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**The metabolic diversity, biological activity, and stability of the
steady state condition in closed ecosystems**

Brittain, Andrew Marsh, Ph.D.

University of Hawaii, 1993

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Ann Arbor, MI 48106

**THE METABOLIC DIVERSITY, BIOLOGICAL ACTIVITY,
AND STABILITY OF THE STEADY STATE
CONDITION IN CLOSED ECOSYSTEMS**

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
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ABSTRACT

It is proposed that the closure of small but diverse collection of microorganisms on a sub-liter scale can result in a model ecosystem useful for the study of natural ecosystems and can eventually lead to the elucidation of general ecological laws. For a closed ecosystem (CES) to be a useful model system some basic questions must be answered. This thesis focuses on three questions: 1. Do Closed Ecosystems (CES) maintain biological activity and diversity on the same level as natural ecosystems? 2. How predictable and reproducible are CES? 3. What are the characteristics of the steady state condition for CES, and are CES stable?

A pair of CES which had been sealed for six years was opened and eighteen biological parameters were measured. These were compared to the results of the analysis of samples from the Kaneohe Bay site from which the CES inoculum was originally obtained. Direct measurements of organic carbon, living carbon and daily carbon production showed the CES were as biologically active or slightly more active than the Kaneohe Bay samples. Elective culture was used to enumerate microorganisms capable of different reactions of the carbon and nitrogen cycles from the two sample types. The functional diversity of the two sample types was similar.

The predictability and reproducibility of CES were investigated with 20 ml CES, established as 500 replicate ecosystems. Subsets of these were sacrificed over time and biological activity was measured. Three distinct phases were identified over 144 days: development, steady state and decline. A steady state period which persisted over many live carbon turnovers was found to be resistant to perturbation by gas exchange (i.e. removal of the ambient gas phase and replacement by N₂ or H₂) but susceptible to carbon pool perturbations (addition of glucose or bicarbonate). The variability of the replicate CES was also quantified. The effects of similar but not identical starting conditions for replicate CES were determined to judge the uniqueness of the steady state. No evidence for alternate steady states was detected unless different carbon pool concentrations were used to establish the CES.

In summary CES can retain biological activity and functional diversity over long periods of closure. Replicate CES can also be established with a low degree of variability and with predictable patterns of behavior. This behavior includes a steady state period that is stable to perturbation. These results allow CES to be used as benchtop models of ecosystems with predictable results to address significant ecological questions.

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CHAPTER 1, INTRODUCTION

Ecology has evolved into a science from its early beginnings in natural history. Descriptive accounts of plant and animal communities have been replaced by quantitative descriptions that utilize sophisticated measurement and statistical techniques. Experimentation is even used although it is not as common as quantitative description. For an outdoor experiment, the design of adequate replicates and controls becomes increasingly difficult as the size of the ecosystem increases. Much work has been done with laboratory systems but problems with pertinence and validation to "real" ecological systems remain. This study will attempt to validate a laboratory model ecosystem, referred to as a closed ecosystem (CES), as a general ecosystem model. This model system is to be applied to ecological problems in the same manner as E. coli is used as a general biological model system in the study of biology.

CES differ from previous ecological model systems by being materially-closed and energetically-open. Light and heat enter and leave the systems but matter does not. This is a significant difference from previous experiments with microcosms which were allowed to exchange carbon dioxide and oxygen with the atmosphere. A microcosm does not develop into a bioelementally stable system but

continuously changes as the exchange of elements with the atmosphere alters bioelemental ratios (Shaffer, 1991). In CES material closure forces bioelemental cycling which results in a bioelementally steady state ecosystem.

This introduction will provide a brief summary of the historical background for the study of closed ecosystems and then review the work done in this laboratory on CES.

Early work relevant to closed ecosystems. Closed ecosystems can trace their ancestry to 1851 when Robert Warrington presented his work with twelve gallon glass microcosms containing gold fish, snails and water plants to the Chemical Society of London (as reported in Beyers, 1964). Warrington claimed to have established a "wondrous balance" between the plant and animal kingdoms in terms of bioelemental cycling. This early work is instructive in that the role of respiration, photosynthesis and nutrient regeneration is clearly stated, yet the system is open to the atmosphere to permit gas exchange. After this presentation efforts were made by aquarium hobbyists to produce a balanced aquarium with plant growth producing O₂ to supply the needs of the animals. Atz (1950) rightfully objected to the concept of balanced aquaria as the addition of fish food or fertilizer and the gas exchange, permitted through the unsealed tops of the aquaria, prevented a real

balance from being attained. These were not closed ecosystems, but could be described as microcosms.

The inability to create the perfect balanced aquarium did not hinder ecologists from using microcosms to study ecological phenomena. Serious scientific study of microcosms began in the late fifties with a series of papers by H. T. Odum and his students (Odum and Hoskins, 1957; Odum et al., 1963). Microcosms were used to study phosphorus cycling (Whittaker, 1961) carbon productivity and metabolism (Beyers, 1963; Beyers, 1965, among many others), temperature effects (Beyers, 1962) and replicability of microcosms (Abbott, 1966). Microcosms are still a very useful model system used to address specific questions in limnology and oceanography. Recent studies include investigations of nutrient cycling (Roman et al., 1988), O₂ and CO₂ metabolism (Oviatt et al., 1986), turbulence on plankton assemblages (Estrada et al., 1987) and eutrophication (Oviatt et al., 1986) among others.

Work on biologically based life support systems. A major impetus for the study of closed ecosystems has been in connection with life support in space. Once the hatch has been closed a closed ecosystem has been created. The degree of closure depends on how efficiently wastes are recycled and to what extent stored supplies are required. During the late 50's and early 60's biological links for

life support systems for submarines and space ships were actively investigated. Efforts concentrated on the growth of pure cultures of algae at high densities to produce O₂ and consume CO₂ (Matthern and Koch, 1964). These efforts led to the model systems in which mice were coupled to algal reactors for various periods of time (Golueke and Oswald, 1964). In some of these systems mouse waste products were returned to the non-axenic algal culture for nutrient recycling. Although some attempts to recycle algae as mouse food were made, all long term successful closures relied on stored food (Taub, 1974).

In 1962 a conference (Space Biology: Ecological Aspects) was held at the University of Oregon specifically to promote communication between engineers and ecologists in regards to biologically based life support systems (Odum, 1963). Little communication or understanding was achieved (Taub, 1974) and the dichotomy between the engineered subsystem approach and the balanced ecosystem approach became entrenched. The repercussions of this conference still affect the study of bioregenerative life support today.

The importance of closure to the atmosphere was of obvious concern for mouse-algae recycling schemes and the fact that a spacecraft recycling food and water is a closed ecosystem was elucidated early on (Odum, 1963). The importance of complete closure to material exchange in

experimental model ecosystems was not appreciated until later (Taub, 1974; Botkin et al., 1979; Folsome and Hanson, 1986). By isolating a subset of the biosphere from material input and allowing radiant energy to flow in and out, an entirely new type of system is created, in both thermodynamic and biological terms (Folsome and Hanson, 1986; Barlow and Volk, 1990).

Although the United States government and NASA have not shown much interest in closed ecosystems as such the former Soviet Union has maintained a long term research effort (Gitel'son et al., 1989; Perchurkin, 1989). In the U.S. a privately supported experiment has spent over 100 million dollars on CES and is currently in its second year of a two year partial closure of a complex ecosystem that includes six humans (Allen, 1986).

Work done in C. E. Folsome's laboratory. C. E. Folsome, along with his graduate student Clarence Fraser, began sealing seawater and sediment samples in round-bottom flasks in February 1974. Two 2-liter round-bottom flasks were partially filled with sand, coral rubble, and seawater from Waikiki beach. These flasks were sealed with a glass stopper fitted with a Geiger-Mueller tube to count gas phase radioactivity. The systems were labeled with $^{14}\text{C-CO}_2$ and $^{14}\text{C-acetate}$ and the CPMS monitored at intervals for 6 months. Although the data are largely unanalyzed, it was

apparent that diurnal respiratory activity continued for six months. These two systems still exhibit overt signs of life (green and red pigments, slow changes in bubbles and other features) at this writing.

Beginning in 1980, E. A. Kearns under the guidance of Folsome established many 2-to-5 liter round bottom flask CES with Kaneohe Bay sediment and overlying water. These flasks were sealed with glass stoppers fitted with gas-tight septa that allowed the repeated sampling of the headspace gas for gas chromatography (G.C.). Generally 50 ul of gas was analyzed for N₂ and O₂ on a molecular sieve column and a thermoconductivity detector. Kearns used percent O₂ in the headspace as an indicator of biological activity (Kearns and Folsome, 1981; Kearns, 1983). Kearns used O₂ concentration as a metric because of its obvious biological importance and the fact that a small gas sample could be removed without greatly perturbing the systems.

Her work demonstrated the ability of CES to exceed the atmospheric O₂ concentration and called into question the stability of the earth's atmosphere at 20.95% O₂, a lack of stability that has just been confirmed (Keeling, 1992). Kearns calculated that this type of CES compared favorably with natural ecosystems in terms of photosynthetic production and determined a CES of 833 m³ could support one human (Kearns and Folsome, 1982). Next, Kearns determined

the quantum efficiency of CES to be 1.3% and attempted to calculate other thermodynamic values for these systems (Kearns, 1983).

The G.C. measurement of O_2 is not a particularly sensitive metric to apply to CES, so other metrics were investigated. D. C. Obenhuber determined the eucaryote-to-procaryote ratio (E/P ratio) by microscopic examination of the water phase over the development period of a 2-liter undefined CES (Obenhuber and Folsome, 1984). He found the E/P ratio increased from an initial value of 10^{-3} to 10^{-1} and remained at that value concurrent with attainment of steady state for the O_2 values. C.T. Takano measured particulate ATP concentrations in the liquid phase of 2-liter CES and detected levels 2-3 times higher than those of the Hawaiian pelagic ocean water (Takano et al., 1983). The ATP levels appeared to oscillate on a 50-day cycle which corresponded to observed cell counts. Her work demonstrated the applicability of ATP determination to CES and the existence of significant levels of living biomass after 9 months of closure.

Two major problems with the type of CES studied above were the size and the chemically undefined nature of the contents. A more useful model ecological system would be replicable in content and behavior and more economical in terms of space and materials.

Obenhuber developed smaller CES with a chemically defined medium using 20-ml scintillation vials as containers. This allowed hundreds of replicate systems to be made and incubated on a desk top. He also attempted to produce a biologically defined system by mixing pure cultures of various types of algae. These CES were not successful as they were either not persistent or stable by his own analysis. Obenhuber used ^{14}C -bicarbonate additions in replicate systems sacrificed over time to study carbon cycling and persistence of carbon cycling in CES (Obenhuber, 1986). He also measured diurnal pressure changes in headspace gas in isothermal CES to determine daily respiration and carbon fixation (Obenhuber and Folsome, 1988). Carbon fixation was determined to be 5 micromoles carbon day^{-1} at steady state in his most productive systems, and he calculated, through slope analysis, that the systems would be persistent for up to 6.5 years.

A series of experiments were begun by Folsome in collaboration with Joe Hanson at Jet Propulsion Laboratory in 1982. These experiments involved enclosing Halocaridina rubra (a small endemic Hawaiian shrimp) in 1- and 2- liter chemically defined systems. These CES were monitored for O_2 concentration and number of shrimps for years but no results have yet been published. Anecdotally, some of these shrimps are still surviving 10 years later attesting

both to the capability of CES to support macrofauna and the longevity of the shrimps, as the shrimps do not breed under these conditions.

Jon A. Shaffer recently finished a long series of experiments with replicate small scale CES (Shaffer, 1991). He directly compared gas-open microcosms with closed ecosystems which were identical in terms of inoculum, medium and incubation. The CES achieved a steady state within 50 days with respect to the parameters he followed. The microcosms did not achieve a steady state with regard to reduced carbon but continued to take up carbon from the atmosphere for over 150 days. The rate of carbon reduction remained greater than the carbon oxidation rate thus the open systems were never stable by the criteria of Beyers (1963).

Shaffer also studied the effect of dilution on the inoculum used to start a CES. The Kearns type CES were established with at least 100 cm³ of sediment assuring a great diversity of microorganisms. As it became important to chemically define the CES it became necessary to restrict the unknown nutrients added with the inoculum. Shaffer and I began using an inoculum derived from the thin cyanobacterial mats found in the intertidal zone as more concentrated sources of a diverse microbial inoculum. The amount of inoculum needed to establish robust and

persistent CES was determined empirically. Approximately 25 g wet weight of mat was blended in 200 ml of a medium and then further diluted to a final volume of 6 l. This would result in an inoculum with 2 micromoles reduced carbon and about 150 nanomoles of live carbon (by ATP analysis) in each 20 ml CES. Shaffer found that if this level of inoculum was diluted in a 1 to 10 dilution series the ability of the CES to achieve steady state was lost by the third dilution. The second dilution would not consistently reach steady state but would exhibit a boom and bust behavior. This results implies that the diversity of the organisms in the inoculum is important for ecosystem function.

Finally Shaffer examined the effects of varying the medium composition in terms of bioelements on the steady state of biologically similar systems. The results could not be explained by simple Redfield ratios and Liebig type limitation but definite empirical relations were determined with regard to total reduced carbon, live biomass and respiration in relationship to the levels of input C:N:P. The biological diversity in these CES remained essentially constant, while the carbon pool and metabolic rates changed in response to the different levels of inorganic bioelements. The overall level of carbon in the CES had the largest effect on how the system was organized while

phosphate had the least effect. Combined nitrogen levels had the greatest effect on respiration rates.

In conclusion, persistent and productive biological systems that are materially closed can be easily constructed and monitored for experimental study. Further, these systems go to a steady state for various parameters and cycle carbon between the oxidized and reduced condition. With the development of chemically defined, replicable, miniature CES, an experimental model system becomes available to address questions of ecology in an experimental manner (Folsome and Hanson, 1986).

Purpose of this work. CES have steadily developed as an experimental system. The understanding of the time-course behavior of CES and applicability of different methodologies allows more sophisticated experiments to be designed. Unfortunately, no other laboratories have decided to use this model system. With this dissertation I hope to advance the utility of CES as a bench-top model ecosystem for ecological investigations and to use the model system to address some significant questions in ecology.

To use CES as an experimental system, some basic questions need to be addressed. I propose to answer the following:

1. Are CES really ecosystems? This question depends as much on the questioner's definition of ecosystem as on the systems under discussion. I will address specific aspects of the question. Do CES maintain significant biodiversity after prolonged closure? Do CES maintain complex metabolic capabilities for bioelemental cycling? Do CES maintain biological activity at levels comparable to the biosphere for prolonged periods? Do CES exhibit other ecosystem-like behavior?
2. How reproducible are CES? An experimental model system must have a known degree of replicability to be useful. The quantification of variability will help define limits for CES as an experimental system.
3. What time-course behavior can be expected out of this model system and of what ecological significance is the behavior?
4. If CES are ecosystems, how resistant to perturbation are they? What does their reaction to perturbation reveal about ecosystems stability? Are there multiple steady state end points for CES? How sensitive to starting conditions is the steady state?

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CHAPTER 2

THE MAINTENANCE OF FUNCTIONAL DIVERSITY AND HIGH LEVELS OF BIOLOGICAL ACTIVITY IN MATERIALLY-CLOSED ENERGETICALLY-OPEN ECOSYSTEMS AFTER SIX YEARS OF CLOSURE

Abstract

Materially-closed energetically-open ecosystems (CES) have previously been shown to support life for years, cycle carbon rapidly, and attain a steady state with respect to biologically significant parameters. Two CES were analyzed for reduced carbon, biomass (living) carbon, and biomass carbon production after six years of closure. The microbiota of the CES were enumerated by elective culture and dilution to extinction in 13 different selective media. Sediment samples from the same site as the initial inoculum of the CES were also analyzed and found to be remarkably similar to the CES. No significant loss of functional diversity or biological activity occurred after six years of material closure. These results indicate microbial activity and functional diversity remain high after closure and provide further evidence that closed ecosystems can properly be called ecosystems.

Introduction

Materially-closed energetically-open ecosystems (CES) have been proposed to fill an important void in experimental ecology, that of reproducible, stable, model ecosystems (Folsome and Hanson, 1986). These authors emphasize the important distinction between microcosms (materially-open, energetically-open, but biologically isolated model systems) and closed ecosystems (energetically-open but materially-closed systems). Much of the previous work done with microcosms has been to address specific questions, using the microcosm as a simulation of a specific ecosystem. Proponents of the study of CES suggest closed ecosystems are models of ecosystems in general, and that the study of CES can reveal general laws of ecology.

In order to justify the use of CES as an experimental tool in ecology certain minimal criteria must be satisfied. In this study the maintenance of biological diversity was investigated as one criterion. A general consensus maintains that ecosystems isolated from the biosphere are doomed to degeneracy and failure (Taub, 1974). This has been a macroscopic viewpoint with a system being considered degenerate when the macro-organism species list changed or contracted after closure (Maguire, 1978). This concept has

been elaborated into a restatement of the Gaia hypothesis in terms of the perceived conflict between persistence of life and the material closure of the earth (Barlow and Volk, 1990). At this point in the study of closed ecosystems, it cannot be determined if this is not simply a question of scale and macroscopic prejudice. A two-liter flask populated with bacteria and photosynthetic algae does not lend itself to the generation of a species list. A CES may contain 10^9 organisms (Obenhuber and Folsome, 1984, Takano et al., 1983) with a collective turnover time of a few days (Obenhuber and Folsome, 1988).

It has previously been shown that CES develop a steady state condition for biologically significant parameters. Gas phase oxygen concentrations can exceed the Earth's atmospheric concentration and remain stable for months (Kearns and Folsome, 1981). Gross microbial community parameters, such as eucaryote-to-procaryote ratio (Obenhuber and Folsome, 1984) and dominant algal concentrations (Wright et al., 1985), achieve steady state values. Particulate ATP concentrations remain detectable for long periods of time at biologically significant levels in the liquid phase of CES (Takano et al., 1983). Diurnal community respiration approaches a steady state value and the rate of change of that value has been used to predict a minimum of six years of persistence for those particular CES (Obenhuber and Folsome, 1988). These previous studies

of CES have all indicated life persists but they have not determined whether a post closure degeneration of biological complexity occurs. The present study was undertaken to determine if the metabolic functional complexity of the initial inoculum is maintained in a CES after lengthy closure. For this largely procaryotic ecosystem, metabolic functional diversity in terms of carbon and nitrogen cycles is assumed to reflect overall complexity and biological diversity.

