Coupling Plant Growth and Waste Recycling Systems in a Controlled Life Support System (CELSS)

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ABSTRACT

The development of bioregenerative systems as part of the Controlled Ecological Life Support System (CELSS) program depends, in large part, on the ability to recycle inorganic nutrients contained in waste material into plant growth systems. One significant waste (resource) stream is inedible plant material. This research compared wheat growth in hydroponic solutions based on inorganic salts (modified Hoagland's) with solutions based on the soluble fraction of inedible wheat biomass (leachate). Recycled nutrients in leachate solutions provided the majority of mineral nutrients for plant growth, although additions of inorganic nutrients to leachate solutions were neccessary. Results indicate that plant growth and waste recyling systems can be effectively coupled within CELSS based on 1) equivalent wheat yield in leachate and Hoagland solutions, and 2) the rapid mineralization of waste organic material in the hydroponic systems. Selective enrichment for microbial communities able to mineralize organic material within the leachate was neccessary to prevent accumulation of dissolved organic matter in leachate-based solutions. Extensive analysis of microbial abundance, growth, and activity in the hydroponic systems indicated that addition of soluble organic material from plants does not cause excessive microbial growth or "biofouling", and helped define the microbially-mediated flux of carbon in hydroponic solutions.

INTRODUCTION

The Controlled Ecological Life Support System (CELSS) program is a long range National Aeronautics and Space Administration (NASA) effort to develop a bioregenerative life support system which would reduce reliance on external material supplies by utilizing photosynthetic organisms as CO_2/O_2 converters and sources of food. Development of a functioning CELSS depends, in large part, on the ability to recycle inorganic nutrients contained in inedible plant material and human wastes into

plant production systems. Garland and Mackowiak (1990) found that the majority of most inorganic nutrients contained in inedible wheat biomass was readily soluble (or leachable) in water. In addition to the inorganic elements, the leachate contained significant quantities of organic carbon (approximately 15% of the carbon in the inedible biomass). Garland and Mackowiak (1990) proposed that leachate could be used as a hydroponic nutrient solution with admendments of certain inorganic nutrients (particularly nitrate and iron), thereby reducing the reliance on external supplies of inorganic nutrients required in inorganic salt-based media such as Hoagland's solution (Hoagland and Arnon 1938). While preliminary bioassays using wheat in static hydroponic systems indicated no significant difference in plant growth between leachate-based (LBS) and Hoagland-based systems (HBS), several concerns with full-scale use of organic-rich nutrient solutions based on plant leachates remained: 1) The presence of potentially phytotoxic organic compounds in leachate, 2) The persistence (accumulation) of organic material within plant growth systems, and 3) The proliferation of deleterious microbial populations (phytopathogenic, biofouling, etc.) within plant growth systems as a result of organic enrichments.

In order to address these concerns, full growouts of wheat were conducted in recirculating hydroponic systems containing 1) wheat leachate-based nutrient solution, and 2) inorganic salt-based Hoagland's solution. The first two concerns discussed above were addressed by comparing plant biomass and dissolved organic carbon concentration, respectively, between the two types of systems. The question of the effects of leachate on microbial populations within hydroponic systems required the development of a more comprehensive understanding of microbial processing of carbon in hydroponically-based plant growth systems.

It is important to realize that significant microbial activity is expected in HBS due to the release of organic material from plant roots. The rhizosphere, or area in the vicinity of plant roots, has long been known to be a region of enhanced microbial

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activity due to the release of root-derived carbon (Hiltner 1904, Lyon and Wilson 1921). Low molecular weight compounds (primarily organic acids, amino acids, and sugars) released into the surrounding environment by healthy, intact roots were defined as root exudates by Rovira (1969). Martin (1977) proposed the term cell lysate to describe organics released upon the death of epidermal and cortical root cells, a common occurrence during the life cycle of plants (Holden 1975, Van Vuurde and Schippers 1980). The total amount of carbon released from roots represents a significant fraction of the total carbon fixed by plants, estimated at between 12-24% of net primary productivity for crop plants during early periods in the life cycle (Barber and Martin 1976, Martin 1977, Merckx et al. 1986, Whipps and Lynch 1983, Martin 1971).

The rate of carbon transfer into the rhizosphere steadily decreases with plant age (Keith et al. 1986, Martin and Kemp 1986, Merckx et al. 1986). The cause of the decrease has not been elucidated, but has been proposed to be related to reduced root growth (Merkcx et al. 1986) and the onset of seed formation (Martin and Kemp 1986, Nooden 1984).

The processing of root-derived carbon in recirculating hydroponic systems has not been investigated. Existing models of rhizosphere carbon-flow in soil-based systems view transport of root exudates as a balance between microbial uptake and molecular diffusion, resulting in a spatially limited rhizosphere (<2 mm) caused by relatively rapid utilization of exudates near the root surface (Newman and Watson 1977). In recirculating hydroponic systems, advective forces could increase the rate at which root-derived organics are removed from the root zone, thereby expanding the zone of microbial stimulation, or rhizosphere, to include the nutrient delivery system. Barber and Lynch (1977) found that approximately a third of the total bacteria (as estimated from plate counts) in small, static hydroponic systems were associated with solution and surfaces. Strayer (1991) reported that over 95% of the microbial load in

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large-scale recirulating hydroponic systems was associated with the plant roots. However, no studies have comprehensively evaluated the spatial distribution of microbial abundance, activity, or growth in hydroponic systems.

Additions of soluble organic material (e.g. leachate) to nutrient solutions represents a potentially very different direction of carbon flow than the release of organic material from roots. First, the spatial extent of microbial communities in LBS will depend not only on competition for root-derived carbon, but also on competition for soluble leachate organics directly added to the nutrient delivery system. Secondly, additions of organic material in leachate will most likely occur in pulses related to replenishment schedules of inorganic nutrients. Finally, the leachate may be comprised of different types of organic molecules than root exudates. In particular, the brownish color of leachate indicates that it may contain a higher percentage of compounds more recalcitrant to microbial mineralization (e.g. - tannins and other cyclic hydrocarbons). Supplementing hydroponic systems with leachate organics represents an effective experimental approach for evaluating carbon-limiting growth conditions in different microbial habitats within hydroponic systems based solely on root-derived carbon, thereby helping define the pathway of carbon flux from the root.

The source of the microbial community within hydroponic systems may significantly affect the degradation of organic material in LBS. Plant leachates appear to contain a heterogeneous mixture of organic molecules, the mineralization of which requires some level of adaptation within microbial communities. McArthur et al. (1985) found that downstream microbial communities could utilize dissolved organic material from different types of upstream riparian vegetation, but upstream communities could not utilize dissolved organic carbon from riparian vegetation found only in the downsream region. These results suggest genotypic or phenotypic adaptation within the microbial community involving resistance to inhibitory compounds or utilization of recalcitrant molecules. Murray and Hodson (1986) found that leachate from an aquatic

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macrophyte caused a short term reduction followed by subsequent stimulation in productivity of bacterioplankton. Bacterial types resistant to inhibitory compounds in the leachate developed, and displayed luxuriant growth on the labile portion of the leachate.

It follows, then, that microbial community composition with hydroponic systems, especially leachate-based ones, can influence the processing of carbon. Hence, it is important to understand whether functionally similar microbial communities will develop in CELSS regardless of the inoculum used.

The influence of microbial inoculum is a particularly relevant question in CELSS. Microbial inoculum in a closed system can be controlled to a much greater degree than in most biological systems open to the continuous invasion of microorganisms. However, unlike other levels of biological organization within CELSS, bacterial inoculum cannot be completely controlled. While a specific number of plant and animal species will be introduced into CELSS, many different microorganisms will enter the system unless stringent, probably impractible, decontamination procedures are employed (e.g. - use of gnotobiotic plants, animals, humans). If the composition of the microbial inoculum to a CELSS cannot be completely controlled, than the effects of different inocula on the system must be understood.

The research described in this document involved the intensive spatial and temporal analysis of microbial abundance, activity, and growth in recirculating hydroponic systems using nutrient solution type (HBS vs LBS) and microbial inoculum as experimental factors. This approach was considered the most effective since it addressed the potential effects of leachate organics on microbial communities within hydroponic systems, and helped define how both root-derived and leachate organics are processed by microbial communities in CELSS. This area of carbon flux was identified by Garland (1989) as being very poorly understood, yet potentially,

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quantitatively significant in CELSS. Potential use of the plant growth systems as a site of waste processing increases the importance of understanding the pathway of carbon flux in hydroponic systems. Finally, this research involved the first evaluation of the importance of microbial inoculum in CELSS.

