	1	20–28		—	—	0
32.	2	2	39–56, 56+				
33.	3	3	28–39, 39–56, 56+				
34.	1	1	9		—	—	0
35.	2	2	9–13, 37+	0, 22–30			
36.	3	3	5–8, 37+, 37+	0, 30–37, 30–37			

explanation of symbols: + = alive at termination of experiment; 0 = none; — = no data

bits of the original fertilizer.

Older animals that appeared pink-red were assumed to have been oxygen stressed. Younger animals were mostly clear but sometimes had a red tinge perhaps due to red light reflected or transmitted from the algal chloroplasts (i.e., uncoupled fluorescence from electron transfer). However, we could not be sure, based on color, when hemoglobin was largely responsible for the red appearance of animals. The animals were always moving and tended to swim in a circular fashion, from top to bottom, as opposed to swimming strictly in a horizontal plane. There were a small number of experiments where the animals were much thinner than the average, for unknown reasons.

The following paragraphs present some of the details of selected experiments from Table III, those with fertilizer added. The number of the chamber is equivalent to the experiment number.

MDA chambers 4–6

In experiment 4, two cysts were added to the chamber, and both hatched within 24 hrs. The experiment was ended on day 4. Most often, brine shrimp nauplii emerged 18–24 hrs after cyst submersion in the medium. However, experiments 5 and 6 were exceptions. On day 1, two nauplii hatched in experiment 5, then on day 2 another emerged. The same held true for experiment 6, with three nauplii hatching on day 1, and another on day 2. This was the only block of experiments in which delayed hatching occurred.

MDA chambers 10–12

In experiment 10, one of two cysts hatched. The animal became very red before it matured. By day 7 it had reached 8 mm and died. The obvious differences between experiments 10 and 12 was that the algal suspension of experiment 10 began to turn yellow rapidly at day 4, whereas the suspension of experiment 12 remained green and became less viscous. Also, by day 5, suspended particulates in experiment 10 became more conspicuous, and this condition also increased much more rapidly than in experiments 11 and 12.

In experiments 11 and 12, adult brine shrimp were observed at days 10 and 9, respectively. In experiment 11, three cysts were placed in the chamber with only one cyst producing a nauplius. By day 4, the metanauplius of experiment 11 appeared very red with the algal suspension very green. At day 7, the algae began to yellow and particulates were observed. By day 10, a 9–10 mm adult male brine shrimp was conspicuous.

In experiment 12, four cysts were placed in the chamber and two hatched. After day 3, the algal suspension remained green but one metanauplius had died. In contrast to other animals in experimental block 10–12, this animal was brown rather than red. On day 5, half the medium in the chamber had leaked out and was trapped between the Lexan™ plates. By day 9, this remaining medium was still bright-green and a 9–10 mm adult male brine shrimp was present.

MDA chambers 13–15

Two cysts were placed in the chambers of experiments 13 and 14, and three cysts in the chamber of experiment 15. The color of the algal suspension was similar among all chambers throughout the experiments: bright-green up to day 11, rapidly turning yellow after that. Initially, there was considerable settling of *Chlorella* in all chambers, but settling almost completely subsided by day 11. Two nauplii hatched in experiment 13 and the animals grew to ca 6 mm length at the time of their deaths on day 12.

In experiment 14, both cysts initially hatched, but one metanauplius died on day 2. The remaining animal grew at a rate similar to those in experiment 13, and by day 12 had reached 7 mm; sometime between days 12-17 it developed into an adult female. In experiment 15, all three cysts hatched, but by day 2 all three animals had died.

MDA chambers 22–24

In these experiments, two cysts were added to each chamber. The algal suspensions remained similar in color among the chambers throughout the experiments, but appeared less viscous on day 4. The cultures turned a dark, off-green color by day 7, indicating that many algae were senescent or dead. At day 12 the medium had become yellow.