Materials and Methods

Carbon and nitrogen cycle reactions were combined into 13 function-based categories of organisms and selective media for each category was prepared. The number of culturable organisms for each functional category was determined by most probable number assay (MPN) for 2 CES and 2 separate (winter and summer) samples of the Kaneohe Bay (Hawaii) sediment from which the CES were originally derived. Additional direct measurements to determine total reduced carbon, biomass carbon, daily biomass carbon production, and direct bacterial count were done to compare the samples.

Sample description and preparation. Dr. B. Kearns, a previous student in this laboratory, had established 2 CES in 2-liter round-bottom flasks each containing 300 cm³ of

Kaneohe Bay sediment and 300 ml overlying water (Kearns and Folsome, 1982). The flasks were maintained at room temperature (19-23°C) with natural light from a northern exposure for 6 yr (maximum measured light level of 1000 micro-einsteins $m^{-2}sec^{-1}$). The two CES were individually sampled by cleaning the outside of the flasks with 70% isopropanol, unsealing them and decanting the overlying water into sterile beakers. The remaining sediment was removed by breaking the flask into a surface-sterilized plastic bucket, large pieces of glass were removed, and the content was homogenized with a spatula. Sediment samples from Kaneohe Bay were obtained by diving to the same location (± 10 m) from which the original inoculum for the CES was obtained. The sediment sample (from the sediment surface to approximately 100 mm sediment depth at a water depth of 3 m) was collected in an acid-washed plastic bucket, transported back to the University of Hawaii and the analysis begun within 4 h of sampling. For each sample a slurry was prepared by blending 25 g (Kaneohe Bay) or 35 g (CES) sediment samples, wet weight sediment, with 200 ml of overlying water for 2 min at high speed in a sterile blender. This sediment slurry became the undiluted sample and further 1 to 10 dilutions were made into autoclaved 80% seawater.

Direct measures of biological parameters. Triplicate tared, precombusted, glass fiber filters (GF/F, Whatman) were used to determine dry weight and reduced carbon content by combustion for each sample. Two ml of the undiluted slurry were filtered, the filters washed with 1 ml of distilled water, and then dried at 60°C under vacuum for 7 days. After determining the dry weight, the filters were combusted at 550°C for 24 h to determine reduced carbon content by weight loss.

Biomass carbon was determined by measuring particulate ATP and using the conversion factor of Karl (1980). ATP was extracted in triplicate by the method of Karl and Craven (1980). Five ml of the undiluted slurry was filtered through GF/F (Whatman) filters and the ATP extracted from the filters in boiling phosphate buffer. The extracted ATP was measured in a photometer (Biospherics) using firefly luciferase (Sigma) as per Karl and Holm-Hansen (1978).

Biomass carbon production was determined by the incorporation of tritiated adenine into DNA (Karl, 1982). An 80-ml aliquot of the undiluted slurry was incubated with 100 uCi (³H) adenine (16.6 micro-Ci mmol⁻¹) for 1.5 and 3 hr. Two sets of triplicate subsamples were filtered after incubation (5 ml, GF/F) and processed to quantify either the radioactivity of the ATP or of the DNA. The radioactive ATP was extracted with boiling phosphate buffer

and isolated by ion exchange thin layer chromatography. The separated ATP was eluted from the chromatography plate and the radioactivity quantified by scintillation counting. The filter for DNA analysis was homogenized with a hand-held homogenizer and the labeled DNA was separated from other labeled material by differential hydrolysis and precipitation as described by Karl (1982). The specific activity of ATP, the immediate precursor to the adenosine residues in DNA, was calculated from the radioactive and chemical ATP determinations. The DNA production rate was calculated assuming a .25 ratio between adenosine residues and total residues and using the radioactivity in the isolated DNA and the specific activity of the ATP. The total biomass carbon production was calculated assuming DNA makes up 2% of the biomass carbon (Karl, 1980).

Direct counts were done with acridine orange-stained bacteria retained on irgalan black-dyed polycarbonate filters (Hobbie et al., 1977).

Enumeration of culturable microorganisms. Thirteen functional groups of organisms were enumerated by MPN in selective media. The common name of the groups and the energy, carbon, and nitrogen sources used to define the groups are outlined in Table 2-1. A simple artificial seawater formulation based on ASN II (Rippka et al., 1979) was made up to a salinity of 28 g l⁻¹. This base medium

was amended with trace minerals, EDTA-chelated iron and a vitamin mixture. The medium was then made selective for the different functional categories. These media were not meant to isolate species but rather to enumerate organisms capable of the metabolic transformations required for growth in the particular medium. The additions to the basal medium, the incubation conditions and the criteria for positive growth are summarized in Table 2-2. The MPN dilution series was a 1 in 10 dilution with 3 or 5 tubes at each dilution. Most incubations were done in 24-well tissue culture plates, with 20-ml vacutainer^R tubes used for the heterotrophic nitrogen fixer medium and the proteolysis medium. End points were determined at appropriate time intervals ranging from 5 days for proteolytic heterotrophs to 4 weeks for cyanobacteria.

Plate counts of heterotrophic bacteria were done on marine agar 2216 (Difco) with 1 in 10 serial dilutions of the sediment slurry in triplicate. Bacterivorous protozoa were enumerated by elective culture in a rice grain enrichment (Cynar et al., 1985).

Results

The directly measured parameters (Table 2-3) show that, overall, the Kaneohe Bay samples and the CES samples are similar. The reduced carbon was 1%-2% of the dry weight while the biomass carbon was 1%-2.5% of the reduced

carbon. The carbon production values show that both sample types were quite active biologically. The direct counts of bacteria were high and similar in both samples. The data indicate that the CES samples were slightly more active and contained slightly more living biomass than the Kaneohe Bay samples.

The MPN enumeration of the photosynthetic microorganisms (Table 2-4) imply eukaryotic algae dominated primary production. The algae were not different among the sample types while the cyanobacteria were less numerous in the CES than in Kaneohe Bay. This difference was less than the standard error of the analysis. The phototrophic bacteria also appeared less numerous in the CES but again the difference was less than the standard error.

The MPN enumeration of chemotrophic organisms (Table 2-4) revealed functionally diverse ecosystems with only 2 of the 13 functional types sought not found. Overall, no dramatic differences were seen between the sample types. The Desulfovibrio-type organisms were found in high numbers and no difference in recovery was observed with or without the addition of combined nitrogen. The CES 1 sample had fewer Desulfovibrio-type organisms than the other three samples.

The organisms capable of oxidizing ammonium were found in slightly higher numbers in the CES samples than in the

Kaneohe Bay samples. Organism capable of partial denitrification (reduction of nitrate to nitrite) were the most abundant organisms; with higher numbers in Kaneohe Bay than in the CES samples. No organisms capable of complete denitrification (nitrate to dinitrogen gas) or chemosynthetic growth via nitrite oxidation were cultured. These negative results indicate either these types were absent or the medium was unable to support the growth of the particular species or strains present in the samples. The proteolytic heterotrophs (bacteria able to hydrolyze gelatin) were enumerated in similar magnitude in both sample types, as were the heterotrophic bacteria as enumerated by plate count. The bacterivorous protozoa are found in similar quantity in the three samples tested.

The largest difference observed between the sample types was in the recovery of heterotrophic organisms capable of nitrogen fixation. More microorganisms with this capability were recovered from the Kaneohe Bay samples than the CES samples.

Discussion

The directly measured environmental parameters of Table 2-3 are similar to previously reported values for the same parameters in Kaneohe Bay sediment except for the organic carbon content. Hanson and Gundersen (1977) measured organic carbon in Kaneohe Bay sediments between

0.2 and 0.5 mg C g⁻¹ dry wt by a different method. Although the organic carbon values reported here are higher these data do show that both the Kaneohe Bay and CES samples have similar amounts of organic carbon despite the unlimited CaCO₃ (coral sand) of the sediments.

The amount of biomass carbon in the Kaneohe Bay sediments determined in this study were lower but comparable to previously reported values for Kaneohe Bay, which ranged from 215 to 432 ug biomass C g⁻¹ dry wt. (Burns et al., 1984; Craven and Karl, 1984). The biomass carbon in the CES was higher than the biomass carbon as determined in this study for the Kaneohe Bay sediment but more in the range determined by Burns et al. for Kaneohe Bay. The value of 42 ug C g⁻¹ dry wt, is low when compared to the other biomass carbon values and in relation to the carbon production value (see below) determined for this sample. The reduced carbon values found in the CES samples were similar to those in Kaneohe Bay and indicate that the CES are not continuously building up reduced carbon. The biomass carbon values show that the CES were not existing at a much reduced living biomass.

The carbon cycle activity measurements for the Kaneohe Bay samples agree surprisingly well with those determined by Craven and Karl (1984). The average value for three Kaneohe Bay sites as determined by Craven and Karl was 331 ug C day⁻¹ g⁻¹ dry wt, with a high value of 593 ug C day⁻¹.

The Kaneohe Bay value determined in this study was comparable. The CES carbon production was higher than the Kaneohe Bay values but by less than a factor of two. It could indicate a slightly higher biological activity level in the CES. Although the assumptions and conversion factors needed to convert the results of an incorporation of tritiated adenine determination into a carbon production value are not without dispute (Fuhrman et al., 1986; Karl and Winn, 1986) these results indicate that the activity was high and comparable between Kaneohe Bay and the six year old CES.

The comparison of the directly measured parameters in Kaneohe Bay with the literature values indicates that Kaneohe Bay has not changed greatly between the time when the CES inoculum was obtained to when the samples analyzed in this study were obtained.

Dividing the biomass carbon by the carbon production gives a biomass carbon turnover time. A value of 9 hours implies these CES were quite biologically active even after 6 years closure. With the active biomass turnover and any respiratory efficiency value, the reduced carbon pool must also be turning over. The reduced carbon value divided by the daily carbon production gives an average turnover time of the reduced carbon. The value was 20 days. Although the entire pool needs not be uniformly active some portion

of the reduced carbon pool of the CES must be actively cycling. As the CES had been sealed for 6 y and the reduced carbon, biomass carbon, and carbon production are similar to those in the original inoculum, the system must be in a steady state condition with regards to these parameters.

The determination of the number of microorganisms by culture can only yield a minimum value for the actual number of organisms present. The direct count of organisms generally gives a figure 10 to 1000 times the cultural count (Jannasch and Jones, 1959; Mills and Bell, 1986). It has been suggested that cultural counts are useful for comparative purposes (Tate, 1986) and which was what was intended in this study. The acridine orange direct counts give a figure 100 to 1000 times the heterotrophic plate count and again that is not unusual (Anderson et al., 1981).

The phototrophic component in the two sample types was dominated by algae. There was a slight decrease in both the cyanobacterial and phototrophic bacterial count between Kaneohe Bay and the CES. In an earlier study at five sites in Kaneohe Bay Hanson and Gundersen (1976) enumerated various classes of bacteria by MPN assay. They reported between 5 and 50 purple nonsulfur bacteria and between 2 and 25 purple and green sulfur bacteria per gram sediment. These results are consistent with the results reported here

and indicate these types of organisms are authentic members of the microbial community. Using different media Hanson and Gunderson were unable to isolate any cyanobacteria study in contrast to our results.

The heterotrophic components cultured from the CES are not greatly different from the Kaneohe Bay samples in any of the media tested. In the earlier study by Hanson and Gundersen between 25 and 120 heterotrophic organisms capable of nitrogen fixation g^{-1} dry wt. and between 10 and 50 Desulfovibrio-type organisms capable of nitrogen fixation were enumerated by their MPN assay. The Desulfovibrio -type microorganism counts are lower than the data reported here but the medium used by Hanson and Gundersen contained lactate as a carbon source as opposed to the ethanol used in this study.

This study demonstrates that the components of the microbiota of the CES are numerous, active and diverse. After 6 years of material closure, representatives of a multi-tiered food web are present. The fact that the elective culture results are similar in the two independent CES and the source inoculum after 6 y closure, implies that the CES are in a steady state condition for the microbial populations examined. The implication is that homeostatic forces keep the CES from large random population swings

which would cause stochastic eliminations of some metabolic functions.

Although the observed differences between Kaneohe Bay samples and the CES samples are small, a pattern is discernible. Microorganism requiring anaerobic conditions were recovered in decreased number in the CES while aerobic organisms were recovered in slightly higher numbers. This trend includes a decline in the numbers of organisms capable of partial denitrification and Desulfovibrio-type organisms and an increase in the numbers of ammonia oxidizing organisms and heterotrophic protozoa. These changes could be related to the more aerobic conditions in the CES. The Kaneohe Bay sediment extends down an indeterminate distance resulting in a large reservoir of anaerobic material. During sampling care was taken to sample only the top 100 mm of sediment, however the oxygen concentration was not measured at that depth. The CES sediment was no thicker than 50 mm and light could enter the sediment through the flask sides directly into the sediment column. From the outside of the flask, black regions of sulfide deposition were not observed.

The observed decrease in anaerobic organisms includes heterotrophic nitrogen fixing bacteria. This decrease coupled with the decrease in cyanobacteria could indicate changes in the nitrogen cycle in the CES after closure. Even though, it is impossible to determine the in situ

rates of the various biochemical transformations from available data, it could be argued the importance of nitrogen fixation is diminished in the CES.

Because of this apparent change in the nitrogen cycle, CES 2 was analyzed for nitrogen fixation by testing for in situ acetylene reduction (Hardy et al., 1968). The headspace of the CES was made up to 10% with acetylene and 24 h later the headspace was sampled for ethylene and analyzed by gas chromatography. Although no extensive calibration and controls were run to produce a detection limit, no ethylene reduction was detected indicating that no nitrogen fixation was occurring in the CES. Sediment samples from Kaneohe Bay were reported to exhibit an acetylene reduction rate (over 4 h incubations) corresponding to an average nitrogen fixation rate of $3 \text{ ng N h}^{-1} \text{ g sediment}^{-1}$ (Hanson and Gundersen, 1977).

Perhaps the CES cycle nitrogen without dinitrogen involvement, i.e. the nitrogen is retained in combined forms. Such a nitrogen cycle could have developed in the CES for several reasons: 1. a lack of anaerobic niches for denitrification and nitrogen fixation, 2. a lack of complete denitrifying bacteria in the original inoculum, or 3. it could be a consequence of closure. The first reason seems unlikely as some anaerobic bacteria were present including Desulfovibrio type microorganisms and

photosynthetic bacteria. It also seems unlikely that the 300 g of inoculum used to start the CES would contain no denitrifying bacteria. Nitrogen fixation is an energetically expensive process. Odum (1969) presented evidence that, as an ecosystem approaches a climax condition, it decreases its production to biomass ratio resulting in a more energy-efficient ecosystem. Rice and Pancholy (1972) proposed that denitrification rates progressively decrease during the course of succession for terrestrial ecosystems for reasons of energy efficiency. I propose that, as a consequence of closure and the establishment of a steady state, the denitrification-nitrogen fixation loop is diminished in favor of an energetically more efficient nitrogen cycle. This proposal is in contrast to the Kaneohe Bay situation where Hanson and Gundersen (1977) concluded a large amount of heterotrophic nitrogen fixation was required to balance the large input of allochthonous carbon to the bay.

In conclusion, the functional diversity of the CES is still as high as that determined for the Kaneohe Bay sediment from which it is derived. The overall amount of biology and biological activity is similar in the two sample types. Although some slight differences are apparent, it is clear that the six year closure of the CES did not result in a degenerate ecology, at least for the parameters measured here. Furthermore the fact that the

parameters in the CES similar to the original inoculum indicates that a steady state condition exists for these parameters and strongly implies that homeostatic mechanisms are at work.

The small changes observed between the Kaneohe Bay sediment and the Closed Ecosystems derived from the sediment can be explained by the more aerobic conditions of the CES and the establishment of the steady state climax ecology of the CES.

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TABLE 2-1. Metabolic function categories as defined by carbon, nitrogen and energy sources and electron acceptor + or donor -. These categories served as a functional basis for the enumeration of microorganisms by elective culture.

Metabolic Functional Category	Carbon Source	Nitrogen Source	Energy Source	Electron Acceptor or Donor
1 Photosynthesis				
Oxygenic				
a. Algae	CO ₂	NO ₃ ⁻	Light	-H ₂ O
b. Cyanobacteria	CO ₂	N ₂	Light	-H ₂ O
Anoxygenic				
c. Purple and Green Bac.	CO ₂	N ₂	Light	-H ₂ S
d. Purple Nonsulfur	Organic	N ₂	Light	-Organic C
2 Heterotrophic				
N ₂ fixation	Glucose	N ₂	Glucose	+Organic C
3 Denitrification				
a. Nitrate Reduc.	Organic	Fixed	Organic	+NO ₃ ⁻
b. N ₂ liberation	Organic	Fixed	Organic	+NO ₃ ⁻
4 Nitrification				
a. Ammonia Oxi.	CO ₂	NH ₄ ⁺	NH ₄ ⁺	+CO ₂ , +O ₂
b. Nitrite Oxi.	CO ₂	NO ₂ ⁻	NO ₂ ⁻	+CO ₂ , +O ₂
5 Ammonification				
a. Bacterivory	<	Live Bacteria	>	+O ₂
b. Proteolysis	Protein	Fixed	Protein	+O ₂
6 Desulfurication				
a. w/combined N	Organic	NH ₄ ⁺	Organic	+SO ₄ ⁼
b. via N ₂ fixation	Organic	N ₂	Organic	+SO ₄

TABLE 2-2. Elective culture media amendments and culture conditions for selection of microorganisms capable of the metabolic functions described in Table 1. Additions to the basal medium, incubation conditions and criteria for positive growth are outlined.

Metabolic Functional Category	Media Additions	Incubation ^a Conditions	Criteria for Positive
1 Photosynthesis			
Oxygenic			
a. Algae	NO ₃ ⁻	Aerobic	Visible Growth ^(b)
b. Cyanobacteria	Nothing	Aerobic	Visible Growth
Anoxygenic			
c. Purple and Green Bac.	Na ₂ S	Anaerobic	Visible Growth
d. Purple Nonsulfur	Ascorbate Succinate	Anaerobic	Visible Growth
2 Heterotrophic N ₂ fixation	Glucose + Yeast ex.	Anaerobic	Acetylene Reduction ^(c)
3 Denitrification	Nitrate		
a. Nitrate Reduc.	Peptone	Anaerobic	NO ₂ ⁻ (d)
b. N ₂ liberation	Yeast ex.	Anaerobic	No NO ₂ ⁻ NO ₃ ⁻
4 Nitrification			
a. Ammonia Oxi.	(NH ₄) ₂ SO ₄	Aerobic	Visible Growth
b. Nitrite Oxi.	KNO ₂	Aerobic	Visible Growth
5 Ammonification			
a. Bacterivory	Rice grains	Aerobic	Visible Growth
b. Proteolysis	Gelatin	Aerobic	Liquefaction
6 Desulfurication			
a. w/combined N	EtOH, NO ₃ ⁻ , SO ₄ ⁼	Anaerobic	SO ₄ ⁼ prec. (e)
b. via N ₂ fixation	EtOH, SO ₄ ⁼	Anaerobic	SO ₄ ⁼ prec.
7 Plate Count	Marine Agar	Aerobic	Visible Growth

footnotes on next page

^a The photosynthetic media (1a-d) were incubated in indirect light while all the other media were incubated in the dark. All media were incubated at 20-22°C.