METHODS

Plant Growth Systems

Four, replicate nutrient delivery systems were used in the research. Each system consisted of the following elements: 1) a plant growth tray (29 cm^2), 2) a nutrient solution reservoir (29 cm x 8 cm x 9 cm), and 3) a solution collecting tray constructed out of 1/4" thick polyvinylchloride (PVC). Plant growth trays contained nine channels separated by PVC inserts. Solution was gravity fed through sections of Masterflex neoprene tubing (11.11 mm O.D., 7.94 mm I.D) connected to the nutrient solution reservoir and attached to separate inlets at the back of each channel. Solution from the collecting tray was pumped back to the reservoir through similar tubing using a bellows-type metering pump (Gorman-Rupp Industries, model 14251-007).

Plant growth systems were located on a benchtop within a modified environment. Two high pressure sodium (HPS) lamps (Lucalox 400 watt bulbs) were placed 70 cm above the tops of the plant growth trays. A 20 h light:4 h dark diurnal cycle was maintained during all growouts. While direct measurements of light intensity were not taken, the instantaneous photosynthetic photon flux was estimated at between 500-690 mol m^{-2} d^{-1} based on values for similarly designed benchtop plant growth systems at KSC (Ray Wheeler, pers. comm.). Based on a 20 hr light period, this instantaneous light intensity corresponds to an integrated PPF of 36-50 mol m^{-2} d^{-1}. Relative humidity (60-66%), solution pH (5.8 pH units), liquid level (total solution volume/system of 2 L) were maintained using computer contol. Air temperature in the

plant growth region ranged from 23-27° C during the light period, and decreased to approximately 20° C at night. Estimated average daily temperature was 24.5° C. Based on Bruce Bugbee's wheat maturity formula (pers. comm.), plants were harvested after 63 days (i.e. - 62 day life cycle for continuous lighting at 23° C, add 1 day for each 30 min reduction of photoperiod, substract 1 day for each 0.2° C increase in temperature).

More complete details of the plant growth systems and all methodologies used in this research are presented in Garland (1991).

Cultural Techniques

Two types of nutrient solutions were used: 1) inorganic-based modified Hoagland's solution (Hoagland and Arnon 1938, Mackowiak et al. 1989), and 2) a plant leachate-based solution with inorganic admendments. Hoagland's solution was prepared from stock solutions of separate inorganic salts as outlined in Table 1. All stock solutions were prepared using deionized water filtered through a 0.2 micron pore size Supor-200 membrane filter (Gelman) and autoclaved before use. Plant leachate was prepared by soaking 50 g of oven-dried (90° C) wheat residue (9 parts straw to 1 part root, cut into approximately 5 cm sections) in 1 L of deionized water for 3 hr at 25° C. Wheat residue was obtained from KSC-Biomass Production Chamber (BPC) growouts. Leachate was prefiltered through a Whatman GF/C filter and filter sterilized by passage through a 0.2 micron pore size Gelman Versacap filter. A 10% leachate solution was admended with stock solutions to approximate the chemical composition of modified Hoagland's solution (Table 2). Admendments were based on chemical analysis of similarly prepared wheat leachate (Garland and Mackowiak 1990). The relative contribution of leachate and admendments to the total supply of different elements in the leachate-based nutrient solution are reported in Table 3.

Replenishment of nutrient solutions over the course of plant growth was performed based on monitoring of the electrical conductivity of the nutrient solution. An appropriate volume of replenishment solutions (Table 4) were added to reservoirs when conductivity dropped below 1000 uS/cm to increase the conductivity to approximately 1500 uS/cm. The chemical makeup of replenshment solutions was developed by KSC-CELSS horticulturalists by estimating the relative utilization of different nutrients by wheat. The rate of replenishment is most intensive (approximately every 3 days) during the 3rd-7th week of plant growth (Figure 1).

Thirteen wheat seeds (*Triticum aestivum* cv *Yecora roja*) surface sterilized with 10% bleach were planted per channel. Seeds were supported by two strips of polyurethane foam, and moistened during seedling development by Versapor membrane filter "wicks" in contact with nutrient solution at the bottom of the channels. Clear plexiglass covers were placed over the plant growth trays for a 3 day germination period. The membrane wicks were removed after 14 days when a continuous root mat had formed on the bottom of the channels.

Microbial Decontamination and Inoculation

This research compared inocula which might arise from different approaches to controlling microbial populations in CELSS. Microbes which survived antimicrobial treatments of seeds and systems (see below) simulated the type of microbial community which would develop using a decontamination approach in CELSS. This treatment will be termed "uninoculated" in following discussion. It is important to realize that the potential diversity of microbes within the uninoculated systems in this study is much greater than that expected with similar treatment in a closed system like CELSS since these experimental systems were only partially closed. Microbes could have entered the system during the growout around the base of plants or during sampling procedures (see below).

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A second approach to controlling microbial populations in CELSS is inoculation of the system with organisms adapted for growth under environmental conditions within plant growth systems. An inoculum for this research was prepared from two separate environmental samples: 1)wheat roots collected from Hanover County, Va., and 2) sediment and rocks from the South Fork of the Rivanna River north of Charlottesville, Va. These habitats were selected because the hydroponic system represents a flowing water system containing wheat roots. A microbial suspension was collected from each sample type by shaking in 0.1% sodium hexametaphosphate. Separate 125 ml Erlenmeyer flasks containing 50 mls of 10% leachate were inoculated with 5 ml of suspensions and shaken at 100 rpm for 1 or 4 days. The four different culture types (river/1 day, river/4 day, rhizosphere/1 day, rhizosphere/4 day) were concentrated by centrifugation, treated with glycerol, and stored at -70° C in glass vials (Gherna 1981). Frozen cultures were prepared so that replicate inocula could be produced for different plant growouts. The 1 or 4 day cultivation step was performed to enrich for microbes capable of degrading leachate organics, and to produce cells in log phase which show greater survival when frozen (Gherna 1981).

Prior to each of the three growouts, a subsample of vials were thawed, and 0.1 ml of each type of culture (0.4 ml total) was added to a flask containing 50 mls of sterile 10% leachate solution. The flask was shaken as above for 24 hr, at which time 5 ml of suspension was removed and added to the "inoculated" systems.

Sampling

Nutrient solution was sampled through a serum stopper located 2 cm above the base of each solution reservoir using a 1", 18 gauge needle attached to a 60 ml plastic syringe. Sampling was performed at day 0, 1, 2, 4, 8, 16 , 21, 34, 46,and 63.

The root mat contained within a single channel could be removed in one piece

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without any cutting of root material. Sampled root mats were excised from the shoots, and sectioned transversely into 1-cm lengths for subsequent analyses (see below). Roots were sampled four times over the life cycle of wheat: 1) late tillering at day 21, 2) flowering stage at day 34, 3) seed fill stage at day 46, and 4) harvest stage at day 63.

Surfaces within the nutrient delivery system were sampled concurrently with root material. Rectangular chips (2.5 cm by 1 cm) of 1/8" PVC were incubated in the solution reservoirs and removed over the course of the plant growout to estimate microbial abundance and activity on PVC surfaces within the nutrient delivery system. Chips were placed in holders contructed out of PVC so that that the large chip "faces" were vertically oriented 1 cm above the bottom of the solution reservoirs. One-centimeter sections cut from the tubing between the pump and and nutrient solution reservoir were sampled to estimate microbial abundance and activity on the inside of tubing walls.

Sampling at days 21, 34, 46, and 63 was always performed two days after replenishment. This approach was utilized so that short term effects in microbial communities caused by pulse addition of organic material within the replenishment solutions were ignored. While these effects are probably of interest, the goal of this research was to evaluate temporal effects in microbial communities from the point of view of a wheat life cycle rather than replenishment cycles.

Measurements

Plant biomass from sampled channels (days 21, 34, 46, 63) was separated into root, shoot, and head fractions. Dry weight estimates were obtained after drying overnight at 90° C. Total root weight/channel was estimated by taking the mean dry weight of three 1-cm root sections, and multiplying by the total number of sections.

Plant biomass in unsampled channels remaining at harvest (5/system) was also separated into root, shoot, and head fractions, dried, and weighed as above. After heads were dried, seeds were separated from the chaff, redried, and weighed.