In experiment 22, both nauplii hatched; growth ceased at 3 mm length. Experiment 23 had both cysts hatch, with one 4 mm animal dying at day 11, and the other reaching only 3 mm in length by day 14. Experiment 24 also had two cysts hatch, with the animals reaching a length of 3 mm earlier than those of experiment 22. All the animals previously mentioned in experiments 22 and 23 were much thinner and more active than most animals of all other experiments. By day 6 in experiments 22–24 there was an unusually large amount of suspended particulates which continued to accumulate until day 8. However, on day 9 much of the suspended material had been degraded and the chambers remained low in particulates until termination on day 14.

MDA chambers 25–27

The results from these experiments are somewhat similar to those of experiments 22–24. Two cysts were placed in each chamber. However, the cultures re-

mained bright green until day 9, then began to yellow slightly, and on day 11 became the off-green characteristic of senescent cultures. Only one nauplius hatched from chamber 25; it reached 6 mm on day 9 and then died on day 11 in the presence of considerable suspended material. Throughout this experiment the animal was observed carrying the same piece of fertilizer; it appeared to be developing into a female before its death. No cysts hatched in experiment 26.

In experiment 27, both cysts hatched, and both animals survived the course of the experiment, reaching lengths of 5 mm and 6 mm; they appeared to be developing into females. Both specimens were the brightest red of any observed. Similar to experiments 22–24, there was a dense suspension of particulates by day 5. On day 6 however, the water looked clearer, and by day 8 there was no obvious sign of suspended material.

MDA chambers 31–33

The single cyst placed in the chamber of experiment 31 hatched. However, the animal reached only 2 mm by the time it died, sometime between days 20 and 28. This culture had begun to turn yellow by day 5 and remained so until the animal died.

Both cysts of experiment 32 hatched, with the animals showing slow steady growth. One animal died between days 39 and 56, reaching a length of 8–9 mm, and the other was 10 mm at 56 days when the experiment was terminated.

In experiment 33, three cysts were placed in the chamber and all hatched. The smallest animal was 4 mm and died between days 28 and 39; the second died between days 39 and 56 and reached a length of 8 mm; and the third, alive at termination of the experiment at 56 days, reached a length of 7 mm. At 20 days, all animals of experiment 33 were moving very slowly. At day 5, the *Chlorella* suspension began to yellow but retained this color until day 19. However, the culture of chamber 33 was not as green as the cultures in the other two experiments. Also, at day 5, much suspended material appeared, increasing in density by the time the chamber was again viewed at day 19. This suspended material never completely disappeared, however. By day 28 the culture suspension was almost completely yellow and the particles of fertilizer were covered by a green coating of *Chlorella*. On day 39, the medium was clear, and remained that way until termination of the experiment on day 56.

MDA chambers 34–36

As in the preceeding experiments 31–33, one, two, and three cysts were placed in chambers 34–36, respectively. In experiment 34, the single cyst hatched, with the animal reaching only 3–4 mm by day 8 when it died.

Two nauplii hatched in experiment 35, with the first reaching a length of 4 mm and dying between days 9 and 13. The other animal, whose growth began more slowly, picked up growth after day 16 and growth continued throughout the remainder of the experiment. This animal developed into a female sometime between days 22 and 30. It was last observed on day 37 and had reached a length of 11 mm. The two animals in this chamber were both thinner than usual.

Three nauplii hatched in chamber 36, with the first animal dying sometime between days 5 and 8 and reaching a length of only 4 mm. The other animals had virtually no growth between days 8 and 21, and then slow growth to day 37 when they reached a length of 7–8 mm. Sometime between days 30 and 37 both animals developed into males.

In the very beginning of the experiments when the Lexan™ plates were being slid, a piece of fertilizer was lost between the plates of chamber 34; however, there was still some fertilizer present in that chamber. The medium remained dark green until day 5 and then very slowly began to yellow, which it did throughout the remainder of the experiment.

On day 16, suspended material was evident in experiment 35, but that disappeared by day 21; suspended material reappeared by day 30 and then remained until termination. At day 5 there was suspended material in chamber 36, but by day 9 it was no longer evident. Suspended material then reappeared in chamber 36 on day 21 and became more evident as the experiment continued.