(b) A tube or microtiter plate well was judged positive if turbid or if sediment in excess of uninoculated controls was present. Rice grain enrichments were scored under an inverted microscope for the presence of flagellates or other protozoa.

(c) After incubation for 2 weeks, the headspace of the vacutainer was made up to 10% acetylene and after 24 h analyzed by gas chromatography for ethylene, any tube with ethylene above background was considered positive.

(d) Nitrite was detected by the addition of a sulphanilamide hydrochloric acid solution followed by a n-(1-naphthyl) -ethylenediamine dihydrochloride solution directly to the microtiter plate well. Nitrate was detected after being reduced to nitrite with zinc powder and the well was tested for nitrite.

(e) A microtiter well was scored positive if a black precipitate was visible after incubation.

TABLE 2-3. Directly measured parameters from two Kaneohe Bay sediment samples, (K-bay 1 and 2) and two six-year old closed ecosystems (CES 1 and 2).

Sample type Month	Reduced Carbon ^a mg C g ⁻¹	Biomass Carbon ^b ug C g ⁻¹	Carbon Production ^c ug C d ⁻¹ g ⁻¹	Direct Count Bacteria ^d no. g ⁻¹
K-Bay 1 December	n.d. ^e	42.3±10	475±67	n.d.
K-Bay 2 July	15.5	207±20	n.d.	8±4x10 ⁹
CES 1 January	n.d.	343±99	943±254	n.d.
CES 2 July	18.4	436±79	n.d.	5±0.6x10 ⁹

^a Reduced carbon was measured by pyrolysis. Average of duplicate determinations.

^b Biomass carbon was determined by assay of particulate ATP and conversion to biomass carbon. Average of triplicate determinations ± standard deviation.

^c Carbon production was determined by the incorporation of tritiated adenine and assay of radiolabeled DNA produced. Average of triplicate determinations ± standard deviation.

^d Direct counts of bacteria determined by the acridine orange staining technique.

^e Not Done

TABLE 2-4. Log of the number of culturable microorganisms per gram dry weight sediment as determined by selective culture in a most probable number assay. Functional categories are described in Table 2-1, culture conditions in Table 2-2 and sample preparation for dilution series described in the methods section.

Functional Category	Log of organisms per gram sediment			
	K-Bay 1	K-Bay 2	CES 1	CES 2
1 Photosynthesis				
Oxygenic				
a. Algae	5.2 (1.7) ^a	7.0 (2.3)	5.7 (1.9)	6.4 (2.1)
b. Cyanobacteria	1.9 (.64)	5.7 (1.9)	<1.4 (.47) ^b	3.2 (1.0)
Anoxygenic				
c. Purple and Green Bacter.	2.5 (.84)	n.d. ^c	1.4 (.47)	n.d.
d. Purple Nonsul.	2.5 (.84)	n.d.	<1.4 (.47)	n.d.
2 Heterotrophic N ₂ fixation	n.r. ^d	2.2(.74)	<1.4(.47)	0.9(.3)
3 Denitrification				
a. Nitrate Reduc.	7.2(2.4)	7.1(2.4)	5.3(1.8)	3.9(3.9)
b. N ₂ liberation	<1.6(.54)	<1.0(.33)	<1.4(.47)	<0.9(.3)
4 Nitrification				
a. Ammonia Oxi.	1.6(.54)	3.0(1.0)	3.8(1.3)	2.8(.94)
b. Nitrite Oxi.	<1.6(.54)	<1.0(.33)	<1.4(.47)	<0.9(.3)
5 Ammonification				
a. Bacterivory	n.d.	3.8((1.3)	4.9(1.6)	4.6(1.5)
b. Proteolysis	3.7(1.2)	5.3(1.8)	3.1(1.0)	4.8(1.6)
6 Desulfurification				
a. w/combined N	n.d.	4.1(1.4)	n.d.	3.2(1.1)
b. N ₂ fixation	3.3(1.1)	4.1(1.4)	1.4(.47)	3.2(1.1)
7 Plate Count	7.0	n.d.	6.5	n.d.

^a Numbers in parenthesis are standard error after Cochran, 1950.

^b No positive tubes in MPN series, number indicates the detection limit of the assay

^c Not done

^d Not reported, all MPN tubes were positive either indicating more than 10⁸ heterotrophic N-fixers g⁻¹ dry wt or improperly sterilized media

CHAPTER 3

THE STEADY STATE BEHAVIOR AND VARIABILITY OF CLOSED ECOSYSTEMS

Abstract

A subset of microcosms, closed ecosystems, have been proposed as a model experimental system for ecology. To be useful, a model system must be reproducible and exhibit behavior significant to the study of the system modeled. This study determined the variability of photosynthetic, microbial closed ecosystems (CES) with regards to total biomass, live biomass and carbon production in replicate systems over 114 days. The coefficient of variation of the CES for the measured parameters was between 1 and 50%. This is similar to previously measured variability in microcosms and natural systems. The kinetics of the development and the maintenance of a steady state in the CES are analogous to the bioenergetics of succession and climax seen in natural ecosystems. These results confirm the utility and applicability of CES in the study of ecology.

Introduction

Laboratory scale simulations of ecosystems have been used for many years in experimental ecology. Odum promoted microcosm studies (Odum and Hoskin, 1957) as more convenient and more amenable to experimental manipulations than similar studies performed in nature. In an early review Beyers (1964) detailed the advantages of microcosm studies for investigating both general theoretical questions and specific questions pertaining to specific ecosystems. Since then, few researchers have attempted to systematically justify the basic assumptions implicit in their use.

The utility of laboratory scale models of ecosystems as experimental model systems depends on how accurately these laboratory systems do model natural ecosystems and how reproducible the systems are. The question of reproducibility is basic to experimental science and straightforward to study. Yet few authors have examined replicability in microcosms (for the exceptions see Beyers, 1963; Abbott, 1966; Isensee, 1976).

Whether laboratory-based model systems are accurate models of natural ecosystems is more controversial. What is meant by natural? If natural excludes any ecosystem perturbed by mankind then the anthropogenic elevation of the carbon dioxide level of the atmosphere effectively

eliminates all habitats on the planet as natural (with the probable exception of mid-ocean ridge hydrothermal vent ecosystems).

What is meant by ecosystem and what constitutes ecosystem behavior? No universally accepted definition of ecosystem exists but most definitions involve the stable interaction of the resident biology of some biogeographically identifiable locale with itself and its environment. A major difficulty arises when attempting to define the boundaries of a natural ecosystem (Beyers, 1964; Margalef, 1968). The accuracy of a laboratory ecosystem model in exhibiting ecosystem behavior can be judged in both the specific and the general sense. Although most microcosm studies attempt to establish a microcosm that is a correlate to a specific natural ecosystem, no rigorous verification that the model system accurately mimics the parent system is generally performed or even possible. Although the same can be said of general ecosystem models, certain of their general attributes of ecosystems have been observed in laboratory-scale models (Cooke, 1967; Odum, 1969).

This work will examine the suitability of a subset of microcosms, closed ecosystems (CES), as a general model of ecosystems with regard to the development and characteristics of the steady state and reproducibility of

the systems. It is important to distinguish between the microcosms as introduced by Odum and Hoskin (1957) and reviewed by Wimpenny (1988) and CES as defined by Folsome and Hanson (1986). Microcosms are a physically isolated subset of the biota, open to gas exchange with the biosphere but with no or limited biological and non-volatile exchanges. CES are sealed to all but light and heat exchange with the outside environment, materially-closed energetically-open systems. This closure to material exchange results in a qualitatively different type of model ecosystem (Folsome and Hanson, 1986; Barlow and Volk, 1990) better suited to model general ecosystem behavior than microcosms (Morowitz, 1978). Practically, this closure also creates unambiguous boundary conditions, the ecosystem ends at the edge of the glass vessel. This simplifies quantification of materials flux, gas exchange and other experimental parameters.

Although CES can be studied on different scales (i.e. the entire biosphere of the earth or the Biosphere II project in Arizona) our group has met with considerable success in establishing CES on spatial scales of 0.01 to 0.30 m or 20 to 5000 cm³ volumes and studying them on temporal scales of several months to several years. These CES are easily and economically established from standard laboratory materials and maintained under bench-top conditions with added artificial lights. Biologically, the

CES are complex photosynthetic microbial ecosystems with clearly defined but enormous boundary conditions in time and space. For a bacterium, 300 mm and 100 days is comparable to 600 km and 2000 years (assuming 20 yr generations) for a human-scaled organism.

Of the list of attributes that have been proposed to describe an ecosystem, CES exhibit many. They have been reported to exhibit overt signs of biological activity for years (Kearns and Folsome, 1981; Obenhuber and Folsome, 1988) thus exhibiting persistence (Holling, 1973). The measurement of significant diurnal carbon cycling over time (Obenhuber, 1986) indicates continuous biological activity and a balanced carbon fixation-respiration condition. CES have been shown to utilize radiant energy as efficiently and be as productive as natural ecosystems (Kearns, 1983). Evidence of homeostatic ecosystem behavior has been reported (Takano et al, 1983, Obenhuber and Folsome, 1984; Obenhuber, 1986; Kearns and Folsome, 1982). Finally CES have been shown to maintain biological diversity in terms of microbial carbon and nitrogen cycling capabilities and organisms indicative of a multi-tiered trophic structure over 6 years of closure (chapter 1).

This report will discuss steady state behavior in ecosystems, not equilibrium or stability. All living things exist in a nonequilibrium condition (Morowitz, 1978)

utilizing a flow of energy to maintain a displacement from thermodynamic equilibrium (Nicolis and Prigogine, 1977). Equilibrium for a living organism or ecosystem is death or extinction (Maurer, 1987). Stability has been discussed at length and is generally considered the tendency of an stressed or perturbed ecosystem to return to its pre-perturbation state (Pimm, 1984; Harrison, 1979; Holling, 1973 and others). Although there is some agreement on the general definition of stability, no operational definition or metric has been accepted. Indeed the very existence of stable ecosystems has been questioned (Connell and Sousa, 1983, Whittaker, 1953). Steady state behavior in this work refers to unchanging values for ecologically significant parameters (Whittaker, 1953; Margalef, 1968) over time scales pertinent to the system (Connell and Sousa, 1983, Sutherland, 1990). Clearly defined and widely accepted terminology is needed to untangle some of the semantic controversies of ecology. The terminology should also be consistent with the other physical sciences, and specific operational definitions need to be established. Thermodynamics would require an unchanging state function over time to establish the steady state condition, but the nature of the state function for ecosystems has not yet been elucidated.

Materials and Methods

In this experiment total reduced carbon, living carbon and carbon production were measured over 140 days in replicate CES on triplicate samples, sacrificed at each time point. This experimental design eliminated pseudoreplication and allowed the calculation of coefficients of variance for the measured parameters.

Establishment of CES. An artificial sea water algal growth medium [based on ASN III, (Rippka, 1974) and described in detail in the appendix] was prepared at 25 ‰ NaCl. The medium contained EDTA-chelated iron, trace metals and a plant vitamin mix (Sigma, Gamborg's Vitamin Solution). Concentrations of major mineral nutrients were 64 mM NaHCO₃, 8 mM KNO₃ and 100 μM NaH₂PO₄. A thin (average 1 cm thick) cyanobacterial mat was collected from the intertidal beach rock shelf near Makaha, Oahu, as a complex photosynthetic microbial inoculum. A fresh 25 g (wet weight) sample was blended with 200 ml medium at high speed for three minutes and heavier pieces were allowed to settle out for five min. The top 160 ml of this inoculum was added to 6 l of medium and stirred vigorously by magnetic stirrer while 10 ml aliquots were dispensed to 20 ml Vacutainer tubes. The inoculum added to each tube contained 27 micromoles of organic carbon (analyzed by pyrolysis) accounting for less than 5% of the total carbon in the

medium. The CES were all sealed within 4 hours of filling with the red butyl rubber stoppers supplied with the Vacutainer tubes and the stoppers were taped down to prevent the generated gas pressure from lifting the stoppers. The CES were incubated at 22°C in a continuous, uniform fluorescent light field of 200 micro einsteins m⁻² sec.⁻¹. Every effort was made to assure the uniformity of the CES in terms of composition and incubation.

CES Sampling. Every few days three of the CES were nonselectively removed for analysis. The determination of gas phase oxygen concentration, particulate fraction of adenosine tri-phosphate (ATP) concentration and of the incorporation rate of tritiated adenine were performed in triplicate. Inorganic nutrient analysis for soluble reactive phosphate (SRP), nitrite, nitrate, and ammonia were done on the pooled filtrates of the samples.

Gas Analysis. From each CES 100 ul of gas was removed through the Vacutainer stopper and 50 ul of gas was injected into a Perkin-Elmer gas chromatograph. A 2 m by 2 mm i.d. molecular sieve column (Mole Sieve 5A, Porapak) at 50°C separated the N₂ and O₂, which were detected with a thermal conductivity detector. The detector response was quantified by peak height measurement of chart recorder data after day 30 of the experiment, by a Hewlett Packard integrator.

The ATP and adenine incorporation analyses were combined so that one CES sample would suffice for both determinations. Labeled adenine ([2,³H]-adenine, 15.5 Ci mmol⁻¹, New England Nuclear Corp.) was added as 0.25 ml of a 10 micro Curie ml⁻¹ solution. The adenine was injected through the stopper of the CES. The CES was vortexed for 3 sec and put back in the incubation enclosure. After 2 hours, the CES were opened and poured into a filter manifold with a glass fiber filter (2.5 cm diameter, nominal 1 um cut off, Millipore) the vacutainer tube was put on ice and 10 ml of 0.5 M (4°C) phosphoric acid was added. The filter manifold was then connected to gentle vacuum and filtered. Immediately upon completion of filtration the filter was placed in the tube containing the phosphoric acid and briefly vortexed. The filtrate from the CES was pooled and frozen for later nutrient analysis.

Cold carrier DNA and RNA (2 mg each tube⁻¹) was added to assist in macromolecule recovery and the samples were precipitated on ice for at least one hour. The tube contents were then ground with a hand-held tissue grinder inserted into the Vacutainer tube. The tubes were centrifuged at 2,000xg for 8 minutes at 4°C to separate the precipitated macromolecules from the soluble ATP. A sample of supernatant was removed, neutralized to pH 7 and diluted to 90 mM phosphate with 5 M NaOH and distilled

water. These samples were frozen for later radiolabel (Karl, 1981) and chemical ATP quantification (Karl and Holm-Hansen, 1978). The precipitated macromolecules were processed to determine the radioactivity in the RNA, DNA and protein (Karl, 1979).

Nutrient Analysis. Ammonia, soluble inorganic phosphate, nitrite and nitrate were analyzed by standard colorimetric methods (Grasshoff et al., 1988). The nitrite was reacted with sulfanilamide and N-(1-naphthyl)-ethylenediamine to form a diazo dye which was photometrically quantified. The nitrate was first reduced to nitrite with a cadmium reduction column and then measured as nitrite. Unfortunately the nitrite and nitrate analyses were largely unsuccessful due to interference by unknown compounds in the CES. The ammonia was reacted in alkaline solution with hypochlorite in the presence of phenol and nitroprusside ions to form indophenol blue. Some interference in the form of green product was seen in a few samples. The soluble reactive phosphate was complexed with molybdenum and the resulting blue product photometrically quantified. This analysis worked well although occasional samples were apparently contaminated with the phosphoric acid used to extract the filter.

Data Analysis. The peak heights (or peak area) were converted to percent O₂ in the gas phase. This percent was used to calculate the increase in O₂ in the tube. As it was sealed, the increase was pressure corrected with the assumption that no N₂ gas was either added or removed from the gas phase and that CO₂ was insignificant. Attempts were made to quantify CO₂ in the gas phase with a Silica gel column (Folsome, personal communication) and the TCD detector. Although this method would detect approximately 10% of ambient atmospheric levels of CO₂ (35 ppm) no CO₂ was detected in these systems. This limit of detection means that not more than 8 micro moles carbon as CO₂ was in the gas phase, less than 1.3% of the carbon in the system. The O₂ produced was converted to CO₂ reduced assuming a respiratory quotient of 1.

The particulate fraction ATP concentration was converted to living biomass carbon (live carbon) by multiplying by 250 (Karl, 1980). The rate of incorporation of tritiated adenine into DNA was corrected for specific activity of the precursor ATP and recalculated for a daily rate. The DNA produced per day was calculated assuming adenine was 25 mole percent of the DNA. Live carbon produced per day (carbon production) was calculated assuming DNA was 2% of the total live carbon by weight (Karl and Winn, 1984).

These analyses allowed the quantification of three carbon pools, reduced carbon, live carbon and inorganic carbon (by subtraction as the system is sealed) and one transfer rate, the rate of production of live carbon. The net photosynthesis rate could be determined by oxygen concentration changes over periods of a few days during the development phase of the CES but at steady state the changes were small or zero so this was not possible.

Results

The total reduced carbon in the system increased steadily over the experiment (Figure 3-1). Over the first 28 days a development period occurred during which the reduced carbon pool increased rapidly at an average rate of 19.2 micromoles day⁻¹ (Table 3-1). After day 28 until day 144 the rate of increase of the reduced carbon pool declined to less than 1 micromole C day⁻¹. During the period between day 28 and day 140 the change in the reduced carbon pool was small, varying from a low of 304±22 micromoles carbon (± 1 standard deviation) to a high of 444 ±31 micromoles carbon on day 140 (Figure 3-1). The percent O₂ in the gas phase during this time period varied between 50% and 57% O₂, greatly elevated from ambient levels (data not shown).

The live carbon pool increased from $1.1 \pm .11$ micromoles carbon of the inoculum on day 0 to 29 ± 5.7 micromoles carbon on day 27, (Figure 3-2). The rate of increase of the live carbon pool over this development period was 1.6 micromoles carbon day^{-1} (Table 3-1). Over the stable period the live carbon pool varied from a high of 37 ± 1.3 micromoles carbon on day 35 to a low of 9.9 ± 6.5 on day 114.