Bacterial abundance was determined using the acridine-orange (AO) method with epifluorescence microscopy (Hobbie et al. 1977). Suspensions of rhizosphere microoganisms were prepared by shaking root sections in 0.1% sodium hexametaphosphate solution containing glass beads (5-mm diameter) for three minutes). PVC and tubing samples were dislodged from surfaces by scraping with a razor blade. Bacterial suspensions from all habitats, including nutrient solution samples, were sonicated for 30 sec at 100 W using a Fisher Sonic Dismembrator MOdel 300. The sonication step dispersed aggregates of cells, thereby reducing the variation among cell counts (Velji and Albright 1986).

Microbial activity was assayed by measuring the turnover of a mixture of ^{14}C-labelled amino acids (NEC-445 Amino Acid Mixture, NEN Research Products). The specific activity of the mixture was 2.23 uCi/ugC, which, assuming an average molecular weight of the amino acids in the mixture of 150, is equivalent to 334 uCi/umol. The tracer method of Williams and Askew (1968) was utilized using a concentration of 0.04 nCi/ml, or 3.33 nM amino acid. Assimilation was defined as the radioactivity associated with particulate fraction (filtration through 0.22 um pore size MF membrane filters). Respiration was defined as the radioactivity associated with CO_2 released after acidification of samples with 1 ml 15% HCl/10 ml of samples, and trapped using phenethylamine. Nutrient solution samples were incubated for 30-60 min. Root samples (1-cm sections) were incubated for 30 min in 5 ml of nutrient solution taken at the time of sampling and filtered through a Supor-200 (0.2 um pore size) filter. Four replicate samples (2 live and two killed) were analyzed for each sampling.

Microbial growth was estimated by estimating the rate of tritiated thymidine (^{3}H-TdR) incorporation into bacterial DNA using the method of Pollard and Moriarity (1984) as modified by Blum and Mills (1991). Four replicate samples from each habitat (5 ml nutrient solution, PVC chip, 1-cm tubing section, 1-cm root section cut in half longitudinally) were placed in small glass vials. Five ml of filtered (0.2 um pore size) nutrient solution was added to vials containing PVC chips, tubing sections, and root samples. ^{3}H-TdR (20 uCi) and cold thymidine were added to vials to yield a final concentration of 60 nm. Two of each set of four replicates were immediately fixed with buffered formalin (4% v/v final concentration). The remaining replicates were incubated at 25° C for 20 min, then similarly fixed.

Dissolved organic carbon (DOC) concentration in nutrient solution samples were estimated using the UV-assisted persulfate oxidation method with a Dohrman DC-80 Total Organic Carbon Analyzer. Nutrient solution samples were filtered through PTFE membrane syringe filters (0.22 um pore size) into acid washed borosilicate glass scintillation vials. Samples were acidified to pH 3.0 with 15% nitric acid and refrigerated at 5° C until analyzed.

Data Analysis

Data were analyzed by repeated measures multivariate analysis of variance (MANOVA) using the SPSS-PC statistical software package. A 2 x 2 full factorial model was employed with inoculation (inoculated vs uninoculated) and solution type (leachate vs Hoagland's) as factors. The number of replicates was 3, corresponding to the number of growouts. Complete tables of MANOVA results are not reported; probability values for statistically significant effects ($p < 0.05$) are reported in the results section.

RESULTS

Plant Biomass

Neither the solution nor inoculation treatments had a significant effect on plant growth (Figures 2 and 3). Total harvested dry wt per channel was approximately 8 g seed, 8 g shoot, and 2 g root for all treatments (Figure 3). Total plant dry wt on an area basis (channel size of 67.6 cm^2) was approximately 2700 g/m^2, or 43 g/m^2/day. Seed production on an area basis was approximately 1200 g/m^2, or 19 g/m^2/day.

Wheat growth within these systems was similar to or greater than that in BPC growouts at KSC. Total biomass production rates for BPC growouts ranged from 27-39 g m^{-2} d^{-1}, while seed yields were 8-15.6 g m^{-2} d^{-1} (Wheeler et al. 1990). Using the estimated integrated PPF values of 36-50 mol m^{-2} d^{-1}, growth rates were 80-100% of those reported by Bugbee and Salisbury (1988). These comparisons indicate that not only was wheat growth in LBS similar to that in HBS in this study, but that average growth rate in all systems was similar to that expected for near-optimum, controlled environment conditions.

Dissolved Organic Carbon

DOC concentration in LBS was almost five times higher than that in HBS at day 0 (240 mg/L vs 50 mg/L, respectively), but rapidly declined to roughly equivalent values by day 7 (60-75 mg/L). This decline reflects mineralization of leachate organics by microbial populations within the plant growth system, and/or significant adsorption of organics to surfaces within the NDS (Figure 4). DOC concentrations in LBS rose slightly during the middle phases of plant growth as replenishment (and concomitant additions of leachate organics) increased, but the effect was inconsistent among growouts. DOC concentrations were consistently higher in uninoculated LBS systems vs inoculated LBS at day 63 for all three growouts. Average concentration in inoculated LBS systems at harvest was similar to that in HBS systems (approximately

75 mg/L), but average concentration in uninoculated systems was over twice as high. These results indicate that accumulation of leachate organics in hydroponic systems is not significant, but only if systems are inoculated with microbial communities selectively enriched for growth on leachate. The effect of inoculation on carbon mineralization will be discussed further below.

Microbial Abundance and Activity

Habitat Comparison

The rhizosphere habitat dominated both microbial abundance and production (^{3}H-TdR incorporation) in hydroponic systems at each of the four dates when samples from all four habitats were obtained. Root-associated microbes accounted for 60-90% of the total cells (Figure 5) and 60-95% of the total ^{3}H-TdR incoporation on a per system basis (Figure 6). Non-rhizosphere cells comprised a larger percentage of the total abundance and production in LBS compared to HBS, and at sampling dates during the middle phases of plant growth.

The greatest proportion of non-rhizosphere ^{3}H-TdR incorporation (up to 30%) was associated with PVC surfaces while the greatest proportion of non-rhizosphere cells were associated with tubing surfaces. The higher activity/cell on PVC surfaces suggested by this relationship is supported by lower calculated cell turnover times (production / biomass) for PVC surfaces (less than a day) compared to tubing surfaces (up to 8 days). The difference in turnover time between surface type was most pronounced at the later sampling dates as cell density continued to increase on tubing surfaces without proportional increases in ^{3}H-TdR incorporation (Figures 7 thru 10). Cell density was similar between surface types at day 21 (10^7/cm^2), but over two orders of magnitude greater on tubing surfaces by day 63 (Figures 7 thru 10).

While considerable variation in values existed among samples, habitats can be ranked based on average cell turnover time as follows: nutrient solution > tubing >

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rhizosphere > PVC (Table 5). The long cell turnover times reported for nutrient solution samples (up to 5600 days) indicate very slow growth of suspended cells.

Rhizosphere

The density of microbial cells (expressed as the number per g dry wt root) associated with plant roots remained relatively constant from day 21-63 (5-9 x 10^{10}), but the total abundance of cells increased with time (p=0.001) as the total root weight increased (Figure 11). These results indicate that the limiting factor to cell density in the root zone is the amount of root surface available for colonization. However, cell densities (p=0.014) and total cell abundance (p=0.084) were slightly higher in LBS compared to HBS, indicating that additions of carbon in some way increases the "carrying capacity" of the rhizosphere.

Microbial growth as estimated by ^{3}H-TdR incorporation/dry wt root declined 5-10 fold from day 21 to the middle stages of the plant life cycle, but increased again at day 63 (Figure 12) (statistical tests of these data were not feasible since the number of replicate data points was 1 for day 21, and 2 for day 34, 46, and 63). Increased amino acid turnover time, another indicator of decreased metabolic activity, was also observed at day 46 in some treatments (Figure 13). This temporal pattern suggests reduced availability of root-derived carbon during the period of seed fill. This effect is supported by the consistent stimulation in ^{3}H-TdR incorporation in the rhizosphere of LBS systems versus HBS at day 46 (remembering that the leachate treatment can be viewed as a carbon-supplementation treatment).

Nutrient Solution

Measurements of microbial abundance and activity in the nutrient solution were marked by significant variation among growouts, resulting in few statistically significant temporal or experimental effects. Abundance, growth (^{3}H-TdR incorporation), and

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metabolic activity (amino acid turnover rate) were greater in the nutrient solution of LBS versus HBS for growouts 1 and 2, but equivalent or less in LBS compared to HBS for growout 3 (Figures 14-15, Table 6). In fact, abundance, growth, and activity in the nutrient solution was much less in all systems during growout 3 compared to growouts 1 and 2.