DISCUSSION

The use of algal or higher-plant-based systems for providing food, oxygen and processing wastes has long been contemplated in the space programs of the United States (Corey and Wheeler, 1992; Holtzapple et al., 1989a, b; MacElroy and Bredt, 1984; Radmer et al., 1987; Schwartzkopf, 1992; Smernoff et al., 1987; Tremor and MacElroy, 1988; Wharton, 1988), Japan (Nitta, 1987; Oguchi et al., 1987; Ohya et al., 1986) and in the European community (Skoog, 1987). The actual use of algae with animals in tests of bioregeneration in closed systems has been limited to experiments of very short duration, with the emphasis mostly on nutrient and gas balance rather than on growth and survival. Although past experiments have been largely benchtop-scale demonstrations, the Biosphere II project in Oracle, Arizona, is an example of a large scale earth-bound attempt at bioregeneration in

enclosed, interconnected biomes (Allen, 1991; Shank, 1991; Stover, 1990). Clearly, closed-system bioregeneration is receiving attention as a means to recycle resources that in extraterrestrial environments could be too costly to import on a continuous basis. Microalgal-based systems appear particularly attractive for the reasons suggested in Table I.

The present study determined if a microalgal-based culture system (Rosowski, 1989) could produce adult brine shrimp from dormant cysts (gastrula stage embryos) in a very small closed culture volume (mostly 0.75–1.5 cc unless leaks occurred), with a bare minimum of exogenous controls (i.e., light, temperature, culture aggitation), in the presence or absence of human fertilizer (Tables II, III). Given the economic incentive to develop systems that operate "as small as possible" and self-sustain (Rummel and Volk, 1987), the success here in growing adult brine shrimp in small volumes in a matter of weeks is of interest, for one can envision microalgae and brine shrimp as potential larval foods for establishment of aquacultured animals in permanent space habitats. The recent demonstration

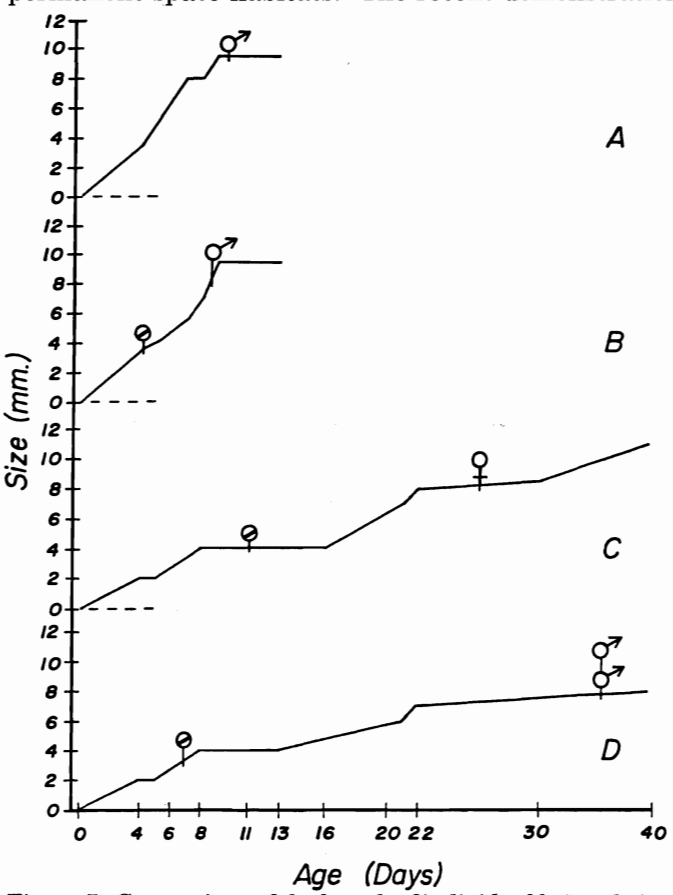

Figure 5. Comparison of the length of individual brine shrimp to their age at maturation. **A**. Experiment 11. One male at day 10, 9–10 mm. **B**. Experiment 12. One male at day 9, 9–10 mm; cohort dead at day 4. **C**. Experiment 35. One female at day 22–30, 8–9 mm (reached 11 mm); cohort died between days 9–13. **D**. Experiment 36. Two males at days 30–37, 7–8 mm each; cohort dead at 5–8 days.

of significant postembryonic development of brine shrimp to preadult stages in microgravity with dried *Spirulina* sp. as food (Spooner et al., 1992) is particularly encouraging in this regard.