The carbon production day^{-1} was both more variable over time and between replicates although the general trends are similar to the other two parameters (Figure 3-2). For the development period an increase in the carbon production rate occurred from day 0 to day 27 but high values 63 ± 23 , 82 ± 30 and 84 ± 5 on days 13, 16 and 21 respectively, indicate a peak and then decline by day 27. Over the stable period, as defined above for reduced carbon, the carbon production values varied from a high of 66 ± 37 on day 62 to a low of 7.2 ± 5.2 on day 114.

The coefficient of variation for the determinations done above were calculated from the triplicate values for each time point. These values were then averaged over the same three periods described above (Table 3-2). The coefficient of variation of the gas samples after day 0 were generally low and ranged between .012 and .15. The average coefficient of variation for ten triplicate air standards was 0.0074. The coefficient of variation on day

0 values represent the combined variation of the inoculum and variation of the method itself. The coefficient of variation for the day 0 samples for biomass carbon and carbon production were 0.11 and 0.7, respectively.

The inorganic nutrient data for SRP and ammonia are presented in Figure 3-3. Ammonia levels were low for the first 27 days, representing less than 1% of the input nitrogen. Between day 35 and day 68, the concentration of ammonia increased by a factor of approximately ten and then declined. The SRP concentration was high initially, as a constituent of the medium, and then declined rapidly to sub-micromolar levels by day 16 and generally remained there for the duration of the experiment.

Discussion

These results demonstrate that steady state ecosystems can be produced with these experimental model systems. Between day 28 and day 89, the measured parameters changed on the average (Table 3-1) less than 0.5 % day⁻¹. The steady state condition for the reduced carbon values during this period indicates that the photosynthetic carbon production to respiration (P/R) ratio is constant, thus meeting the stable microcosm criterion of Beyers (1963). With an average live carbon content of 19 micromoles carbon and an average carbon production rate of

42 micromoles carbon day⁻¹, a live carbon turnover of 1.6 day⁻¹ can be calculated. This rate of live carbon turnover results in a steady state period of $(89-28) \times 1.6 = 97.6$ live carbon turnovers. If the live carbon turnover is interpreted as a generation time, then in terms of live biomass, reduced carbon, and carbon production, these independent ecosystems achieved steady state over a multi-generational period of time and also satisfied Connell and Sousa's (1984) overly-stringent (Sutherland, 1990) criterion for a stable ecosystem.

Most of the literature uses the terms stable or stability for an unchanging set of values for a particular community, but without an explicit test for stability the most that can be said with this data is that the system had achieved a steady state.

Previous work with CES have determined biological parameters that reach a steady state condition with kinetics similar to the parameters measured here. Kearns and Folsome (1981), using 2 to 5 liter CES with 200-1000 cm³ CaCO₃ sediment, observed increases in the oxygen level in newly sealed CES which, after several months, approached a steady state. These levels were also greatly elevated from ambient oxygen levels. This pattern could be repeated, to a certain extent, after removal of the oxygen or depletion of the oxygen by dark incubations. Takano et al., (1983) measured an increase in the particulate fraction ATP in the

aqueous phase of Kearns-type CES and detected apparent steady state oscillations of the ATP concentration. The eucaryote to procaryote ratio in the aqueous phase of Kearns-type CES was found to approach steady state over several months (Obenhuber and Folsome, 1988). Obenhuber later used CES similar to the experimental system used here and determined that the magnitude of diurnal oxygen changes for his most biologically complex systems also achieved steady state after a growth curve similar to the curve determined in this study. The development and steady state kinetics determined in this study are consistent with previous results for CES and also consistent with patterns observed in natural ecosystems.

The growth and development of the CES from a small biological inoculum in an inorganic medium is analogous to the re-establishment of a climax biota in a denuded environment. The change in the parameters of the CES during the developmental period is consistent with a successional series approaching steady state. The increase of reduced carbon from the inoculum level of 27 micromoles carbon (see methods) to 340 micromoles during steady state (figure 3.1) is analogous to the increase in biomass for terrestrial ecosystems during a successional series. In terrestrial ecology biomass is reported as the weight of all living and previously living material; sometimes the

detrital and below ground material is included. This corresponds to the reduced carbon measured in this study and not the live biomass, which is the measure of living protoplasm of the ecosystem. An equivalent measure of total living protoplasm for terrestrial ecosystems is not available, e.g., the living biomass of a tree has not been determined.

The increased in live carbon in the CES also occurs in primary successions. The change from a bare landscape to a climax community obviously involves a large increase in living biomass. The increase carbon production determined in the CES is not exactly equivalent to gross primary production but the two are closely related (Shaffer, 1991) as primary production is the ultimate source of energy for carbon production in the CES. And the increase, peak and steady state decline observed in the carbon production of the CES is also observed in the gross primary production of abandoned agricultural fields over time (Odum, 1963). The decrease of the photosynthesis to respiration ratio from a value greater than 1, to 1 as steady state is reached, is characteristic of a successional series approaching steady state (Odum, 1969). This decrease was observed in the CES. The pattern of development of the measured parameters over time for the CES strongly implies a successional development leading to a steady state climax ecosystem. Remarkably similar

patterns of development for similar parameters have been reported for a 200 year old forest succession in Japan, (Kawaguchi and Yoda, 1988). This pattern of development has been proposed as characteristic for successional ecosystems (Odum, 1969). These results demonstrate that CES model much larger natural ecosystems in that they achieve steady state in a process that is consistent with ecological succession in terms of bioenergetics.

Although most studies of succession and climax have emphasized species lists and community change, those attributes are the most frequently challenged (Gleason, 1926; Drury and Nisbet, 1973) while the bioenergetic patterns of succession proposed by Odum (1969) generated little controversy (Drury and Nisbet, 1973). The methods to study community change in this type of CES have not been developed and while many interesting questions immediately arise, they must await further research.

It is impossible to do experiments with a non-reproducible system: science requires repeatability. Practically, the expected variability of a model system needs to be determined to allow future investigations to be designed with assurance that results will be meaningful and significant. It is important for ecology as a science that multiple independent ecosystems can be established, allowed to undergo 30 generations of successional

development, and lead to quantitatively similar ecosystems (at least for those parameters measured). This argues for the existence of general principles of ecology. Although most ecological studies are implicitly based on the assumption that deterministic relationships underlie ecological phenomena 100 years of the study of natural ecosystems have not elucidated many (uncontroversial) principles.

Lewontin (1969) in his theoretical discussion of ecosystem stability described ecosystem variability as a probability cloud around a stable point and proposed a temperature analog as a variability measure. As a more practical metric, the variability of an ecosystem has been defined by Pimm (1984) as the coefficient of variation of the parameters of interest. If the magnitude of CES variability is much greater than the variability of microcosms or the variability found in natural ecosystems, it implies stochastic processes are more important in CES or CES are more unstable. In either case, it lessens the value of CES as ecosystem models.

Previous determinations of microcosm variability have produced results similar to these. Three early studies that determined production and respiration of the entire microcosm by diurnal pH change reported C.V.s between 12.7% and 14.5% for production and between 7.1% and 32.7% for respiration. These studies used large numbers of

replicates, 12 replicates for Beyers' (1963) study, 18 for Abbott's (1966) study and 6 for the McIntire et al., (1964) study.

In a more recent study Giddings and Eddlemon (1979) determined photosynthetic production by diurnal oxygen change and inorganic chemical parameters in large freshwater microcosms. The C.V.s for production determined for these 8 and 12 replicate experiments were 15% and 21%, respectively. The C.V.s for the chemical data are low for one set of parameters, between 5% and 10% for alkalinity, Na^+ , Mg^{+2} , Cl^- , and Ca^+ , but much higher for another set, between 52% and 87%, for SO_4^{-2} , NH_4^+ , NO_3^- , PO_4^{-3} and K^+). Gidding and Eddlemon also compared the chemical parameters of the pond environment from which the microcosms were derived with the microcosms themselves and found that no significant differences existed except for Na^+ and NH_4^+ and total P. They conclude that the microcosms are reproducible and adequate models of the pond for studying ecosystems properties.

Isensee (1976) used microcosms to study the uptake of radiolabeled pesticides into macrobiota. He reports a range of C.V.s of between 1% and 59% with an average of 27% for the bioaccumulation of various pesticides. He then repeated portions of the experiment several months later and produced similar C.V.s. He compared his data to field

generated data and concluded the microcosms method is as replicable as individual field studies.

A comparison with the variability of similar data from studies of natural ecosystems, although variability is not commonly presented as such, is of a similar magnitude. In his seminal paper on tropho-dynamics Lindeman (1942) reported production determinations for producers, primary and secondary consumers that contain C.V.s of 14%, 15% and 33%, respectively. In an analysis of 30 forest stands Loucks (1970) determined coefficients of variation for production values of between 10% and 30%. Baird and Ulanowicz (1989) presented biomass and production data for the Chesapeake Bay ecosystem. The coefficient of variation for biomass determinations of various types of microorganisms ranged from 25% to 56%. The standard deviations for production were reported for phytoplankton over the four seasons. The corresponding coefficients of variation ranged from 54% to 78%.

The C.V.s above are combinations of the actual variability of the parameter and the random error introduced by the methodology. The actual variability is probably smaller but it is generally impossible to separate the two sources in the literature.

The variability of CES in terms of the parameters measured is similar to both previously determined microcosm variability and compares favorably to the variability found

in nature. This convergence means that CES are adequately replicable model systems and may indicate a lower limit to the reproducibility of ecosystems in general. Perhaps, the stochastic nature of the interactions of the many discrete organisms that comprise an ecosystem places absolute limits on ecosystems replicability.

In conclusion, these photosynthetic microbial closed ecosystems exhibit classic ecosystem behavior in a reproducible manner.

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Table 3-1. Rates of change in reduced carbon, biomass and carbon production values for three time periods.

	Development	Steady State	Decline
	8 to 28 Days	28 to 89 Days	89 to 144 Days
Reduced Carbon			
Slope ^a	19.2	1.1	0.91
R ² b	.989	.467	.778
Average ^c	177	340	414
Percent ^d Change day ⁻¹	11%	0.32%	0.22%
Live Carbon			
Slope	1.6	-0.11	-0.15
R ²	.773	.109	.104
Average	19	27	16
Percent Change day ⁻¹	8.4%	0.40%	0.94%
Carbon Production			
Slope	6.5	-0.06	-0.388
R ²	.777	.022	.386
Average	50	42	19
Percent Change day ⁻¹	13%	0.14%	2.0%

^a A linear regression was performed with the average values from each time point against time (in days) for each period. Expressed as $\mu\text{mol C day}^{-1}$ for reduced carbon and live carbon and $\mu\text{mol C day}^{-1} (\text{day}^{-1})$ for carbon production

^b the r-squared value for each regression analysis

^c the average of the parameters during the time period in $\mu\text{mol C}$ for reduced and live carbon and $\mu\text{mol C day}^{-1}$ for carbon production.

^d the slope expressed as a percent change day⁻¹

Table 3-2. Coefficient of variation values for the measured parameters.

Parameters	Average Coefficient of Variation ^a		
	Development 8 to 28 Days	Steady State 28 to 89 Days	Decline 89 to 144 Days
Reduced Carbon	1.8% (0.68-3.8) ^b	2.3% (0.87-4.88)	3.0% (0.48-5.9)
Live Carbon	24% (14-57)	17% (3.5-35)	39% (25-66)
Carbon Production	45% (26-71)	32% (6.6-66)	47% (9.0-72)

^a Coefficients of variation were calculated for triplicate determinations at each time point and these were averaged over each of the three time periods

^b The range of C.V.s for each time period is presented in parentheses

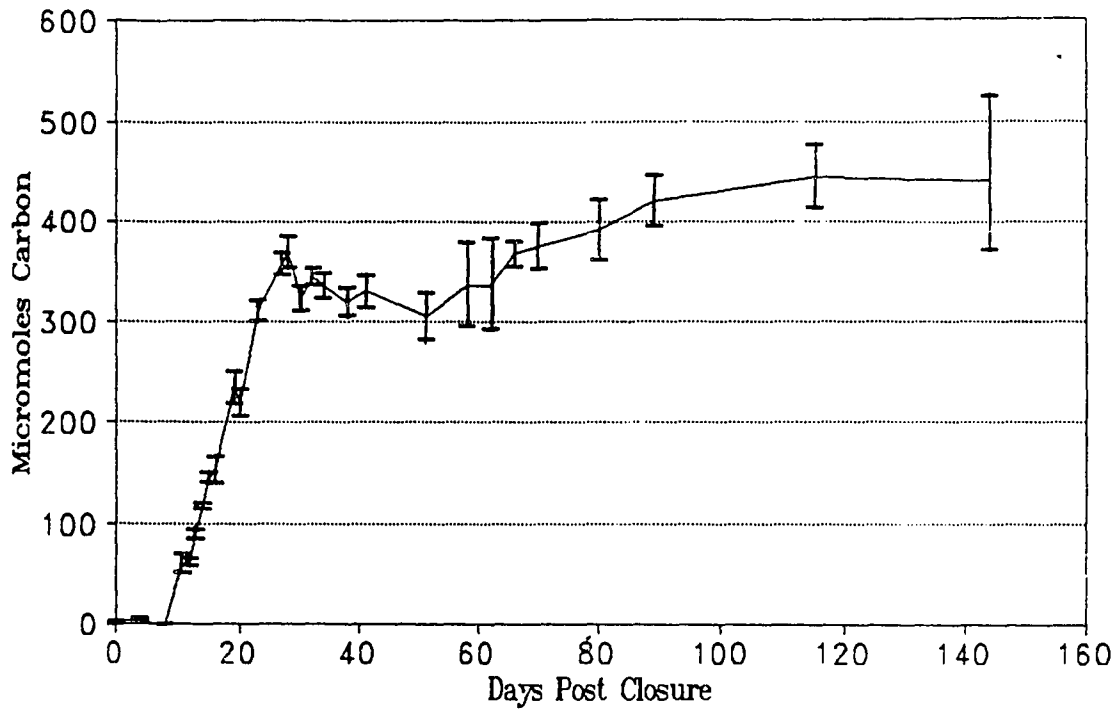


Figure 3-1. Increase in reduced carbon in the CES over 144 days. The gas phase of the CES was analyzed for oxygen concentration and the increase in the reduced carbon content was calculated assuming a respiratory quotient of one. Error bars represent one standard deviation.

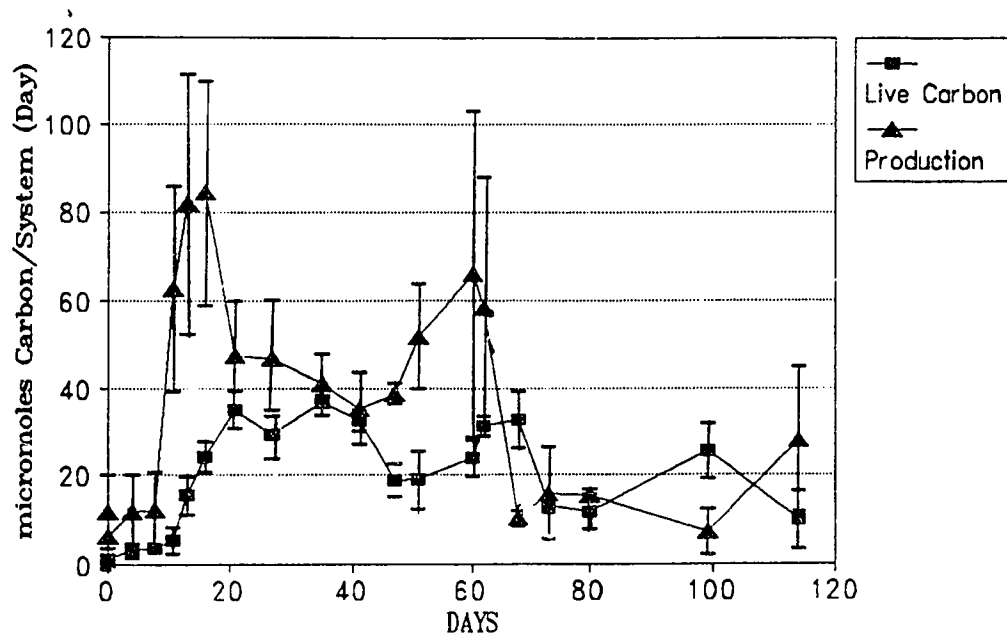


Figure 3-2. Live carbon and daily carbon production over the 144 days of the experiment. Live carbon was determined by measurement of ATP and carbon production was determined by incorporation of tritiated adenine. Error bars are one standard deviation.

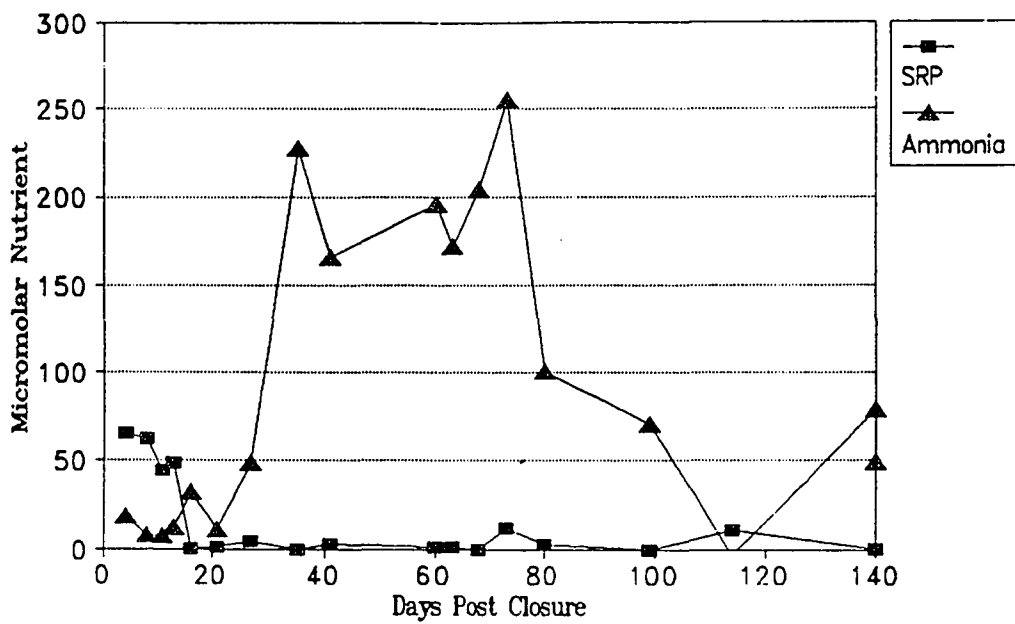


Figure 3-3. Concentration of ammonia and soluble reactive phosphate (SRP) in CES over 144 days. Analyse are of pooled filtrates for each time point.