Consistent temporal ($p = .001$) and solution ($p = 0.011$) effects were apparent in microbial abundance. In general, the number of cells/ml reached a peak in all systems (approximately 10^8/ml) during the first week of plant growth, and declined afterward to values between 10^6-10^7/ml (although density in LBS increased during the middle phases of plant growth for growouts 1 and 2). The only consistent difference between solution type was the greater densities in LBS at days 1 and 2 when DOC concentrations were very high.

Nutrient Delivery System Surfaces

As mentioned above, the density of microbial cells colonizing neoprene tubing surfaces (up to 10^9 cells/cm^2) was up to two orders of magnitude greater than that on PVC surfaces (10^7 cells/cm^2), but the average estimated cell turnover rate of cells associated with PVC surfaces was over ten times faster that of cells associated with tubing surfaces. While cell density and ^{3}H-TdR incorporation on PVC surfaces remained relatively constant over time (neither time effect had $p < .05$) (Figure 16), both these parameters on tubing surfaces significantly increased with time ($p = 0.001$ for both) (Figure 17). Conversely, cell density ($p = 0.047$) and ^{3}H-TdR incorporation ($p = .007$) were significantly greater on PVC surfaces in LBS versus HBS, while no solution effect was found for either cell density or growth on tubing surfaces.

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DISCUSSION

This work represents the first replicated studies evaluating the coupling of CELSS-based plant growth systems and waste recycling systems for complete plant growouts. Results indicate that the soluble fraction of inedible wheat biomass can be *directly* incorporated into recirculating hydroponic systems. Inorganic nutrients within the leachate supplied the majority of the nutritional requirements of the wheat in this study. Organic material within the leachate does not appear to deleteriously affect plant growth. Rather, microbial communities associated with the hydroponic system rapidly mineralize the organic material, suggesting an important role for plant growth systems as sites of waste recyling within CELSS.

Incorporation of leachate in the hydroponic medium significantly reduced the quantity of inorganic nutrients added to plant growth systems from stock solutions. In other words, the use of leachate represents an effective method of nutrient recycling. Leachate provided the majority of all individual nutrients in the original leachate-based solution with the exception of iron (see Table 3 for specific percentages). Micronutrient requirements (with the exception of manganese) were completely supplied by leachate both in the original solution and in replenishments throughout the growout. Stock solution sources of macronutrient were reduced in leachate-based replenishment solutions (see Table 4). However, nearly twice the amount of replenishment solution was added to LBS versus HBS due to more rapid decreases in conductivity observed in LBS following replenishments (see Figure 1 for replenishment schedule). The effect of leachate on the response in conductivity could be related to binding of ions to organic films in the systems, microbial immobilization, or some other factor. Further research is necessary to determine the distribution of elements within the hydroponic system (e.g. plant, microbe, non-biologically bound) in leachate-based versus Hoagland-based systems. Despite the larger volume of replenishment solution added to LBS, the total amount of nutrients from stock

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solutions used in replenishment was lower in LBS versus HBS for most macronutrients (e.g. 50% for nitrogen, 63% for potassium, 45% for calcium, and 10% for magnesium). The present study indicates that nutrient concentrations were sufficient for adequate plant growth; more efficient replenishment schedules using leachate-based solutions will need to be based on further studies involving the monitoring of nutrient pools within the hydroponic system.

From a mass balance perspective, each LBS received between 650-750 mls of leachate solution for the entire growout (200 mls in original solution, 450-750 in replenishment solution). Between 32.5-47.5 g of inedible wheat biomass is neccessary to produce this much leachate (leaching density of 50 g biomass/ L water). The harvested inedible biomass from each system was approximately 50 g, meaning that the ratio of inedible biomass leached/inedible biomass produced was 65-95%. This percentage can be viewed as an index of the closure of the recyling loop. The lack of complete closure (i.e. - ratio < 1) is a result of nutrient imbalance in the leachate. The leachate was diluted to reach adequate levels of micronutrients, creating both the need for greater nutrient supplementation, and an "excess" of leachate. In an operational bioregenerative system, the leachate recycling loop could be fully closed by 1) using the "excess" leachate as nutrient media for other biological systems (e.g. - microbially-based reactors), or 2) reducing the micronutrient concentrations in the leachate so that more concentrated solutions could be used. Either strategy requires the use of other waste recycling streams (e.g. - human waste) in combination with leachate to produce a hydroponic media which provides all plant nutrient requirements.

An alternative configuration of the waste recycling "loop" in CELSS would involve separation of the waste treatment and plant growth systems. In this design, a separate leachate reactor would be used to perform a biological pretreatment step to remove organic material from the leachate prior to the addition of a largely inorganic

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feed stream to plant growth systems. While this research indicates that this step is not neccessary in order to detoxify or mineralize the organic fraction of leachate, a separate bioreactor may more effectively convert soluble organics into microbial biomass suitable for food production (i.e. - aquaculture). The effectiveness of a separate reactor lies not with increased production of microbial biomass, but with increased availability of the biomass to aquaculture systems. The majority of microbial biomass produced in hydroponic systems is associated with the root mat. Methods would have to be developed to transfer the carbon from the root zone since grazing of the root mat itself by fish could be detrimental to plant health. Biomass produced in a leachate bioreactor could be more readily harvested and transfered into aquaculture systems without affecting important processes within CELSS (i.e.- plant growth). Microbial biomass produced from a leachate bioreactor represents only a small fraction (< 3%) of the total amount of waste products available for food input to the aquaculture system (Garland 1989). Therefore, the costs involved with the operation of the bioreactor (e.g. - energy, volume) may exceed the potential benefits.

The addition of leachate organics to the hydroponic systems did not greatly alter the temporal or spatial patterns in microbial growth, activity, or abundance. Rhizosphere communities dominated both microbial production and abundance in both LBS and HBS. Addition of leachate did increase growth and abundance of bacteria associated with PVC surfaces, indicating carbon limitation in habitats removed from the rhizosphere in unsupplemented systems. The stimulatory effect, however, was only limited to periods of intensive replenishment. The dominant temporal pattern in microbial growth in the systems, a decline during the middle stages of the plant life cycle, appeared to be related to decreases in the release of root-derived carbon. This decline was observed in both HBS and LBS, although growth rates were consistently higher in the rhizosphere of LBS at day 46, indicating the development of carbon-limiting conditions in HBS at this time. The observed rise in

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bacterial growth in the root zone at harvest is counter to previous studies involving the pulse-labelling of wheat with $^{14}C-CO_2$, suggesting that the dominant source of root-derived carbon near the end of the plant life cycle was sensecent root cells (i.e. - root lysates), rather than recently fixed photosynthate (i.e. - root exudates). These data provide both the conceptual and empirical basis for a model of carbon flow in hydroponic system. This model is presently under development at KSC.

Total transfer of carbon from plant roots can be estimated by integrating bacterial production rates for all habitats over the plant life cycle in HBS. This calculation assumed 1) a conversion factor of 1.38×10^{18} cells/mole ^3H-TdR incorporated (Pollard and Moriarity 1984, Blum and Mills 1991), 2) an average cell biovolume of 0.2 μm^3, 3) an average C content/cell biovolume of 10^{-13} gC/μm^3 (Bratbak 1985), 4) a growth efficiency of 40% (theoretical yield of energy from glucose), and 5) that recycling of carbon in microbial communities was negligible. Based on these calculations, the loss of carbon from roots expressed as a percentage of NPP decreased from 5-8% at day 21 to approximately 3% at harvest. The higher estimates are based on the assumption that bacterial production increased linearly from day 0 to day 8, then remained steady until day 21. The lower estimate assumes a linear increase from day 0 to day 21. The high suspended cell density and DOC concentration during the early phases of the growout suggest high rates of carbon loss during the early phases of plant growth. On the other hand, total root mass available for microbial colonization was very small during the first week of plant growth. More intensive sampling of bacterial productivity during the early phases of plant growth is clearly necessary to resolve this question.