In this very preliminary study with the MDA, the focus was on providing conditions for rapid development to adults of cyst-hatched brine shrimp, in a sealed environment. The chambers were considered impervious to gas exchange since vacuum grease is a barrier to air when properly applied and the plastic plates of the MDA were held tightly with "C" clamps. The greased area around each chamber was greater in surface area than the chamber itself. Therefore, those chambers that did not leak can be assumed to have had no gas exchange with the surrounding air. Those few that did leak slightly eventually sealed themselves, and liquid never got to the outside of the MDA.

Major independent variables included the number of cysts placed in the chambers, the *Chlorella* density, the particle size of the culture inoculum (occasionally Nitex™ cloth filtered to keep particles below 20 um although protozoans larger than this size were still retained), the protozoan density (periodically reduced in stock cultures largely through centrifugation), and the presence or absence of human fertilizer (when used, held constant at a weight that would not overly stimulate bacterial development). Bacterial species diversity may also be an important factor, but we have no data on its composition and how it might have varied among chambers. There was variation in the particle size of the solid fertilizer and thus the available substrate for bacterial colonization could have varied significantly leading to major differences in bacterial density among chambers. Bacterial density flux was also likely influenced by the filtering activities of the brine shrimp themselves (see discussion in Rosowski, 1989). Cloudiness at times suggested bacterial blooms which would likely have been evidense of aerobes given the green color of the microalgal suspension and the swimming activity of the brine shrimp which was normal (they would likely swim very slowly when oxygen levels approached 0 mg/l, Rosowski, unpublished observations).

One obvious factor affecting the growth rate of the brine shrimp in the MDA microcosms was their own density at the time of hatching and throughout their growth, and this varied among experiments. The growth rate leading to adults was often proportional to the number of animals in a chamber (Fig. 5). For example, the fastest growth was when there was only one animal in a chamber, and growth was clearly slower when two animals were present (Fig. 5D). When adult maturation was reached, in these cases and in all others, there was never more than two adults/chamber. A comparison of the data in experiments 11, 12

and 35, 36 (Fig. 5) is also useful in suggesting that with similar culture stability perhaps predictable development may result.

Because of the inaccessibility of the MDA contents during experiments and the decision to leave these communities largely undisturbed during their development, monitoring had to be mostly qualitative and assessment of events somewhat speculative. The parameters that could be externally monitored included: color of the culture, the fluid volume, and the size and number of brine shrimp; overall culture transparency, and, when mostly free of algae or at low algal density, the degree of cloudiness; relative viscosity of the medium as determined by relative fluid movement (more difficult to assess in the MDA than in larger-volumn microcosms); relative density of suspended detrital particulates including feces and exuviae; relative brine shrimp body length to width; and, approximate growth rate and survivorship of the brine shrimp.

The largest animals in fertilized MDAs developed when there were only 1 or 2/chamber as opposed to 3–5/chamber, and no adults developed in chambers without fertilizer addition although poorly growing, small preadults often developed. The nutrient wastes from the brine shrimp, although likely stimulatory to algal growth, did not sustain the rich-green color characteristic of cultures to which human solid wastes were also added. That is, as *Chlorella* divided in the early days of the experiments without fertilizer, essential nutrients appeared to be lacking as assessed by the culture color. Of particularly significance is that cultures without fertilizer frequently had animals that lived only a matter of days whereas cultures with fertilizer had animals that lived for weeks (cf. experiments in Tables II, III). When the animals of unfertilized cultures did live beyond a week and showed no signs of active growth, their cultures were terminated much earlier than those in which fertilizer was present and growth was obviously rapid. In addition, in unfertilized cultures, the animals frequently died quickly (and young) and there was little opportunity for them to generate nutrient wastes to stimulate algal growth. It should be mentioned that the fertilized cultures, typically very green through 1-2 weeks or more of growth, appeared to have fecal wastes and exuviae mineralized to a much greater extent than those in cultures in which fertilizer was omitted.