CHAPTER 4

THE STABILITY OF CLOSED ECOSYSTEMS AND EXPERIMENTAL EVIDENCE AGAINST ALTERNATE STEADY STATES IN THEM

Introduction

Ecosystem stability has been an explicit attribute of climax community ecosystems since the development of the climax concept (Clements, 1936). Succession and climax as developed by early plant ecologists can be described as a continuous change in the species list (succession) leading to a terminal species list that is resistant to modest perturbations and invasions (climax). Major perturbations, glacier advance, forest fire or climate change can restart the process. This process was seen as an unstable series of communities developing into a stable community. An operational definition of stability was not offered.

There is some confusion in the ecological literature with the terms steady state, stable state and equilibrium. These terms are not synonymous. A steady state system as defined for thermodynamics would be a system with an unchanging state function (Morowitz, 1978). The state function for an ecosystem has not been elucidated. But operationally, for ecology, unchanging parameters of ecological and biological significance can identify an

ecosystem as a steady state system (Margalef, 1968). The stable state has an accepted definition in general; a system at steady state is stable if after a perturbation, the system has a tendency to return to the previous steady state (Pippard, 1985). A steady state system can not be assumed to be stable; these are two distinct conditions. Equilibrium has specific and consistent meaning in chemistry and physics, and no meaning in ecology. An ecosystem cannot exist at equilibrium (Shrodinger, 1944, Maurer, 1987). Ecosystems belong to a separate class of nonequilibrium systems, dissipative structures (Nicolis, 1977), that are displaced and maintained far from equilibrium by an energy flow (Morowitz, 1978).

Stability is quantifiable for equilibrium (Pippard, 1985) and near equilibrium systems (Prigogine and Lefever, 1975; Katchalsky and Curran, 1965) in physics, chemistry and mechanics. Most applications in physics or chemistry describe the system with a series of differential equations and then analyze the stability of the solution. Commonly, a Lypunov function is used to determine stability. Unfortunately ecosystems neither operate near equilibrium nor have they been completely described by system of differential equations.

In the studies of the stability of mathematical models of ecosystems, a hypothetical species assemblage with a specified degree of complexity is allowed to interact over

time (May, 1975). A stable system is usually defined as a model assemblage in which the assemblage becomes invariant with respect to time with at least one surviving species. These works generally confuse the distinction between a stable state and a steady state made above by referring to the steady state results of their models as an equilibrium condition. Recently, nonequilibrium concepts have appeared (DeAngelis and Waterhouse, 1987).

The concept of stability, while much used in ecology, is still not universally defined. A major dichotomy of meaning revolves around whether stable means unchanging values for a particular parameter e.g. the temperatures was stable at 23°C, or whether stable requires a resistance to change for the parameter. As discussed above steady state refers to unchanging parameters and the stable state refers to resistance to perturbation. This distinction is consistent with the reviews of Holling (1973), Harrison (1979), and Pimm (1984). These authors offer several other characteristics to refine the definition of the stable state. An ecosystem's resistance is related to the magnitude of the change in the system parameters in response to a perturbation. Resistance is a measure of how difficult it is to displace the system from a stable state. A system could exhibit zero resistance while being at steady state. A quantitative definition would involve the

specific system parameters under discussion and a quantification of the perturbation (Harrison, 1979). Resilience is a measure of how fast the system parameters return to their original pre-perturbation values (Holling 1973). Resistance and resilience, while they may correlate, are not the same thing. This definition of stability generates some important questions: is there more than one stable state? If the ecosystem is perturbed away from the stable set of parameter values does it have an alternative steady state (or states) in which it is stable? Will it remain in the alternative steady state after the perturbation stops? In the terms of climax theory, can a primary succession lead to alternative climax communities, all else being identical?

These questions have been addressed by traditional ecologists. Connell and Sousa (1983) concluded that there is no evidence for ecosystems' having multiple stable points; furthermore they found there is no evidence for any stable ecosystems at all. They argued that to be considered stable, the ecosystem should be observed for a time equal to at least one generation of its longest lived species. None of the studies they reviewed had done this; obviously for terrestrial arboreal ecosystems this is a daunting task. Others disagreed with specific aspects of their definition, such as what would constitute an identical situation (Peterson, 1984) or what constitutes a

pertinent time scale of study (Sutherland 1990). This dispute is essentially over the boundaries of ecosystems. What are the boundaries (in time and space) for an ecosystem? Where does one draw the line between different ecosystems situated on an elevation or climatic gradient? One ecosystem gradually changes into another sometimes over many miles (Drury and Nisbet, 1973).

In summary, currently no operational definition exists for a stable ecosystem. An operational definition does exist, however, for a steady state ecosystem. A quantitative operational definition for ecosystem stability should measure the effects of a perturbation on the steady state. While it seems straightforward to measure parameter changes of the steady state, it is difficult to devise a universal metric for perturbations to ecosystems. What measure could relate a temperature change and an oxidation manipulation? How would an addition of 0.01% glucose compare to a 0.01% dioxin addition? The perturbations could be measured in terms of how they affect the steady state, this then becomes a relative measure but one that allow comparisons to be made.

The above implies an obvious experimental approach. An ecosystem can be perturbed in a controlled manner and the results recorded. When the requirements for controls and replicates are included, it becomes clear that

laboratory scale ecosystem is desirable. Microcosms have been used as laboratory scale simulations of ecosystems. Odum and Hoskin (1957) have promoted microcosm studies on the basis of convenience. Beyers (1963) detailed the advantages of microcosm studies for investigating both general theoretical questions and specific questions pertaining to specific ecosystems. General attributes of ecosystems have been observed in laboratory scale models (Cooke, 1967; Odum, 1969).

This work will use materially-closed energetically-open ecosystems (CES) as model ecosystems (Folsome and Hanson, 1986). Microcosms are a physically isolated subset of the biota, open to gas exchange with the biosphere but with no or limited biological and non-gaseous exchange. CES are sealed to all but light and heat exchange with the outside environment. This closure to material exchange results in a qualitatively different type of model ecosystem (Folsome and Hanson, 1986; Barlow and Volk, 1990).

CES are economically established from standard laboratory materials and maintainable under benchtop conditions with added artificial lights. Biologically, the CES are derived from complex photosynthetic microbial communities. For the study of ecosystem stability the scale must be sufficient to encompass an ecosystem in space and time (Connell and Sousa, 1983). These photosynthetic

microbial ecosystems are dimensionally huge for a bacterium.

Over the last ten years, CES have been reported to exhibit many attributes of ecosystems. CES are clearly persistent as defined by Holling (1973) and display signs of biological activity for years (Folsome and Hanson, 1986, Obenhuber and Folsome, 1988). The measurement of significant carbon cycling over time (Obenhuber, 1986) implies a high level of biological activity. CES are as efficient and productive as natural ecosystems (Kearns, 1983). Much evidence for homeostatic behavior in CES has been reported (Takano et al., 1983; Obenhuber and Folsome, 1984; Obenhuber, 1986; Kearns and Folsome, 1982). Chapter 1 demonstrated that CES can maintain biological diversity in terms of microbial carbon and nitrogen cycling capabilities and organisms indicative of a multi-tiered trophic structure over 6 years of closure. Chapter 3 described the steady state of replicate CES in terms of carbon pools and carbon production.

It is clear CES achieve a steady state; this chapter will investigate the uniqueness and stability of that steady state.

Materials and Methods

In these experiments total reduced carbon, living carbon and daily carbon production were measured over 140 days in replicate CES on triplicate samples, sacrificed at each time point. For experiment 1 over 400 replicate CES were set up and allowed to establish a steady state condition. On day 59, six sets of the CES were perturbed to examine the stability of the systems at steady state. Three treatment sets had the gas phase removed and replaced with either hydrogen, methane or nitrogen. One treatment set had the light level reduced by half and the final two sets had carbon added as either bicarbonate or glucose. The CES were followed for an additional 80 days to determine the effects of the perturbations on the CES. Experiment 2 was similar to Exp. 1 except the CES were established with the above treatments at day 0 as opposed to day 59. The methods were described previously (Chapter 3).

Establishment of CES. A chemically defined artificial sea water algal growth medium containing EDTA-chelated iron, trace metals and an algal vitamin mix, was used to support the CES. Concentrations of major mineral nutrients were 64 mM NaHCO_3 , 8 mM KNO_3 and 100 μM NaH_2PO_4 . A fresh cyanobacterial mat, 25 grams wet weight, was blended with 200 mls medium at high speed for three minutes. The top 160

mls of this inoculum was added to 6 l of medium and stirred vigorously by a magnetic stirrer while 10 ml aliquots were dispensed to 20 ml Vacutainer tubes. The CES were sealed and incubated at 22°C in a continuous, uniform fluorescent lightfield of 200 micro-einsteins m⁻² sec.⁻¹. Every effort was made to assure the uniformity of the CES in terms of composition and incubation.

In experiment 1, the CES were sampled in triplicate every few days. In experiment 2 detailed examinations were performed at only four sampling periods. Most determinations were done in triplicate; only the inorganic nutrient analysis was done on pooled samples.

Gas Analysis. The headspace gas was analyzed by gas chromatography for oxygen, nitrogen, methane, and in some cases carbon dioxide. The oxygen, nitrogen and methane analyses were performed on molecular-sieve packed column with a thermal conductivity detector (TCD). The carbon dioxide analysis used a silica-gel packed column. The peak heights (or later, peak area) were converted to percent O₂ in the gas phase. This percent was used to calculate the increase in gases in the CES after it was sealed. The increase was pressure corrected with the assumption that no N₂ gas was either added or removed from the gas phase. No CO₂ was detected in these systems (chapter 2) with the exception of the glucose addition treatment which did

produce large amounts of gas phase CO₂. The O₂ produced was converted to CO₂ reduced assuming a respiratory quotient of 1.

The methane was readily detected with the TCD detector but, because hydrogen was the carrier gas for the chromatograph, hydrogen gas from the samples was not detected by the TCD. As the concentration of hydrogen was high in the samples, it was possible to quantify it by the deficit of the volume of gases injected, for example a 50 ul gas sample would upon analysis contain only 25 ul of N₂ and O₂. The deficit in the injection volume was assumed to be H₂ for the hydrogen treatments.

Live Carbon and Carbon Production. The ATP and adenine incorporation analyses were combined so that one CES sample would suffice for both determinations. The details of the procedure have been previously presented (Chapter 3). Briefly, tritiated adenine was injected through the stopper and the CES vortexed and incubated for two hours. The CES was filtered and the filter extracted in 4° C 0.5 M phosphoric acid. The filtrate from the triplicate samples was pooled and frozen for later nutrient analysis.

Cold carrier DNA and RNA was added to assist in macromolecule recovery and the samples were precipitated on ice for at least one hour. The tube contents were then ground and centrifuged at 2,000xg for 8 minutes at 4°C to

separate the precipitated macromolecules from the soluble ATP. A sample of supernatant was removed, neutralized to pH 7 and diluted to 90 mM phosphate concentration with 5M NaOH and distilled water. These samples were frozen for later radiolabel and chemical ATP quantification. The precipitated macromolecules were processed to determine the radioactivity in the RNA, DNA, and protein.

Nutrient Analysis. Ammonia, soluble inorganic phosphate (SRP), nitrite, and nitrate were analyzed by standard colorimetric methods (Grasshoff et al., 1988). The nitrite and nitrate analysis were unsuccessful due to interference by unknown compounds in the CES.

Perturbations. The gas phase perturbation treatments were performed with a gas manifold connected through luer-lok 22 gauge syringe needles pushed through the septa of the CES. The CES were first put under vacuum (15 millibars) and mechanically tapped until the liquid phase degassed. The systems were then flushed twice with the replacement gas and then pressurized to 760 millibars and removed from the manifold. For the light reduction treatment, a test-tube rack containing forty CES was simply wrapped in two layers of cheese-cloth and placed back in the incubation area. This procedure resulted in a measured 50% reduction in photosynthetically active radiation. The carbon

perturbation treatments received 640 millimoles of either NaHCO_3 or glucose in 0.25 ml of an aqueous sterile solution injected through the septa of the CES.

Data Analysis. The particulate ATP concentration was converted to biomass carbon by multiplying by 250 (Karl, 1980). The rate of incorporation of tritiated adenine into DNA was corrected for the specific activity of the precursor ATP and a daily rate of adenine incorporation into DNA was calculated. The DNA produced per day was calculated assuming adenine was 25 mole percent of the DNA. Live carbon produced per day (carbon production) was calculated assuming DNA was 2% of the total live carbon by weight (Karl and Winn, 1984).

These analyses allowed the quantification of three carbon pools, reduced carbon, live carbon and inorganic carbon (this last by subtraction as the system is sealed). The daily carbon production rate allowed the turnover time of the live carbon pool to be calculated. Additionally consumption of methane and hydrogen was determined as well as CO_2 production in the glucose treatment.

Results

The development and characteristics of the steady state were previously examined in detail. This report will concentrate on the effects of the perturbations and evidence for alternative steady states.

Experiment 1. By day 59 of Exp. 1 the CES had been in a steady state condition for about 30 days with unchanging carbon pools, production rates and nutrient levels (Chapter 3). It was at this point that the perturbation treatments were begun. The effects of the perturbations can be grouped in two categories, little to no effect and major changes in the parameters examined. The gas replacement treatments, methane, hydrogen, and nitrogen and the light reduction treatment had very little effect on the CES in comparison to the control set. The bicarbonate and glucose treatments, in contrast, resulted in a large perturbation on the parameters measured.

The live biomass averaged 20 micromoles carbon system⁻¹ (micromol C sys⁻¹) for the post perturbation period (day 60 to day 100) in the control CES while it averaged between 20 and 27 in the four least affected treatments (Figure 4-1). The bicarbonate and glucose treatments both showed a dramatic increase in average value of the live biomass, 51 and 60 micromol C sys⁻¹, respectively, for the same time period (Figure 4-2). The

glucose addition resulted in peak and decline in live biomass while the carbonate addition resulted in a more consistent increase.

The response of the carbon production to perturbation followed a pattern similar to that of the live biomass. The gas and light perturbations had little effect on the carbon production. The control averaged 25 micromol C day⁻¹ sys⁻¹ between day 59 and 144 while the gas and light treatments ranged from 25 to 35 micromol C day⁻¹ sys⁻¹ (Figure 4-3). The bicarbonate addition averaged 66 umol C day⁻¹ sys⁻¹ while the glucose addition averaged 131 micromol C day⁻¹ sys⁻¹ for the same time period (Figure 4-4).

The reduced carbon in the control, nitrogen, and light treatments remained between 450 and 550 micromoles C sys⁻¹ while the CES that received the bicarbonate addition showed an increase in reduced carbon by about 100 micromoles C sys⁻¹ (Figure 4-5) above the curve for the other systems.

The glucose addition was essentially an immediate large increase in the reduced carbon pool that is not reflected by an increase in the oxygen concentration. A large portion of this reduced carbon was oxidized over the 80 days of the post-perturbation period with the concurrent consumption of all the oxygen in the gas phase and the production of up to 45% CO₂ in the gas phase. Some of the CES began to show deposits of black precipitate,

presumably sulfide deposits, indicating anaerobic respiration. The methodology used here cannot quantify the reduced carbon pool at this point.

The gas phase of the methane and hydrogen treatments are more difficult to analyze and the data are not presented as a figure or table. The measurement of hydrogen is indirect and dependent on the volume of gases injected and not the ratio of gases detected. The determination of the concentration of methane relied on two separate analyzes and so is also volume dependent. The coefficient of variation of the volume of replicate dry air standards is 2.3% indicating the method reliable to that degree.

On day 62, 455 micromoles of methane were in the gas phase, constituting 85% of the gas phase. By day 144 only 124 micromoles were in the gas phase while the oxygen in the gas phase had increased by about 50 umoles. The total volume of the gas phase decreased by the equivalent of 280 micromoles of gas. No CO₂ was detected in these CES and the missing gas, by the criteria above, is hydrogen. Regardless of the identity of the gas, the loss of methane and production of oxygen indicates the reduced carbon increased after perturbation.

The hydrogen concentration on day 62 was 87 % of the gas phase and by day 144 hydrogen accounted for 79% of the gas. Over the same time period oxygen increased from 4% to

13%. The total amount of gas increased slightly and no real loss of hydrogen apparently occurred. The 50 micromoles increase in oxygen, if no changes in the average oxidation state of the reduced carbon occurred, would result in a 50 micromole increase in the reduced carbon pool.

The analysis of the inorganic nutrients resulted in useful data for only ammonia and soluble reactive phosphate (SRP). The phosphate, as an ingredient of the media, began at a high level but dropped to very low levels by the time steady state was achieved. Occasional high phosphate levels were attributed to contamination of the filter manifold used to obtain the nutrient samples, as 0.5 M phosphoric acid was used to extract the filters at the same time as the nutrient filtrate was collected. It takes only 20 nl of 0.5 M phosphate solution to result in 90 micromolar phosphate in a ten ml nutrient sample. Discounting the probably contaminated samples the concentration of SRP remained at nearly sub-micromolar levels for the remainder of the experiment in the control set (Figure 4-6). No changes in the phosphate concentrations were observed in the light, nitrogen and hydrogen treatments but the methane, bicarbonate and glucose show a slight increase in phosphate concentrations on day 60 and day 63 but with the above caveats in mind it is difficult judge the validity of these results.

The concentration of ammonia in the control set increased to between 100 and 255 micromolar between day 35 and day 70 and then declined to sub 100 micromolar levels for the remainder of the experiment (Figure 4-7). The treatments all show decreased ammonia levels one day after perturbations but the least effected group of treatments rebounded by day 63 and followed the pattern observed in the control set. The bicarbonate and glucose addition treatments had reduced levels of ammonia for the rest of the experiment.

Experiment 2. A development period has previously been defined for CES (Chapter 3). This period characteristically involves a rapid increase in reduced carbon followed by a steady state period during which the CES maintain an unchanging concentration of reduced carbon. The amount of reduced carbon in all treatments, except methane followed this pattern. They also exhibited a remarkable convergence to between 200 and 250 micromoles C sys^{-1} as the CES approach steady state (Figure 4-8). Between day 37 and day 50, the CES reached a steady state with regard to reduced carbon, with the exception of the methane treatment which increased throughout the 140 days of the experiment. The concentration of reduced carbon in the methane treatment increased rapidly over the development period, from day 0 to day 44 and then the rate

decreased. This pattern is different from the other treatments and may relate to the direct conversion of the methane to reduced carbon in the liquid phase of the CES. The increase in oxygen over this same time period implies that the expected photosynthetic carbon fixation was also occurring.