This estimate of carbon loss from roots is within the lower range of values for soil-cultivated wheat labeled with $^{14}C-CO_2$ (see introduction). This estimate obviously is highly dependent on the conversion factors used in the calculation. While determination of specific conversion factors for hydroponic systems might increase the

accuracy of the estimate, the likelihood that it is an overestimation of the actual carbon loss from roots is low since 1) average or conservative values for conversion factors were used, and 2)a significant fraction of the total habitat in systems (PVC surfaces within plant growth trays, tubing connecting solution reservoirs to plant growth trays) were excluded from the calculations. Significant release of carbon into systems from trays, silicone sealer, tubing, or other materials used in the construction of the system would be a cause of serious overestimation of carbon loss. Estimation of bacterial production in unplanted systems would help quantify non-root sources of carbon. However, it is unlikely that non-root sources of carbon were significant since more than 90% of bacterial production in the system was located in the rhizosphere. One would expect much greater activity on surfaces if they were the source of large quantities of organic material. Therefore, this study indicates that at least 3% of total NPP is released as organic material through the roots of wheat plants grown in hydroponic cultures, and levels in excess of 10% are likely for early periods of plant growth. If one assumes a constant loss of 6% of NPP for a plant growth system with wheat plants under continuous cultivation (i.e. - plants at all different stages of growth), the flux of carbon from roots represents approximately 25% of seed production. This estimate is based on Garland's mass balance model of carbon flux in CELSS.

The major conclusion to be drawn from the data for the purposes of the present study is that addition of soluble organic material of plant origin does not cause excessive growth of microorganisms, resulting in problematic "biofouling" of the system (e.g. - large biological oxygen demand resulting in anoxic conditions in the root zone, clogging of root mat pores, etc.). Rather, the microbial communities in hydroponic systems appear to rapidly mineralize the additional organic material with only relatively minor changes in microbial abundace, growth, or activity. Inoculation of the systems with microorganisms selectively enriched for growth on leachate organics is neccessary to prevent the accumulation of the more recalcitrant organic fraction.

Accumulation of DOC in uninoculated LBS versus inoculated LBS was only observed at the day 63 sampling. If one assumes that the effect of inoculation was the selective enrichment of the enzymatic capacity necessary to degrade a recalcitrant fraction of leachate organics, a gradual elevation in DOC in uninoculated vs inoculated systems with time might have been expected. The fact that this effect was limited to day 63 may be related to the fact that carbon did not limit microbial growth in LBS until the rate of replenishment declined (after approximately day 50). Differences in catabolic potential with inoculation would not have been apparent as excess carbon (probably the most recalcitrant molecules) accumulated, both in solution and bound to surfaces (estimates of bound carbon were not obtained, but a brownish color was apparent on surfaces within LBS, particularly on tubing surfaces and root material). When carbon became limiting after day 50 and the microbial communities turned to the more recaltitrant molecules as carbon sources, the increased catabolic potential of the inoculated communities would have become apparent. This entire argument could be tested by 1) more intensive temporal sampling when replenishment rate decreases, 2) characterization of the DOC into labile and recalcitrant fractions, and 3) analysis of potential carbon accumulation on surfaces.

The results of the present study reveal several areas of future research related to the microbial ecology of plant growth systems in CELSS. First, microbial biofilms which develop on hardware surfaces within the nutrient delivery system represent a potentially significiant site of microbial abundance and activity in the system which has been largely ignored. The microbial communities associated with tubing surfaces showed more distinctive temporal and experimental responses than those associated with PVC surfaces. The present study does not allow for the separation of the effects of flow and surface type. However, the significant differences between the two habitats in the present study indicate that both factors should be evaluated in future studies.

These results also indicate that while the nutrient solution is the most easily sampled habitat within plant growth systems, it is not an effective site to monitor microbial dynamics within the system. Microbial abundance, activity, and growth in the nutrient solution are all relatively insignificant compared to that in other habitats which show high variability among growouts. This study indicates that biofilms on hardware surfaces represent a much more effective site of microbial monitoring because they 1) constitute a larger fraction of total microbial activity and abundance in the system, and 2) possess high cellular turnover rate, and, therefore, may reflect recent changes in factors affecting microbial activity.

This study also represents the first application of techniques measuring microbial function (growth and metabolism) in CELSS-based plant growth systems. Further work characterizing the role of microbial communities in both the carbon and nitrogen cycle (e.g. - denitrification) are needed to fully understand the flux of materials within CELSS.

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TABLE 1. Chemical composition of modified Hoagland's solution.

Stock Solution	g/L	[stock]	ml stock/L	[working]
$Ca(NO_3)_2$	236.15	1M	2.5	2.5mM Ca 5.0mM N
KNO_3	101.11	1M	2.5	2.5mM K 2.5mM N
$MgSO_4$	246.48	1M	1.0	1.0mM Mg 1.0mM S
KHP_2PO_4	136.09	1M	0.5	0.5mM K 0.5mM P
Fe-EDTA $FeCl_3$ h'EDTA*	 2.42 2.43	 8.96mM Fe 	11	 60μM Fe 0.18mM Cl
Micronutrients H_3BO_3 $MnCl_2$ $ZnSo_47H_2O$ $CuSO_45H_2O$ $(NH_4)_6Mo_7O_{24}^4$ $.4H_2O$	 1.07 0.67 0.083 0.03 g 0.0642 	 17.27mM B 3.36mM Mn 0.29mM Zn 0.12mM Cu 0.04mM Mo 	1.1	 19.00μM B 3.70μM Mn 0.32μM Zn 0.13μM Cu 0.04μM Mo

* - acid form of EDTA

TABLE 2. Composition of leachate-based nutrient solution.

Stock Solution	ml/L soln.
$Ca(NO_3)_2$[1]	1.25
$MgSO_4$[1]	0.42
Fe-EDTA[1]	11
$MnCl_2$[2]	0.28
10% Leachate[3]	100

[1] - stock solutions described in Table 1

[2] - 0.67 g $MnCl_2$/L

[3] - Leachate prepared by mixing 1 part leachate (extract of 50 g wheat straw and root mixture soaked in 1L deionized water for 3 hr) with 9 parts deionized water.

TABLE 3. Sources of chemicals in 10% leachate solution.

Stock	Nutrient[1]										
	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	Mo	B
Stocks	100			50	10	5.6	0.5				
Leachate[2]	80	26	300	39	14	0.3	0.15	0.03	0.02	n.d.	0.3
% Leachate[3]	44	100	100	44	58	5	75	100	100	---	100

[1] - concentration of nutrient in ppm

[2] - Estimates based on chemical analysis of similarly prepared leachate by Garland and Mackowiak (1990)

[3] - % of nutrient supplied by leachate

TABLE 4. Replenishment solutions. Solutions A and B prepared and stored separately, but added in equal volumes to systems depending of solution conductivity (see text).

SOLUTION A		SOLUTION B	
Stock*	ml/L soln.	Stock*	ml/L soln.
HOAGLAND'S SOLUTION			
KNO_3	240	$CA(NO_3)_2$	30
KH_2PO_4	30	$MgSO_4$	20
Micronutrients	40	Fe-EDTA	88.75
LEACHATE SOLUTIONS			
KNO_3	20	$CA(NO_3)_2$	24
KH_2PO_4	20	$MgSO_4$	8
$MnCl_2$	8	Fe-EDTA	140
		10% leachate	600

* - Stock solutions prepared as described in Tables 1 and 2.

TABLE 5. Descriptive statistics of cellular turnover time for habitats.

HABITAT	MINIMUM	MAXIMUM	MEAN	MODE
NUTRIENT SOLUTION	7	5600	298	80
RHIZOSPHERE	1	725	40	8
PVC SURFACES	0.1	10	2	1.2
TUBING SURFACES	0.3	74	20	9.6

TABLE 6. Uptake of ^{14}C-labelled amino acid mixture by nutrient solution samples.