Reasons for poor growth of brine shrimp in unfertilized cultures are subjective since no parameters were directly monitored within the sealed culture cylinders. Nevertheless, based on our experiences with open cultures and the studies of other investigators (reviewed in Rosowski, 1989), the poor survivorship and growth in unfertilized cultures may be due to failure of the culture to maintain an appropriate bacterial density. Unfertilized cultures likely had *Chlorella* at a density of 10^7 cells/ml or more (Table II) in the first week, a density beyond that needed for growth of brine shrimp if this density were sustained and if *Chlorella* were the sole food. Therefore, one can speculate that the lack of growth of the brine shrimp in such cultures was because these cultures failed to sustain the bacterial density which would have developed in the presence of human fertilizer. Oswald (1988) has noted that because of the low ambient concentration of carbon dixoide in the atmosphere, in waste stabilization ponds the algae are "almost entirely dependent on commensal bacteria for their carbon supply." In addition to perhaps providing an essential carbon source to the algae of closed systems, bacteria may be an essential food in early development of brine shrimp in *Chlorella*-based cultures, for many *Chlorella* spp. are normally not a nutritious food for brine shrimp (Rosowski, 1989), particularly young brine shrimp. Alternatively, we speculate that a certain density of bacteria must be reached and maintained for bacterial/*Chlorella* interactions that results in particulate aggregates that are a more suitable food.

Although the present study demonstrates endogenous regulation of brine shrimp-dominated communities leading to their rapid growth in closed cultures, nevertheless, growth to preadult or adult maturity did not always occur even in fertilized cultures. That is, in fertilized cultures, there were cases of long periods in which animals remained alive but showed no increase in length and there were unexplained premature deaths as well. It is likely that when the cultures were dense-green and with very young metanauplii they were producing oxygen to supersaturation levels (Rosowski, 1989). Because the algae were the sole source of oxygen generation in the microcosms, once the cultures began turning yellow-green (senescence), the community was doomed to collapse from the eventual depletion of oxygen through community respiration.

It was encouraging that normal mineralization occurred in the fertilizer-enriched experiments, degrading both feces and brine shrimp exuviae. But, the degradation process was unpredictable and inconsistent when the results of the fertilized chambers were assessed (Table III). That is, in fertilized cultures, although degradation remained somewhat consistent for long periods of time, particle density of the cultures fluctuated and the fecal pellets and other detritus accumulated to a noticeable extent and then disappeared only to reappear. For example, in experiment 33 (Table III), the detritus was dense by day 7 and gradually decreased until day 19 at which time it remained a slightly flocculent suspension until day 56 (termination). Also in experiment 33, the culture be-

gan to clear on day 20, and was no longer green sometime between days 28 and 39; it remained non-green until day 56 (termination). Thus the brine shrimp in experiment 33 remained alive for two weeks in the absence of any significant oxygen-generating capacity within the chamber. In experiments 34–36, there were two distinct cyclings of the detritus. Initially, the medium of the MDAs was green and remained so for up to about day 4. By day 5 it was evident that detritus was building up. As this detritus decomposed, the color of the medium began to turn yellow. The medium retained a green component but there were obviously senescent cells present. By day 9, there was no detritus visible and the color of the cultures stabilized at an off-green color. Once again, by day 16, detritus was very evident and then gradually declined, as the off-green medium turned to yellow, until termination of the experiment on day 37 (Table III). In these experiments then (31–36), the color of the cultures seemed to be correlated with detrital degradation.

Intuitively, conditions which would maintain mineralization without wide fluctuations would appear to be less stressful and hense reliable with respect to brine shrimp growth, but this is speculative. Further information is required on conditions which allow for mineralization of feces, exuviae, dead algae and other community microflora and microfauna. The present results suggest that endogenous regulation of xenic-system dynamics is worthy of further study as a reliable and predictive closed-culture bioregenerative management option.

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