The concentration of oxygen over time in the different CES treatments exhibited independent values and kinetics (Figure 4-9). The control increased from the 20.95% O₂ of ambient air at closure to 35% O₂ as the system approached steady state. In the nitrogen treatment the O₂ concentration started at 0% O₂ and increased to 22% O₂ by day 50, while the methane and hydrogen treatments were intermediate between these two. The glucose treatment in contrast decreased to 2.5 % O₂ by day 44.

The concentration of live carbon in the CES for the different treatments (Table 4-1) was similar. The levels were lower than in experiment 1, but uniform for all time points. The carbon production values (Table 4-2) also showed no dramatic differences among treatments. An increase for day 31 and 47 is obvious for all treatments except for the nitrogen treatment. This could correspond to the peak in carbon cycling activity seen in experiment 1 that occurred as steady state was achieved in terms of reduced carbon (days 15-20, experiment 1, figure 3-2).

The nutrient data for experiment 2 did show some differences between treatments. The SRP values (Table 4-3) were low for the control and gas treatments with the methane treatment averaging approximately 1.2 micromolar and the hydrogen averaging slightly over 5.1 micromolar for the steady state period. The bicarbonate treatment was more than twice the hydrogen treatment concentrations while the glucose treatment was over 8 times that concentration. The ammonia values were also dissimilar for the different treatments (Table 4-4). The control, bicarbonate and methane treatments were in the low micromolar range, the nitrogen and hydrogen were in an intermediate range while the glucose treatment averaged almost 100 times the control average.

Discussion

The parameters measured in this study allow the ecosystems to be studied in terms of bioenergetics after Odum (1969) and not in terms of species list, trophic relationships or other metrics that primarily focus on the individual organisms of the ecosystem. Although the perturbations may have caused major changes in species composition or relative dominance of species, the CES will not be considered changed unless a change in the carbon pools or carbon production rates is observed.

Based on that criterion the CES of experiment 1 were stable to the light, nitrogen, and hydrogen treatments.

The CES showed no departure in steady state behavior in the measured parameters in comparison to controls. The methane treatment resulted in a minimal perturbation to the CES. The only parameter that changed was the reduced carbon pool which apparently absorbed 280 micromoles of methane. Unfortunately, it is impossible to determine in what form the carbon from the methane was in or how biologically active or available it was.

The direct addition of carbon in readily available form resulted in clear changes in the measured parameters. The bicarbonate addition doubled the live biomass and carbon production and increased the reduced carbon by 20%. While the glucose increased the pools and rate even more over the first 20 days, by day 144 the glucose perturbed system had the lowest live carbon and carbon production rate of any treatment. The glucose treatment did not result in a new steady state over the experimental period but resulted in a boom and bust behavior. In contrast the bicarbonate treatment resulted in a more consistent increase, putting the CES into a new steady state with more live carbon and more carbon production.

The addition of 640 micromoles of glucose represents a large input of biologically available chemical energy to the CES. These CES are photosynthetic systems with a carbon production rate of $25 \text{ micromoles C day}^{-1} \text{ sys}^{-1}$

(figure 4-2). If the glucose was used with a 50% respiratory efficiency it could provide for an additional 1920 micromoles C sys^{-1} of carbon production. The actual increase in carbon production seen over the time period was approximately 6000 micromoles C sys^{-1} (area between glucose curve and control curve in figure 4-2). Thus the glucose stimulates carbon production beyond the simple addition of chemical energy, but it apparently also destabilizes the CES.

The effect of the bicarbonate addition in exp. 1, is consistent with the work of Shaffer (1991) which showed a dependence of live biomass and production on overall carbon levels. It is interesting that the ecosystems were able to respond to this addition even after 30 days of steady state behavior. The fact that the different gas treatments and the light treatment did not result in large perturbations of the measured parameters after steady state had been achieved show that the bioenergetics of systems are not extremely sensitive to drastic oxidation level changes. Although the treatments appear severe to our oxic metazoan life style and probably impacted the species list of the CES, the carbon flow and carbon pools were not influenced.

The non-effect of the perturbations on bioenergetics while large shifts in the metabolic and probably species lists may have occurred implies a stability concept that goes beyond the invariant species list concept of

stability. It implies that strong homeostatic forces restrain shifts in carbon pools and production in ecosystems. It is arguable that a carbon (or other nutrient) addition results in a different ecosystem and not in a perturbation to an existing one. Shaffer found different levels of biomass and activity for different levels of bioelements in otherwise similar CES. But it is clear the steady state condition demonstrated in Chapter 2 is also a stable condition as it demonstrates resistance to the gas phase treatments.

Experiment 2 was designed to test the sensitivity of the CES to starting conditions. It is reasonable to expect that even if the perturbation treatments could not change the steady state behavior once it was established (exp. 1), the different treatments could redirect the development of the steady state and result in measurably different steady state conditions. The equifinality principle of von Bertalanffy (1950) would predict otherwise, however.

The data from experiment 2, (tables 4-1 and 4-2, figure 4-8) indicate that the treatments had no effect on the final steady state in terms of reduced carbon, live carbon or carbon production. The reduced carbon pools developed to remarkably similar sizes under different oxidation conditions (figure 4-8) with the exception of methane as discussed above. The principle of equifinality

seems to hold for these CES in terms of carbon pools and production rates.

This finding is in distinct contrast to the oxygen concentrations which vary widely in the different CES (figure 4-9). The oxygen concentration appears to develop independently of the ecosystem. The oxygen concentration is a consequence of the overall oxidation state of the system once the biology has established the reduced carbon level. This result indicates that the reduced carbon level is a thermodynamic system parameter for ecosystems, not oxygen concentration. This ecosystem property has recently been measured in the earth's atmosphere (Keeling and Shertz, 1992) where the oxygen concentration has declined annually by an amount equal to the amount of oxidants required to burn the fossil fuel used annually. Attempts to establish artificial biospheres containing oxygen-requiring species, such as astronauts, must take account of this property of CES. A proper redox balance would have to be predetermined in terms of reduced carbon and residual oxidant. The homeostatic mechanisms are apparently responding to reduced carbon content not to 20.95% oxygen. Indeed the recent trouble in Biosphere II with increasing CO₂ levels and decreasing O₂ can be seen as an other example of this property.

Chapter 3 presented evidence that CES achieve steady

state for a multigenerational time period. The data from experiment 1 (figures 4-1 thru 4-4) show that the steady state is stable to some redox perturbations but sensitive to carbon pool perturbations. In experiment 2, the corollary principle is observed; the CES are insensitive to redox conditions while developing a steady state but the CES will produce similar reduced carbon levels.

The stability of the CES to perturbations as shown in this study leads credence to the possibility of producing stable artificial biospheres with reasonable levels of live biomass and carbon production at steady state. Unfortunately, it does not address the question of which species will be existing at steady state. Evidence for the stability of large biospheres has been presented in a recent study by Falkowski and Wilson (1992). They reviewed the historical data on ocean transparency and concluded the photosynthetic biomass and productivity has not increased over the last 90 years in spite of the increase in CO₂ of the atmosphere.

The data from experiment 2, and the control portion of experiment 1, show no evidence for alternative steady states for the CES. In fact, even under different starting conditions the CES develop remarkably similar steady states. Although it is impossible to disprove the existence of alternative steady or stable states the fact that different starting conditions lead to similar steady

states implies at least in the region of stability spanned by these perturbations no alternative states exist.

In conclusion, CES not only achieve steady state (chapter 2) but they are stable. CES, when established under different starting conditions, exhibit equifinality in these steady states. No evidence for alternative steady states was observed in the CES. Finally, the reduced carbon pool appears to be an important system parameter while oxygen concentration does not.

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Table 4-1. Effect of perturbations introduced at day 0 on live carbon in exp. 2.

Time	Micromoles of live carbon in CES ^a					
Days	Control	Nitrogen	Hydrogen	Glucose	Carbonate	Methane
15	4.53	4.4	2.85	2.58	3.46	3.89
31	1.58	1.32	2.66	3.67	2.26	5.76
Time points during steady state period						
47	1.69	2.87	3.37	3.45	5.32	4.28
81	1.16	1.44	2.2	1.34	1.66	2.43
136	2.48	1.7	2.33	1.51	1.74	2.33
Steady state						
Average ^b	1.78	2.00	2.63	2.10	2.91	3.01
Std. Dev.	0.54	0.62	0.52	0.96	1.71	0.90

^aEach point is an average of three ATP determinations as discussed in the text. Results are presented as micromoles live carbon in the CES.

^bThe average and standard deviation for the last three time points during which the reduced carbon was at steady state is also presented. Treatments are described in the text.

Table 4-2. Effect of perturbations introduced at day 0 on carbon production in exp. 2.

Time	Micromoles of live carbon in CES ^a					
	Control	Nitrogen	Hydrogen	Glucose	Carbonate	Methane
Day						
15	2.96	1.52	0.72	1.46	2.46	2.02
31	26.9	6.67	34.63	21.49	23.55	35.41
Time points during steady state period						
47	4.87	9.59	12.33	26.35	15.29	30.85
81	8.02	9.95	11.64	4.76	10.23	8.7
136	4.36	1.83	3.03	1.48	0.15	4.4
Steady state						
Average ^b	5.76	7.12	9.00	10.86	8.56	14.65
Std. Dev.	1.61	3.75	4.23	11.03	6.29	11.59

^aEach point is an average of three carbon production determinations as discussed in the text. Results are presented as micromoles carbon day⁻¹ in the CES.

^bThe average and standard deviation for the last three time points during which the reduced carbon was at steady state is also presented. Treatments are described in the text.

Table 4-3. Effect of perturbations introduced at day 0 on soluble reactive phosphate (SRP) in exp. 2.

Time	Micromolar SRP in CES ^a					
Day	Control	Glucose	Bicarbonate	Nitrogen	Hydrogen	Methane
15	0.00	7.05	31.83	0.00	2.23	2.23
31	45.77	12.83	28.25	15.21	14.08	1.27
Time points during steady state period						
47	8.84	7.53	39.81	2.23	14.14	1.63
81	2.82	11.28	34.45	1.93	1.33	1.33
136	1.45	17.95	52.50	5.50	0.00	0.50
Steady state						
Average ^b	4.37	12.25	42.25	3.22	5.16	1.16
Std.Dev.	1.19	2.94	7.39	1.48	2.19	0.36

^aEach point is a single determination a pooled sample from that time point as discussed in the text. Results are presented as micromolar SRP in the CES.

^bThe average and standard deviation for the last three time points during which the reduced carbon was at steady state is also presented. Treatments are described in the text.

Table 4-4. Effect of perturbations introduced at day 0 on ammonium concentration in exp. 2.

Time	Micromolar ammonium in CES ^a					
Days	Control	Glucose	Bicarbonate	Nitrogen	Hydrogen	Methane
15	17.65	522.40	12.55	5.93	10.00	6.01
31	4.15	488.44	18.83	5.50	58.14	6.01
Time points during steady state						
47	4.66	496.08	12.13	26.65	31.91	5.16
81	5.33	326.27	7.88	86.67	36.75	5.76
136	2.45	282.97	16.29	20.45	65.79	11.62
Steady state						
Average ^b	4.15	368.44	12.10	44.59	44.81	7.51
Std.Dev.	1.23	91.97	3.43	29.87	14.96	2.91

^aEach point is a single determination a pooled sample from that time point as discussed in the text. Results are presented as micromolar ammonium in the CES.

^bThe average and standard deviation for the last three time points during which the reduced carbon was at steady state is also presented. Treatments are described in the text.

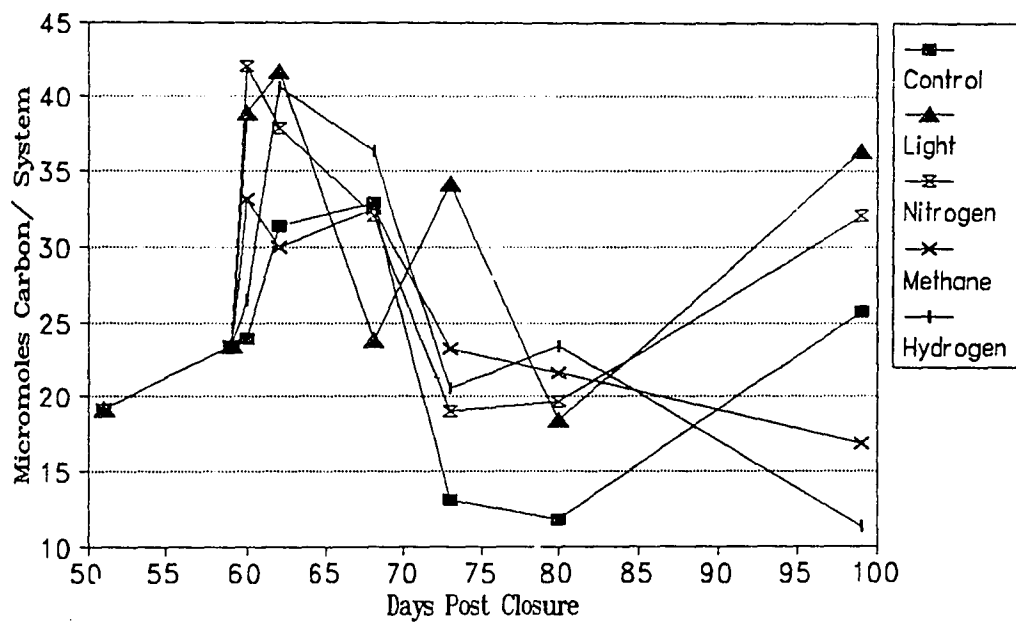


Figure 4-1. Impact of four perturbation treatments (light, methane, nitrogen, and hydrogen) introduced on day 59, on live carbon in experiment 1.

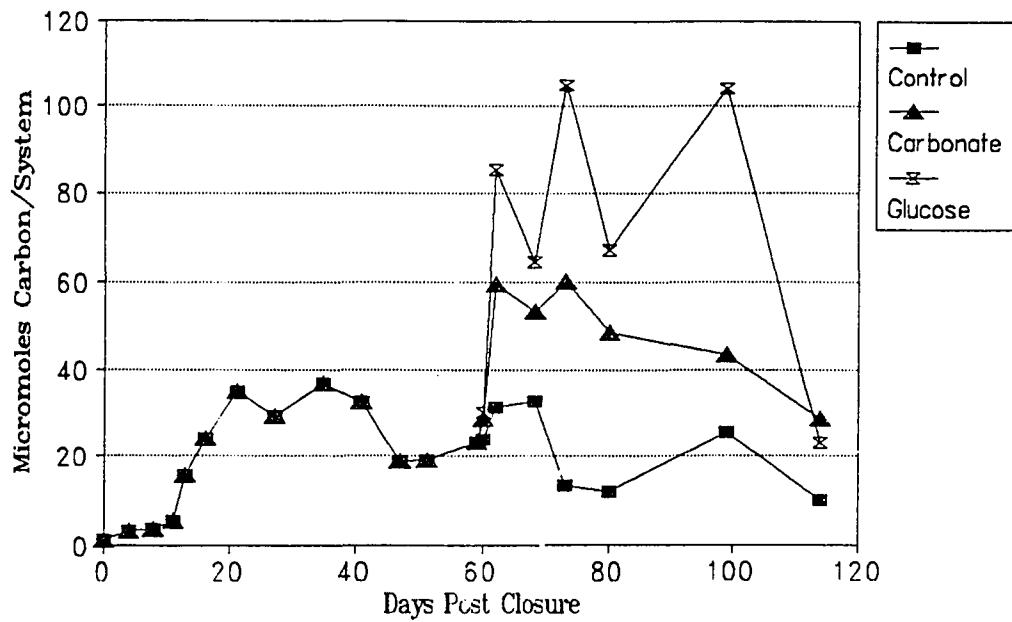


Figure 4-2. Impact of two perturbation treatments (carbonate and glucose) introduced on day 59, on live carbon in experiment 1.

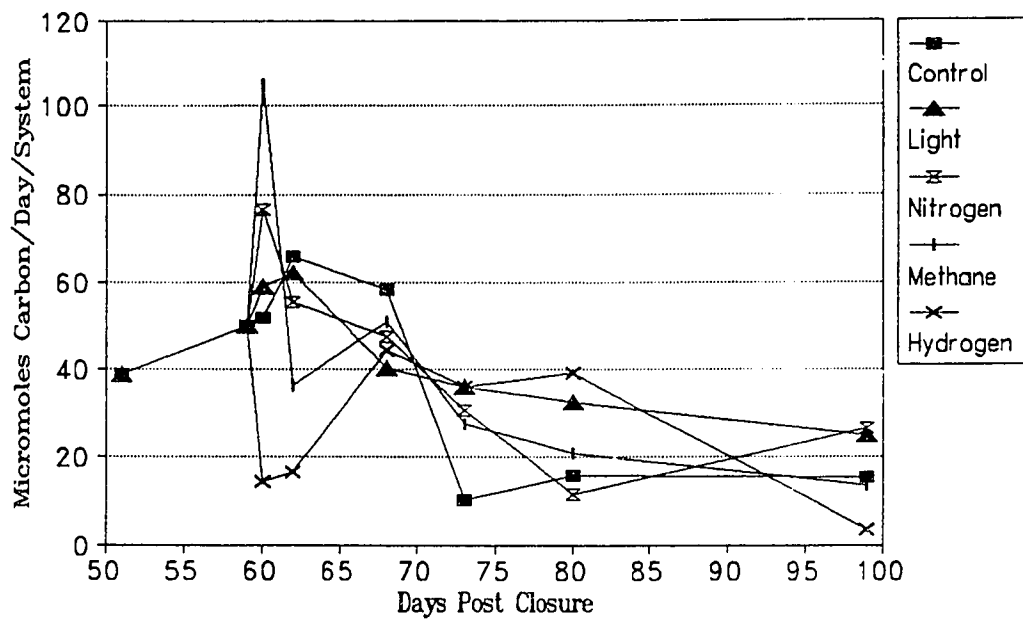


Figure 4-3. Impact of four perturbation treatments (light, methane, nitrogen, and hydrogen) introduced on day 59, on carbon production in experiment 1.