GROWOUT	COMBINED[1]	HOAGLAND[2]	LEACHATE[3]
	TURNOVER TIME (HR)[4]		
1	2.97 (1.35)	3.38 (1.78)	2.58 (0.55)
2	2.96 (1.89)	3.42 (2.41)	2.55 (1.12)
3	9.81 (10.11)	7.23 (7.33)	12.39 (11.74)

[1] - Mean and std. dev. of values for all systems
[2] - Mean and std. dev. of values for hoagland-based systems
[3] - Mean and std. dev. of values for leachate-based systems
[4] - Turnover rate based on both assimilated and respired ^{14}C-labelled amino acid mixutre
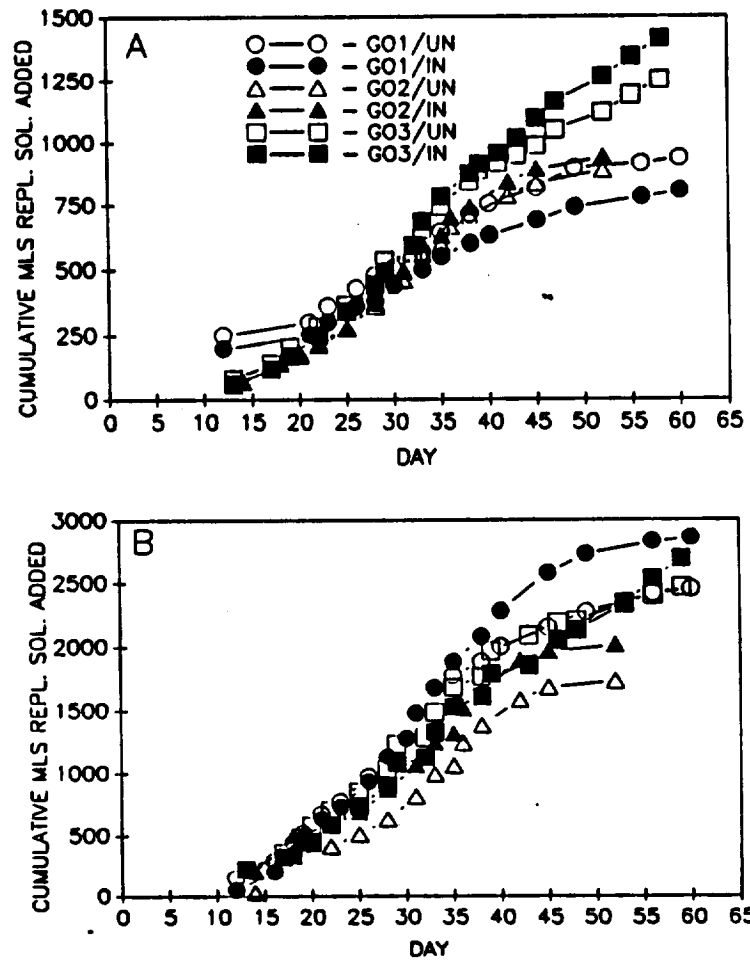
FIGURE 1. Replenishment schedule for a) Hoagland-based and b) leachate-based systems for all three plant growouts.
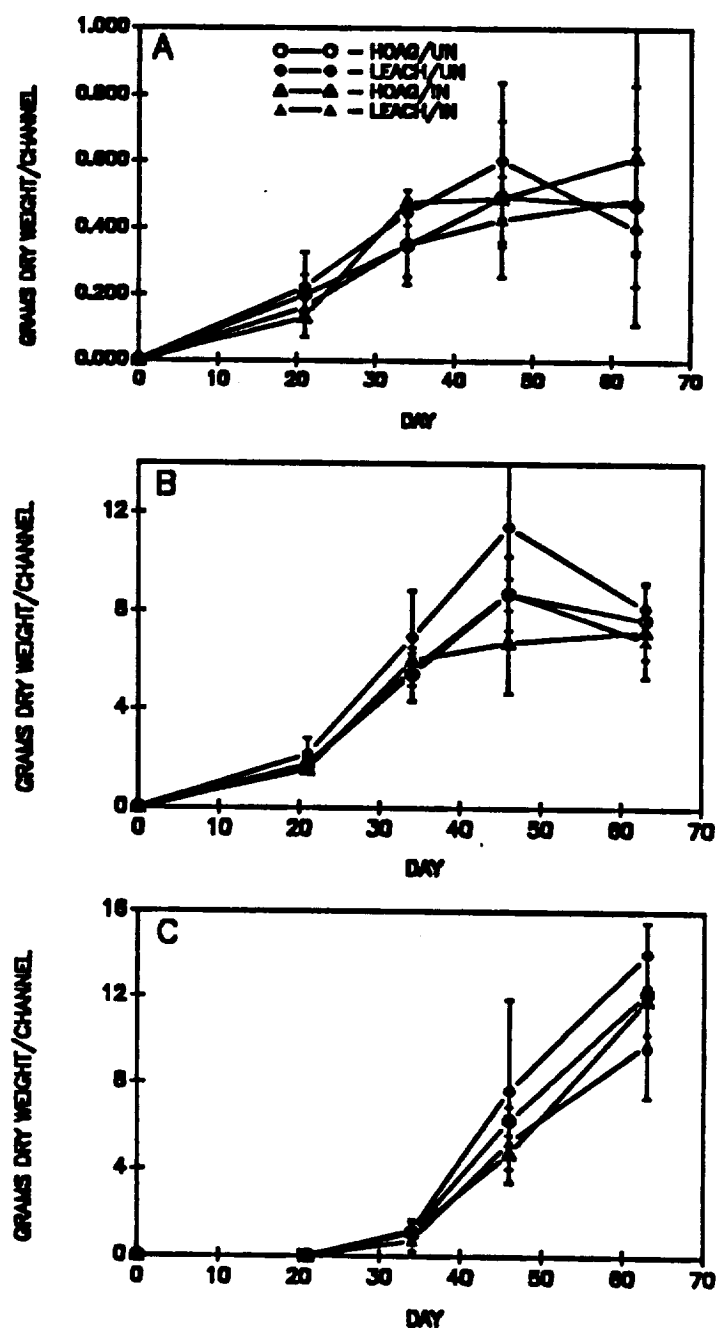
FIGURE 2. Total dry weight of A) root, B) shoot, and c) head biomass in sampled channels. Means and std. dev. based on data from three replicate growouts.
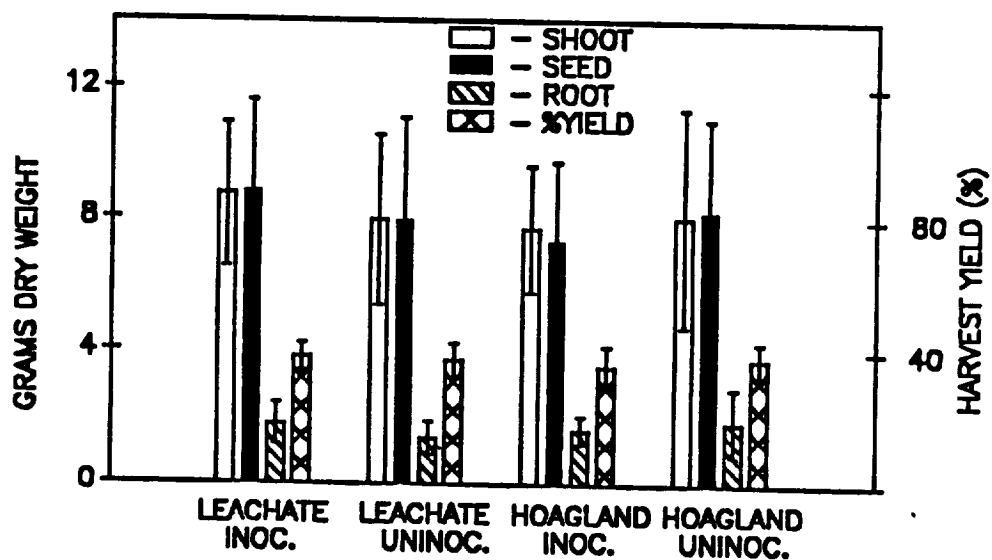
FIGURE 3. Plant biomass (dry weight) in channels not sampled for microbial activity and abundance remaining at harvest. Values based on 5 channels for each of three growouts (N=15). % yield determined by dividing seed weight by total plant weight.