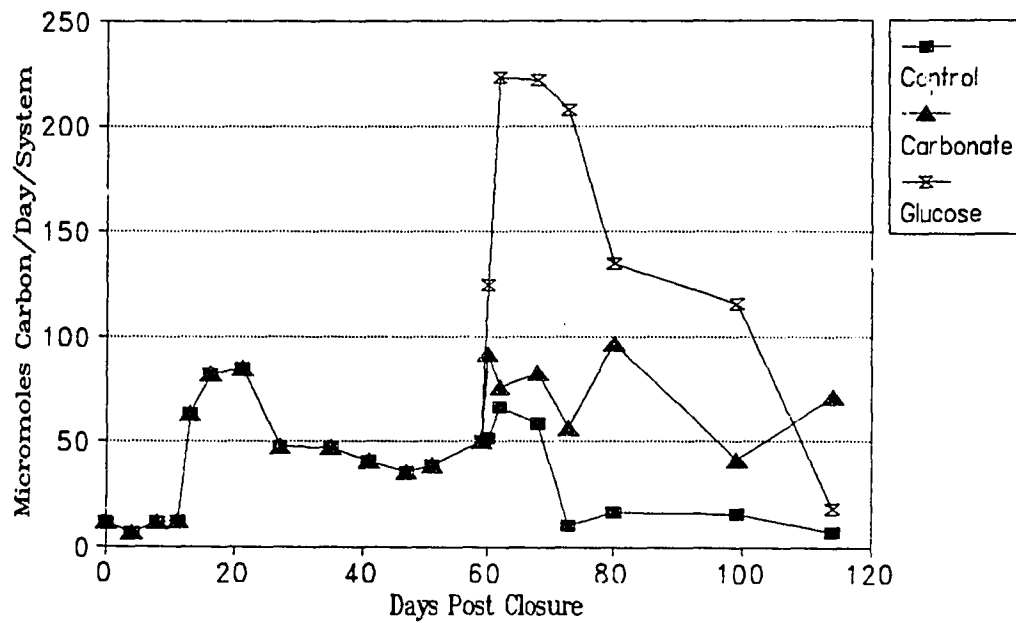


Figure 4-4. Impact of two perturbation treatments (carbonate and glucose) introduced on day 59, on carbon production in experiment 1.

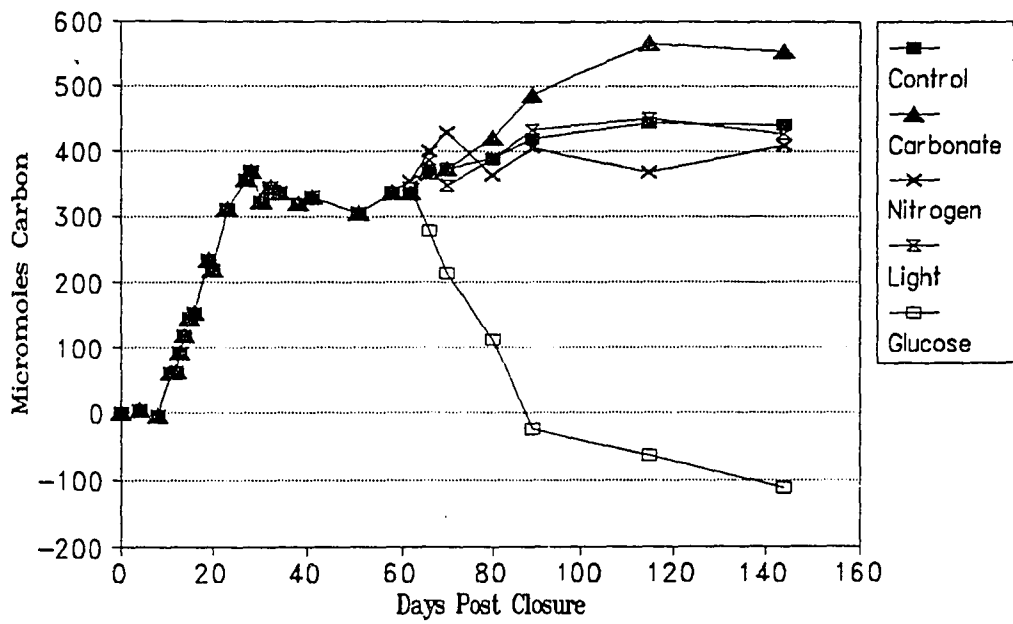


Figure 4-5. Impact of four perturbation treatments (carbonate, light, nitrogen, and glucose) introduced on day 59, on reduced carbon in experiment 1.

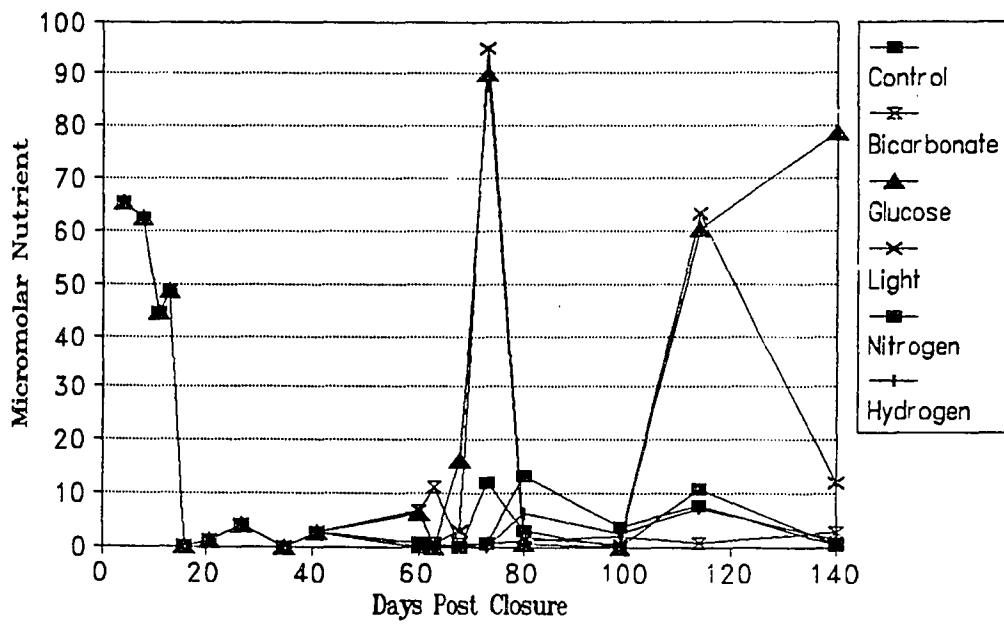


Figure 4-6. Impact of five perturbation treatments (carbonate, light, glucose, nitrogen, and hydrogen) introduced on day 59, on soluble reactive phosphate concentration in experiment 1.

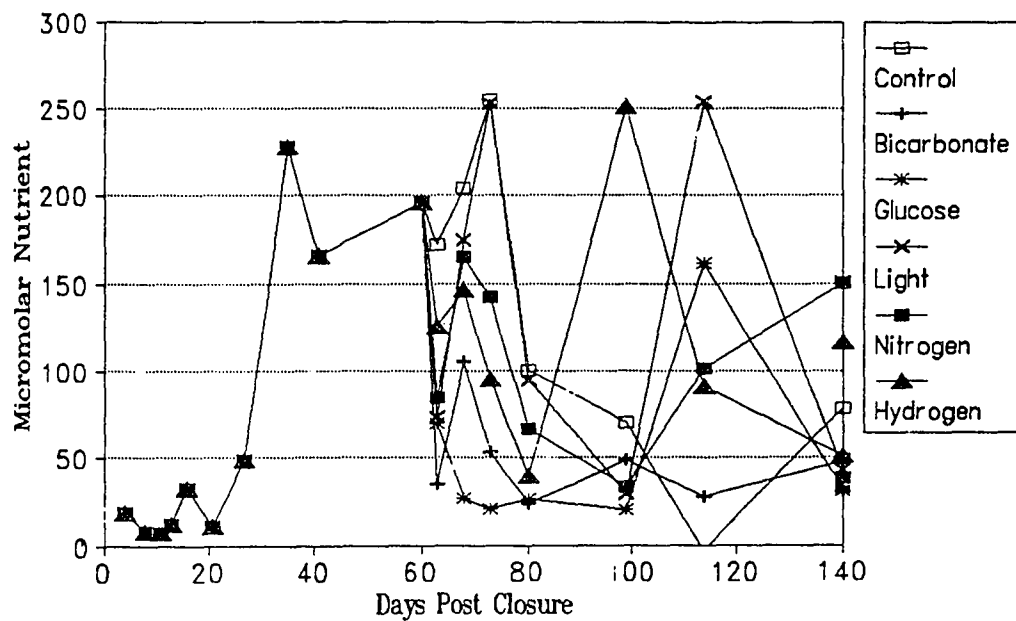


Figure 4-7. Impact of five perturbation treatments (carbonate, light, glucose, nitrogen, and hydrogen) introduced on day 59, on ammonium concentration in experiment 1.

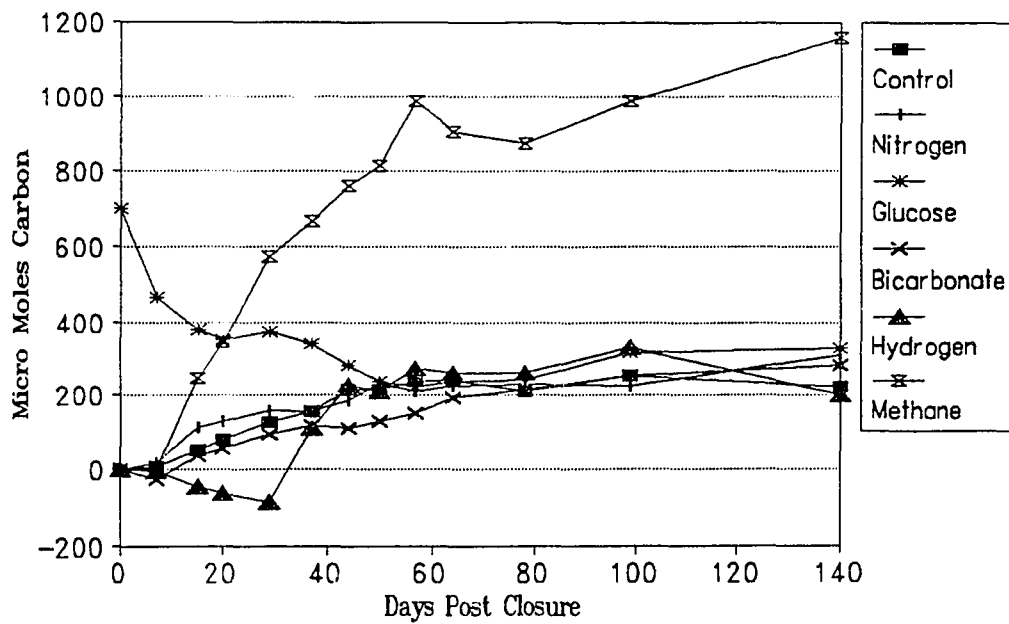


Figure 4-8. Effect of perturbation treatments (nitrogen, glucose, bicarbonate, hydrogen, and methane) introduced on day 0, on the reduced carbon pool in experiment 2.

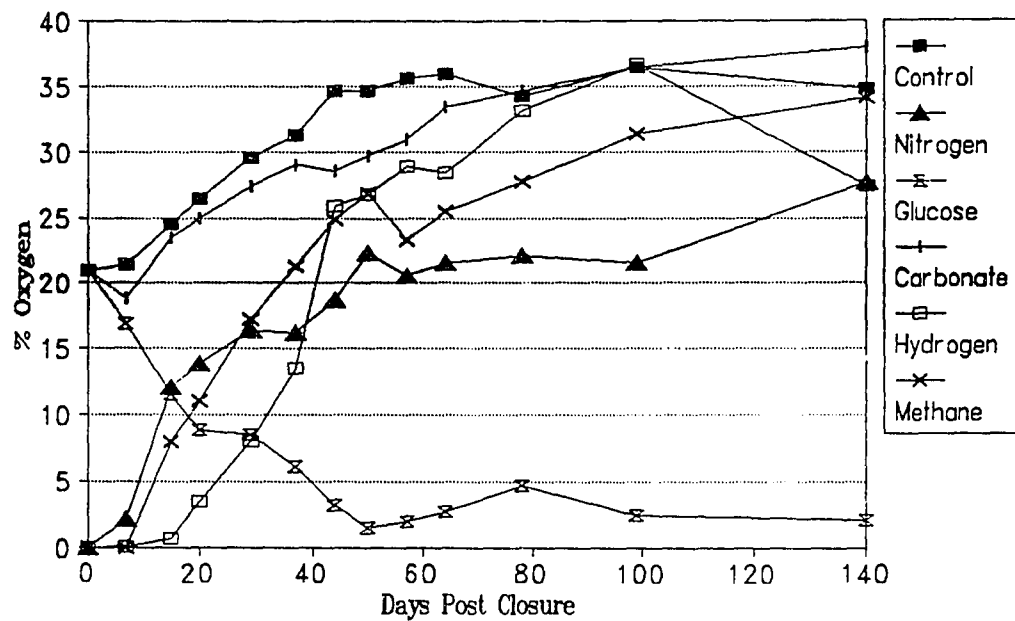


Figure 4-9. Effect of perturbation treatments (nitrogen, glucose, bicarbonate, hydrogen, and methane) introduced on day 0, on the oxygen concentration in experiment 2.

CHAPTER 5

CARBON PRODUCTION IN CLOSED ECOSYSTEMS, AS DETERMINED BY THREE DIFFERENT METHODS

Introduction

The determination of carbon production in the previous three chapters was an indirect measure based on the incorporation of tritiated adenine into DNA. The DNA production rate was used to calculate the carbon production. Using this method, values for carbon production indicate a daily or faster than daily live carbon turnover, even after 100 days of closure. Although much validation work has been done on this method, both in the laboratory (Winn and Karl, 1984) and on field samples (Karl and Bossard, 1985), it has not been universally accepted (Furhman et al., 1986). No validation work has been done using tritiated adenine to measure carbon production in CES. A major problem in microbial ecology is that there is no absolute standard method to determine microbial production in natural samples against which other methods can be compared (Karl, 1986).

Without an absolute standard, the common practice used to demonstrate the validity of a particular technique

has been to compare it to the result of some other technique on the same sample type. Although, there have been many methods proposed to measure microbial production, this chapter will compare tritiated adenine incorporation, adenine pool turnover, and the incorporation of ^{14}C labeled bicarbonate into organic carbon.

The advantages of using tritiated adenine to determine carbon production have been presented by Karl and Winn (1986). The method has been used for over 12 years in many sample types and yields values that are ecologically reasonable, if generally somewhat higher than the results produced by other methods (Burns et al., 1984). The basic assumptions required for this method are that the majority of microorganisms take up exogenous adenine and rapidly equilibrate internal pools, that the addition of the adenine does not stimulate the synthesis of DNA, and that the conversion factors are reasonable for the particular sample type (Karl and Winn, 1984). While the conversion factors have not been measured for CES, they have been determined for many other environments (Karl, 1980; Karl, 1986). The values used here are the standard values for marine samples:

1. **ATP(grams) x 250 = live carbon (grams) or live biomass.**; (Karl, 1986) this is a measure of living protoplasm and does not include cell walls, extensive sheaths or detrital material.

2. **DNA is 25% adenine.**; while the nucleotide residue ratio does vary between species, the mixed nature of the microbial assemblage is assumed to result in a composite DNA with an average adenine content of 25%.

3. **The DNA is 2% of the live carbon.**; the DNA content of cells does vary with growth rate and this figure is an average of measured ratios in natural environments.

To determine the microbial production from the incorporation of tritiated adenine, several quantities must be measured. The total amount of radioactivity in the DNA synthesized during the incubation must be determined without the presence of other labeled macromolecules. This requires a fairly laborious DNA purification procedure. The specific activity of the internal ATP pool must also be determined. To do this the size of the internal ATP pool must be measured as must be the amount of radioactivity in the ATP pool. These three values can then be used to calculate the rate of adenine incorporation into DNA. With these assumptions, a carbon production and live carbon turnover can be calculated.

The determination of the ATP pool turnover relies on the measurement of the specific activity of the internal ATP pool as it changes over time after tritiated adenine is added to a sample. The technique relies on the fact that the time required for equilibration between the

adenine pool and the ATP pool is a function of growth rate. This equilibration period has been measured in laboratory cultures to be between 2% and 2.5% of the live carbon turnover time. The method reduces the assumptions (and bench work) required for the adenine incorporation method. The assumption concerning uptake of exogenous adenine is modified in that accuracy of the method does not require a major portion of the species present in the microbial assemblage to take up exogenous adenine, but the live carbon turnover value determined will pertain only to the microorganisms taking up adenine. To convert the results to carbon production, the inverse of the turnover time must be multiplied by the live biomass.

The incorporation of ^{14}C -labeled bicarbonate has been used extensively in microbial ecology to determine photosynthetic carbon fixation. The method is well suited for use in chemically defined CES at steady state as the specific activity of the inorganic carbon pool is easily calculated and net production is zero. The size of the inorganic carbon pool is larger than the live carbon pool. This allows time courses to be predictable and reasonably long.

The first two techniques discussed above are supposed to determine the production of live biomass, the protoplasm of the living organisms. The last technique measures the reduction of oxidized carbon to reduced carbon. These are

two distinct processes. The reduced carbon is not utilized completely for biomass biosynthesis some (large percentage) is used for respiration. At steady state, ultimately the respiration equals the fixation, but it is not clear how long the residence time is for newly fixed carbon in a steady state system. In a culture of algae the gross production is frequently twice the net production of biomass. For a CES at steady state the net production is zero and the respiratory efficiency is therefore also zero. The importance of the photosynthetic carbon production (PCP) is undeniable; it represents the major input of energy to the biology in these CES. A certain amount of energy could be produced and utilized by the algae and cyanobacteria by cyclic photophosphorylation, but this input is generally ignored.

The carbon production, as measured by adenine incorporation, is of course dependent on the PCP as the biological energy available for carbon production in the CES is dependent on the oxidation of the PCP during heterotrophic processes. But, and this point is important, it is not limited to a value less than the PCP. The recycling of protoplasm from one microorganism to another does not require the complete oxidation of the biomolecules and then their resynthesis. It only requires the hydrolysis of polymers to monomers, their uptake and then

their repolymerization, a much less energy-intensive process (Morowitz, 1979).

If the assumption is accepted that the carbon fixation as measured by ^{14}C incorporation is the major source of energy to the CES and the carbon production as measured by tritiated adenine is reasonably accurate (or at least consistent) then, as proposed by Shaffer (1992), it becomes possible to define a metric for ecosystem energy efficiency. The carbon production divided by the PCP results in a dimensionless number that relates the captured solar energy to the biological activity of the system. The more efficiently linked the energy transfers are in the ecosystem, (i.e. between trophic levels etc.) the more carbon production will be possible from a finite amount of captured solar energy.