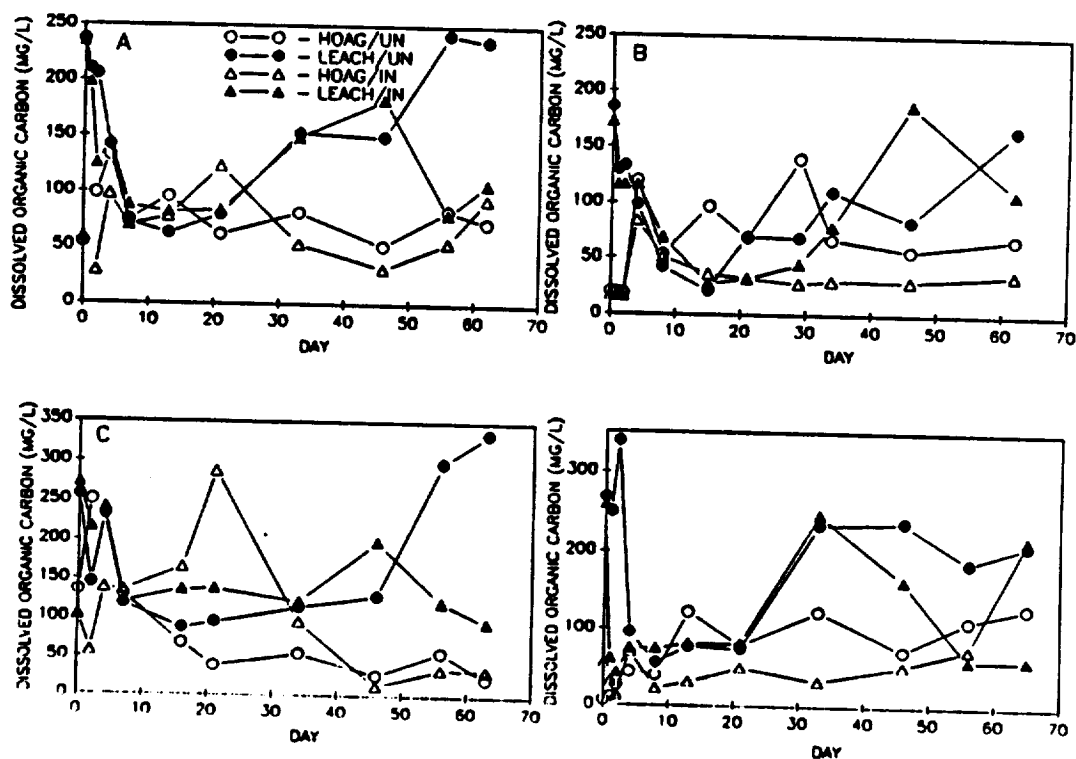
FIGURE 4. Dissolved organic carbon concentration in nutrient solution samples for days 0-63. Numbers represents A) mean value for all three growouts, and B-D) values for growouts 1-3, respectively.
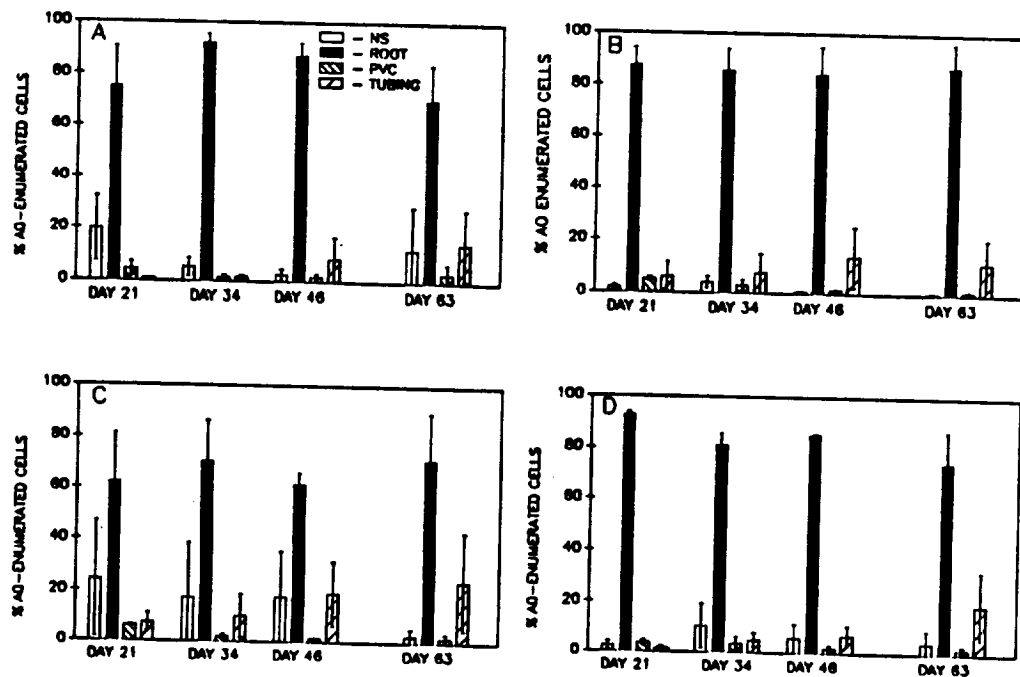
FIGURE 5. Percent of total estimated AO-enumerated cells per system in various habitats in A) uninoculated hoagland systems, B) inoculated hoagland systems, C) uninoclated leachate systems, and D) inoculated leachate systems. Values for days 34 and 63 represent means and std. dev. of all three growouts, values for days 21 and 46 for growouts 2 and 3 only.
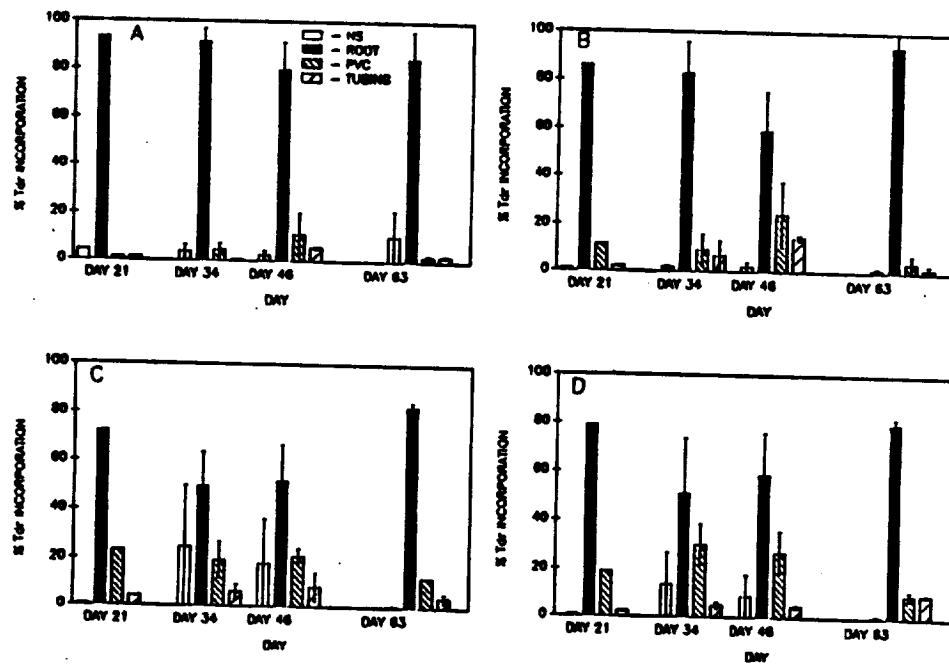
FIGURE 6.   Percent of total 3H-TdR incorporation into cold TCA insoluble
macromolecules per system in various habitats in A) uninoculated hoagland systems,
B) inoculated hoagland systems, C) uninoculated leachate systems, and D) inoculated
leachate systems. Values for days 34, 46, 63 represent means and std. dev. for
growouts 2 and 3, values for day 21 represent values for growouts 3 only.