Materials and Methods

For this experiment the CES established for experiment 2 of chapter 3 were analyzed on days 101, 102 and 140 post-closure. The details of CES establishment and the methods used for the adenine incorporation are in chapter 2. The only modification was that a long time course incubation with tritiated adenine was run. The CES were incubated with tritiated adenine for 12 time periods between 15 min and 72 hr. All the tritiated adenine incubations were begun on the same day, in triplicate, at intervals of

30 minutes to allow time for for filtering. The samples were all processed as described in chapter 2.

The incorporation of ^{14}C bicarbonate was performed on day 102 and 140. Preliminary calculations indicated a 24 hr incubation would be required to produce sufficient amounts of labeled organics. Four hundred μl of labeled bicarbonate (5 uCi ml^{-1} , 1 mCi mg^{-1}) was injected through the stopper of the CES and then the CES were placed back in the incubation chamber. After 24 hr the CES were unsealed and 2 ml of concentrated HCl (11.6 M) was added. CO_2 gas was then bubbled for 20 min with a pasteur pipette into the liquid phase of the CES to displace any $^{14}\text{C-CO}_2$. A 1 ml sample was then added to 10 mls scintillation fluid and the radioactivity in the reduced carbon determined.

The carbon reduced over the incubation period was determined by calculating the fraction of the $^{14}\text{C-CO}_2$ reduced: recovered nanocuries divided by added nanocuries times the inorganic carbon in the liquid phase. The inorganic carbon was calculated by subtracting the reduced carbon, as determined by the oxygen concentration, from the inorganic carbon in the original medium.

Results

The time course results for the incorporation of tritiated adenine into replicate CES are presented in Table 5-1. The specific activity of the recovered ATP quickly peaked after 30 min and then declined for the rest of the experiment (Figure 5-1). The total radioactivity recovered in the DNA fraction increased up to the 24 hr incubation and then began to decrease (Figure 5-2) as did the radiolabel recovered in the protein and RNA fractions. The rate of labeled DNA production decreased steadily with incubation time (Table 5-1). During this time course experiment the live carbon pool of 36 replicate CES was measured 36 times (data not shown) and averaged 2.59 ± 0.77 micromoles carbon system⁻¹. The tritiated adenine incorporation data was used to calculate a carbon production value for each time point (Table 5-1). These values decreased with the length of the incubation from a high value of 38.6 micromoles carbon day⁻¹ system⁻¹ for the 15 min incubation to a low of 0.7 for the 72 hr incubation.

The ¹⁴C labeled bicarbonate incorporation data are presented in table 5-2 for all 6 treatments of experiment 2. The carbon fixation varied from 1.86 micromoles carbon fixed day⁻¹ for the nitrogen treatment on day 102 to 8.31 micromoles carbon fixed day⁻¹ for the glucose treatment on day 140. The carbon fixation rate for the control

treatment treatment was 5.6 ± 2.2 micromoles carbon fixed day^{-1} on day 101.

Discussion

The calculation of carbon production from the tritiated adenine incorporation for each incubation time results in a wide range of values. To put these carbon production values in perspective it is useful to calculate the live carbon pool turnover (live carbon divided by daily carbon production). The first three time points, .25 hr to 1 hr incubations, produce carbon production values from 38.6 micromoles carbon $\text{day}^{-1} \text{system}^{-1}$ to 12.3 micromoles carbon $\text{day}^{-1} \text{system}^{-1}$. These production values, in turn, yield turnover times 1.6 to 5.5 hr for the live biomass. In comparison, the last four time points, the 36 hr to 72 hr incubations, yield live carbon turnovers between 32.7 to 88.8 hr.

A live carbon turnover of a few hours is extremely fast and has not been reported in nature. Live carbon turnovers reported in natural microbial assemblages range from just under 24 hr (Burns et al., 1984) to multi-day periods depending on water temperature and habitat. The colder and deeper the environment the slower the turnover.

The carbon production values for the earliest time points are inaccurate due to the lack of time for the extracellular tritiated adenine to equilibrate with the

intracellular ATP pool. The ATP pool turnover is one thirtieth of the live carbon pool turnover and it takes 5 turnovers for a pool to approach equilibrium (Karl and Bossard, 1984). For a 12 hr live carbon turnover, 2.0 hr would be required for the ATP pool to equilibrate with the labeled adenine (also see below).

The carbon production values calculated from the data for the later time points are low because the degradation of labeled DNA over the incubation time would decrease the recovery of labeled DNA for those incubation times. This method assumes that an insignificant amount of labeled DNA is catabolized during the incubation period. The effect is proportional to the loss of labeled DNA. The turnover of rate of all DNA in the system was not measured, but the fact that the labeled macromolecules decrease after 24 hr indicates the turnover is of that magnitude. For instance for the 24 hr incubation, a 24 hr DNA turnover time would degrade approximately half the labeled DNA over the course of the incubation resulting in a calculated carbon production value that was half the actual value.

The carbon production values determined for the 2 hr to 24 hr incubation periods range from 7.6 micromoles carbon day⁻¹ system⁻¹ to 2.9 micromoles carbon day⁻¹ system⁻¹. The lower values come from the longer incubations where again the degradation of labeled DNA would lower the calculated values for these time points. The live carbon turnover for

these incubation times range from 8.2 to 21.4 hr, similar to values reported for other tropical shallow water systems (Karl, 1986).

The ATP turnover portion of the experiment did not result in specific activity values that could be used to calculate the turnover of the ATP pool. The expected uptake curve for the specific activity (Karl and Bossard, 1984) was not observed. For a reasonable live carbon turnover value, between 12 hr to 24 hr, the corresponding value for the ATP pool turnover would be 15 min to 30 min. As it takes approximately 5 turnovers for the pool to approach equilibrium, the specific activity determined in the time course should increase for at least 75 min and then level off. As can be seen in table 5-1 the specific activity peaked and then declined after 30 min.

This decrease in specific activity indicates that the extracellular pool of labeled adenine was exhausted. After the highest specific activity value at 30 min, the specific activities decreased rapidly at first and then more slowly over the 72 hours of the time course. This is a result of the utilization of adenine for biosynthesis and dilution of the labeled adenine by newly synthesized unlabeled adenine. This decrease in specific activity is then in fact an unlabeled curve for the intracellular adenine pool.

By analogy with the ATP pool turnover method this unlabeled curve can be used to directly calculate the live carbon turnover of the CES if certain assumptions are accepted. First, the only loss of adenine from the adenine pool is by production of RNA and DNA. Second, the decrease in specific activity is exponential. To calculate the turnover of the pool, I multiplied the specific activities by 100 and took the natural log to linearize the results. A linear regression was performed on the points between 30 minutes and 36 hours ($R^2=0.84$). The equation of the line was used to determine an adenine pool turnover time of 15.8 hours and using the average live carbon value of 2.59 micromoles carbon, a carbon production value of 3.9 micromoles carbon day⁻¹ system⁻¹ was obtained.

The ¹⁴C labeled bicarbonate determination produced values of the same magnitude as the other two methods (Table 5-2). The control treatment averaged 6.32 micromoles carbon reduced day⁻¹ system⁻¹. This result is directly comparable to the 3.0 to 7.6 micromoles day⁻¹ from the adenine incorporation time series and slightly higher than the 3.9 calculated from the adenine turnover. If the carbon production data for all treatments of experiment 2 are compared (Table 4-2) to the reduced carbon production data of table 5-2, a reasonable correlation is seen.

The determination of microbial production rates is a highly contentious field. The fact that the three methods

discussed above result in similar values confirms the high metabolic activity of CES. All the data indicate that the live biomass is, on the average, turning over at a greater than daily rate, even after more than 100 turnovers.

These results lend credence to the carbon production figures presented in the first four chapters. Although extensive time series incubations were not done for those tritiated adenine incorporations and, as is clear from the above discussion, incubation period is important, the incubation period used was appropriate.

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TABLE 5-1. Time course data for the incorporation of tritiated adenine into the control CES of experiment 2, on day 101 post closure.

Incubation Period hr	Specific Activity Ci mMole ⁻¹	Labelled DNA rate ^a nanoCi hr ⁻¹	Carbon Production micromoles carbon day ⁻¹ system ⁻¹
.25	0.18	28	38.6 ^b (8.49) ^c
.5	0.23	27	22.5 (2.87)
1	0.18	18	12.3 (1.10)
2	0.18	11	7.6 (1.12)
4	0.12	9.3	7.0 (1.04)
8	0.14	4.7	4.0 (0.54)
16	0.08	3.0	2.9 (1.01)
24	0.074	2.6	3.0 (0.43)
36	0.058	1.5	1.9 (0.05)
48	0.055	1.1	1.6 (0.32)
60	0.052	0.57	0.9 (0.19)
72	0.041	0.40	0.7 (0.10)

^a The amount of radiolabeled DNA recovered at each incubation time point divided by the incubation time.

^b All values are the average of triplicate determinations.

^c Standard deviation for carbon production values.

Table 5-2. Carbon fixation^a in CES of experiment 2.

Sampling Time	Average no. of micromoles carbon reduced day ⁻¹ system ⁻¹					
	Cont. ^b	Gluc.	CO ₂	N ₂	H ₂	CH ₄
Day 102	5.60 (2.19) ^c	5.93 (2.93)	3.81 (0.44)	1.86 (0.58)	2.88 (1.52)	3.49 (2.41)
Day 140	7.04 (1.82)	8.31 (2.38)	5.65 (2.28)	3.15 (0.67)	1.51 (0.77)	5.68 (5.18)

^a The carbon fixation was determined by incorporation of ¹⁴C labelled bicarbonate over a 24 hour light incubation. Radioactivity remaining after acid addition and CO₂ purge was considered reduced carbon.

^b Treatments are control (cont), glucose addition (gluc), bicarbonate addition (CO₂), nitrogen gas phase (N₂), hydrogen gas phase (H₂) and methane gas phase (CH₄).

^c Values in parentheses are standard deviations of triplicate determinations.

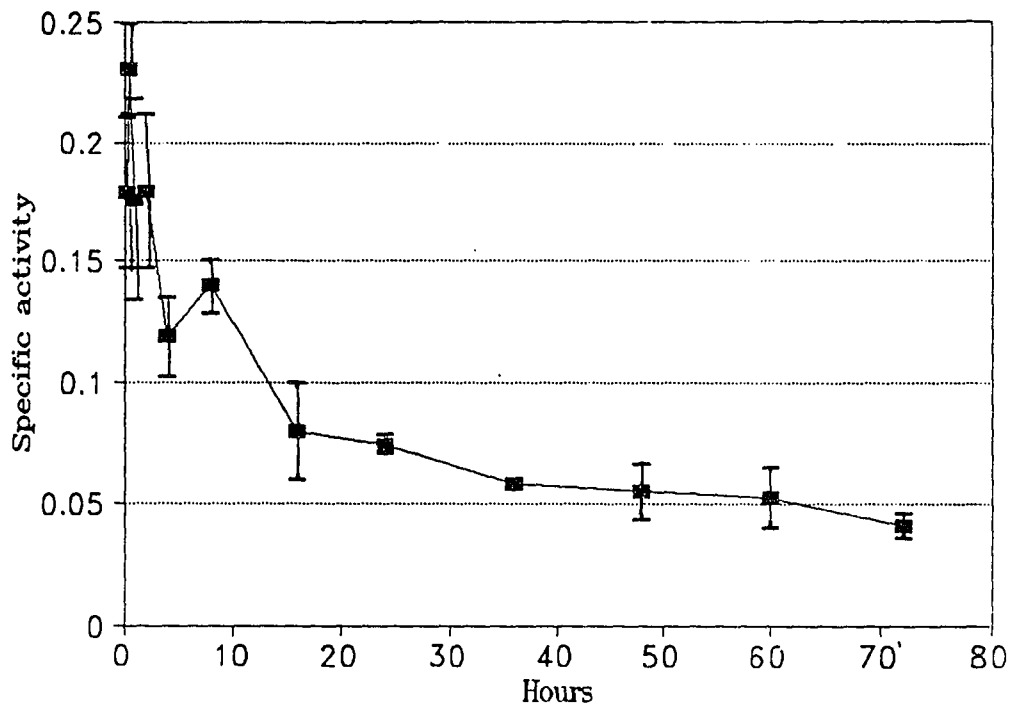


Figure 5-1. Specific activity of recovered ATP over time. On day 101, experiment 2, tritiated adenine, at a specific activity of 16.6 curies millimole⁻¹ was added to 36 replicate CES. The specific activity of the ATP was measured after the CES had incubated for various periods of time. The specific activity is presented in Curies millimole⁻¹ ATP. Error bars represent one standard deviation of the triplicate measurements.

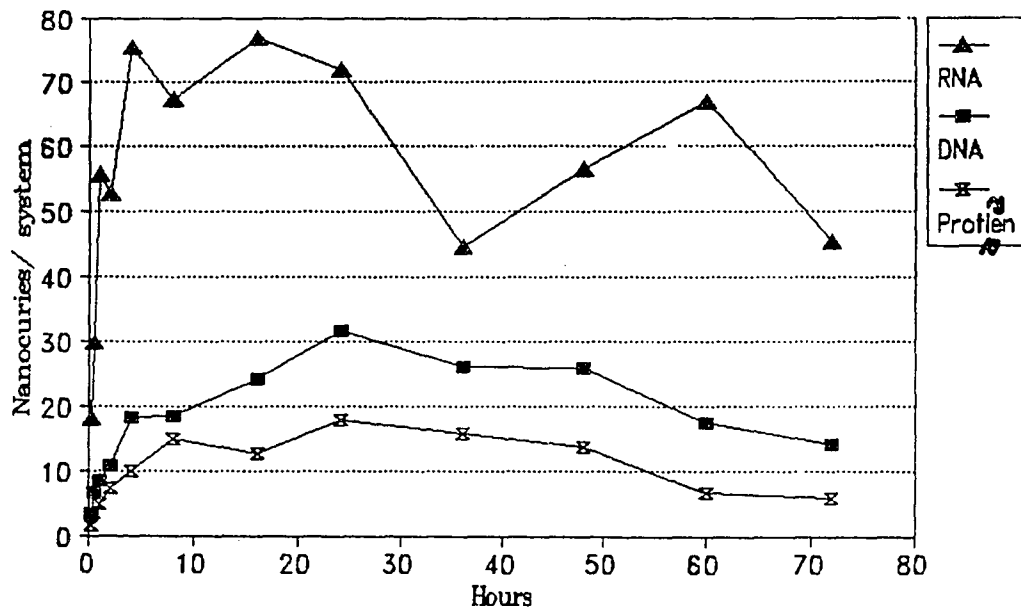


Figure 5-2. Recovery of labeled macromolecules. On day 101 the CES of experiment 2 were labelled with tritiated adenine. After the various incubation periods triplicate CES were processed to quantify the amount of label in each macromolecular pool.

CHAPTER 6

CONCLUSIONS

Are CES ecosystems? Is a 20 ml test-tube with 10^8 microorganisms of diverse trophic types, actively consuming and reproducing, rapidly cycling bioelements for tens of generations, an ecosystem? Although this construct has never been described from nature, I believe it is a bona fide ecosystem. Of course this is a definition dependent question and so I will offer my definition.

Ecosystem: A steady state system of matter displaced far from equilibrium by an energy flow (mediated by life).

Critical points of definition:

1. A system is a group of units composed as a whole working in unison (Merriam-Webster). A system, thermodynamically defined, is also separable from its surroundings. Matter and energy fluxes must be quantifiable across the boundary between the system and its environment.

2. The system must be at steady state; i.e. the state function does not change with time. As the state function for ecosystems is unknown, an operational definition of steady state must utilize unchanging biological parameters. A sealed test tube containing a broth culture of E. coli is not at steady state; it is rapidly going to equilibrium. The oxygen level is dropping, the ATP (live biomass)

content is increasing and then decreasing, the glucose is a steadily dwindling energy source. An open test tube of a complex mixture of algae, bacteria and phage is also not at steady state; it continuously exchanges oxygen with the air and absorbs carbon dioxide thus changing its C:N:P ratio.

3. The system must be further displaced from equilibrium than a similar abiotic system would be if subjected to the same energy flow.

4. Energy flow is generally radiant, but this is not a requirement for this definition. An electrochemical ecosystem can be envisioned in which a 1-2 volt emf maintains a current through a materially closed ecosystem where sulfide oxidizers and sulfate reducers support the food chain. But without some form of energy flow any ecosystem will go to equilibrium, that is die.

5. Life is required as it is the only thing that can produce a steady state system far from equilibrium. Indeed this definition of ecosystem could also be a definition of life.

Are CES ecosystems by this definition? They are clearly a system and they are composed of matter. They have identifiable boundaries and are materially separate from the outside while open to an energy flux. CES are a complex of separate subunits, even if the subunits have not been clearly elucidated, a minimal degree of complexity was demonstrated in chapter 1.

CES achieve steady state and remain at steady state for a significant period of time (Chapter 2), at least for the parameters studied here. The CES at steady state are displaced from equilibrium. The presence of reduced carbon and diatomic oxygen attest to that fact. Furthermore the incorporation of radiolabeled adenine and bicarbonate show that this result is an active displacement from equilibrium and not just an activation energy restricted return to equilibrium (i.e., a CES, if autoclaved at steady state, will not quickly return to chemical equilibrium due to the activation energy barrier). That this displacement is maintained by an energy flux has been demonstrated by Kearns (1983) and Shaffer (1991) who placed CES in the dark and quantified the slow return of the systems to chemical equilibrium. The oxygen was consumed as reduced carbon was converted to CO₂.

CES exhibit many attributes that are considered characteristic of ecosystems. This work has shown that CES maintain metabolic diversity which implies a complex carbon and nitrogen cycling capability. They maintain levels of live biomass and carbon production on par with similar natural environments. In terms of bioenergetics, CES achieve a steady state in a manner analogous with the processes of succession and climax as seen in natural ecosystems.

CES exhibit fundamental properties of life and are worthy of study for that reason alone. They constitute an example of complete life, illustrating integrative properties of which we are only now becoming aware. How do these CES maintain their metabolic diversity for long periods in such a small, relatively homogeneous, disturbance free environments? Why do the CES all establish the same level of reduced carbon and then why does the reduced carbon remain at that level? A forest at climax represents a large supply of nutrients yet the forest remains at more or less steady state. A boom and bust cycle seems more probable. The CES are insensitive to a wide range of oxidation conditions but sensitive to the levels of carbon. According to the Gaia hypothesis the CES (and other ecosystems) maintain their reduced carbon environment (originally produced by the ecosystem during succession) at an optimal level in the face of outside forces.

This study has demonstrated the importance of the reduced carbon pool of these microbial CES and the non-importance, to these CES, of the oxygen concentration. As microorganisms ultimately are responsible for most carbon and mineral cycling on the earth and fully capable of running their own ecosystem, perhaps we, oxygen requiring beings, should realize we are expendable.