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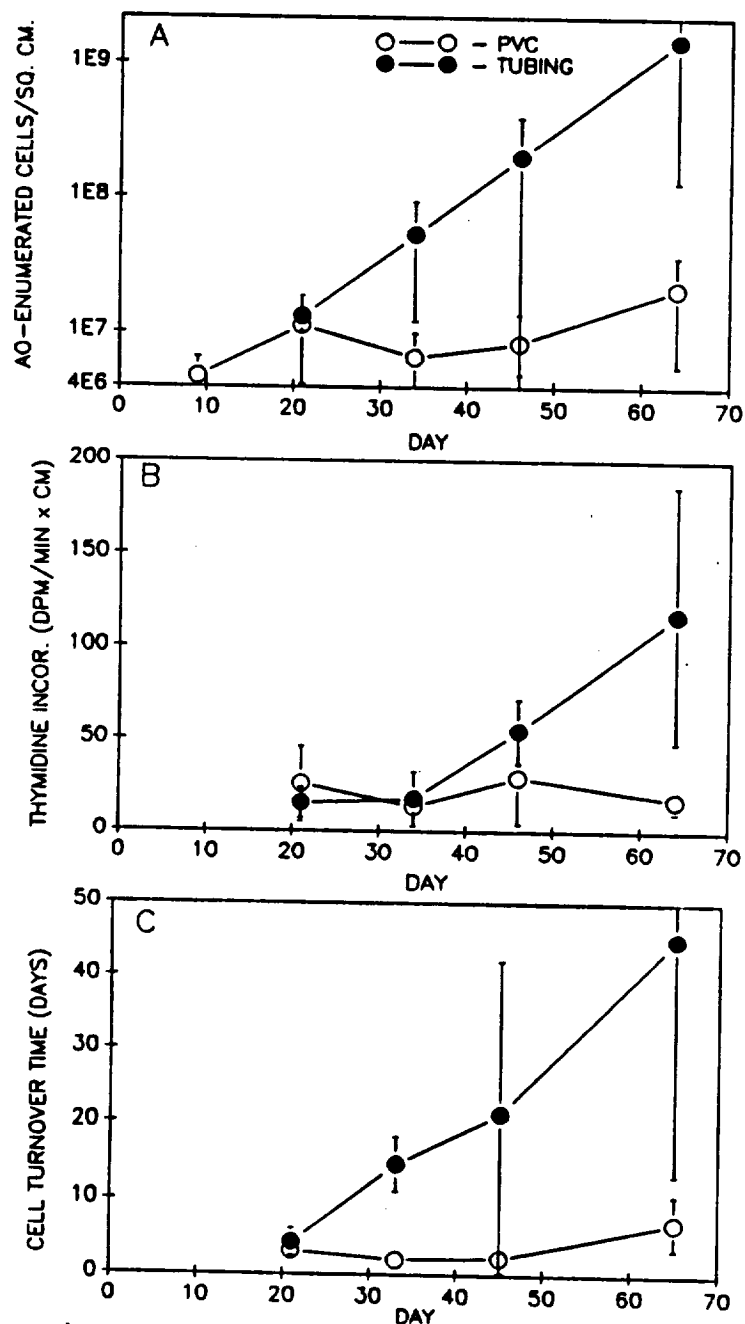
FIGURE 7. Comparison of A) AO-enumerated cell density/cm2, B)3H-TdR incorporation into cold TCA insoluble macromolecules, and C) cell turnover time between PVC and tubing surfaces in uninoculated hoagland-based systems. Values for days 34 and 63 represent means and std. dev. of samples from all three growouts, values for day 21 and 46 represent means and std. dev. of values for growouts 2 and 3 only.
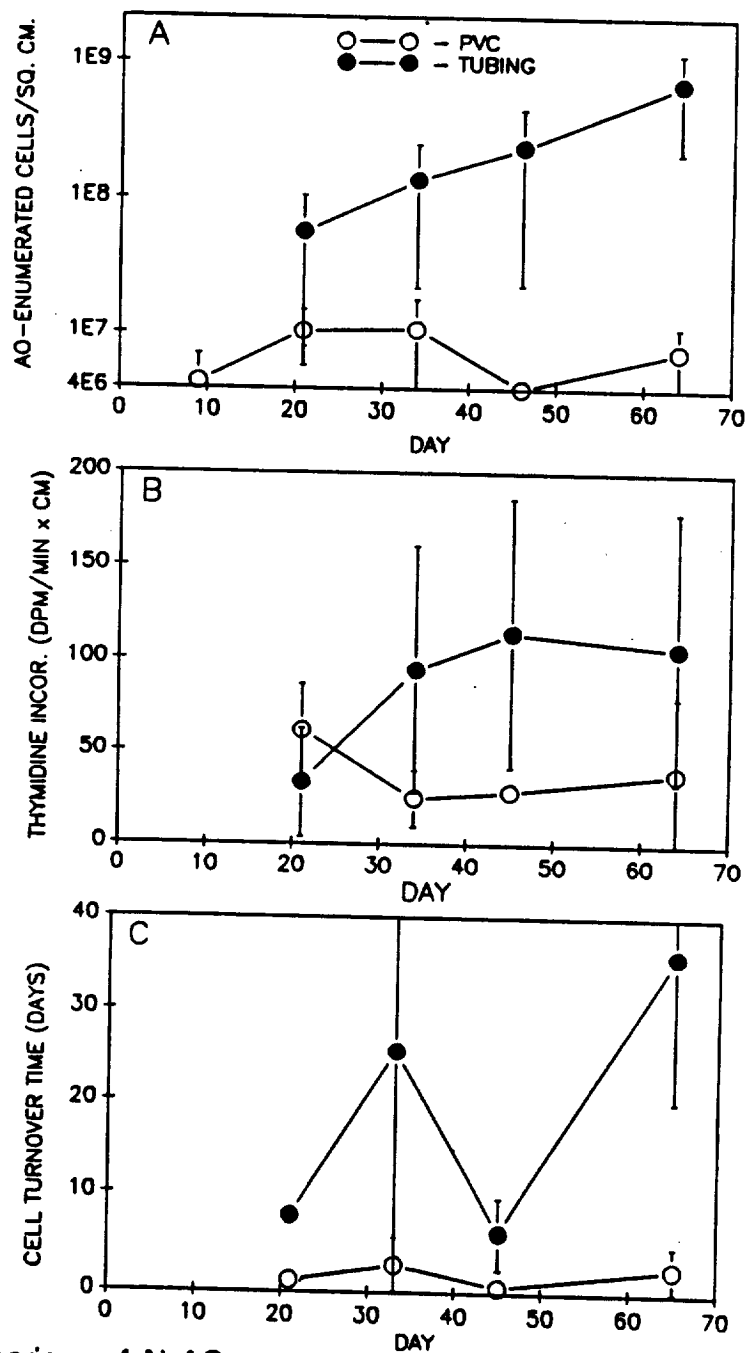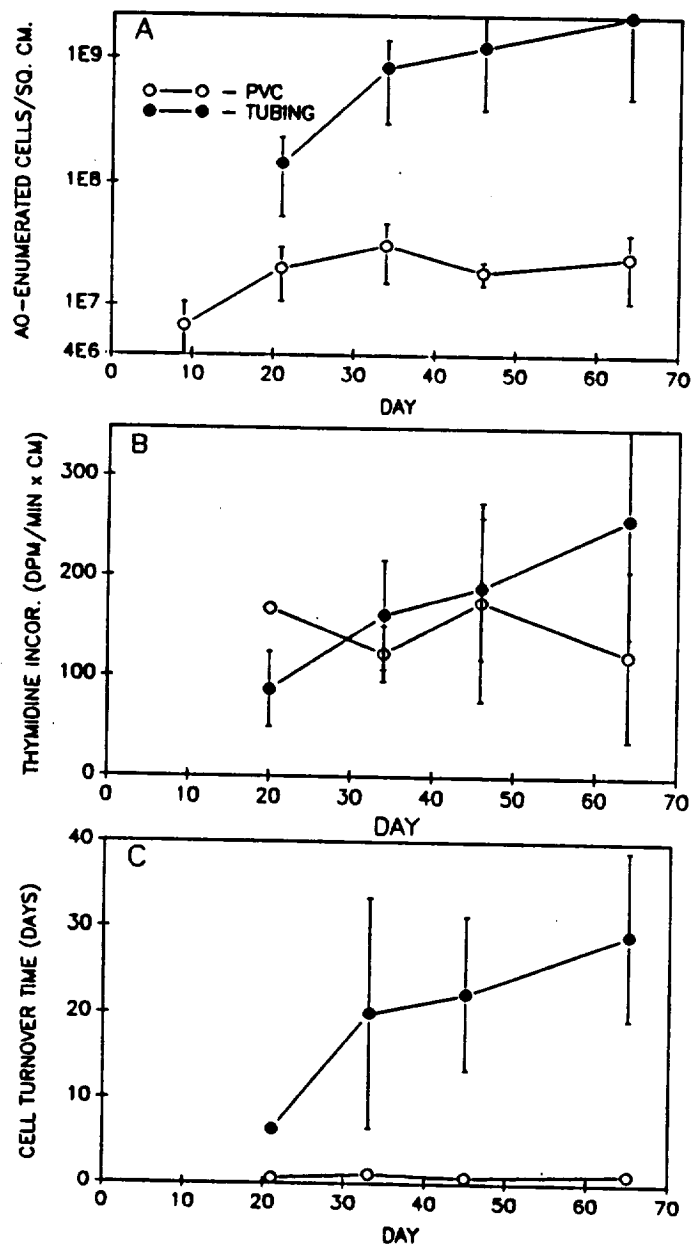
FIGURE 8.   Comparison of A) AO-enumerated cell density/cm2, B)3H-TdR incorporation into cold TCA insoluble macromolecules, and C) cell turnover time between PVC and tubing surfaces in inoculated hoagland-based systems. Values for days 34 and 63 represent means and std. dev. of samples from all three growouts, values for day 21 and 46 represent means and std. dev. of values for growouts 2 and 3 only.

FIGURE 9. Comparison of A) AO-enumerated cell density/cm2, B)3H-TdR incorporation into cold TCA insoluble macromolecules, and C) cell turnover time between PVC and tubing surfaces in uninoculated leachate-based systems. Values for days 34 and 63 represent means and std. dev. of samples from all three growouts, values for day 21 and 46 represent means and std. dev. of values for growouts 2 and 3 only.
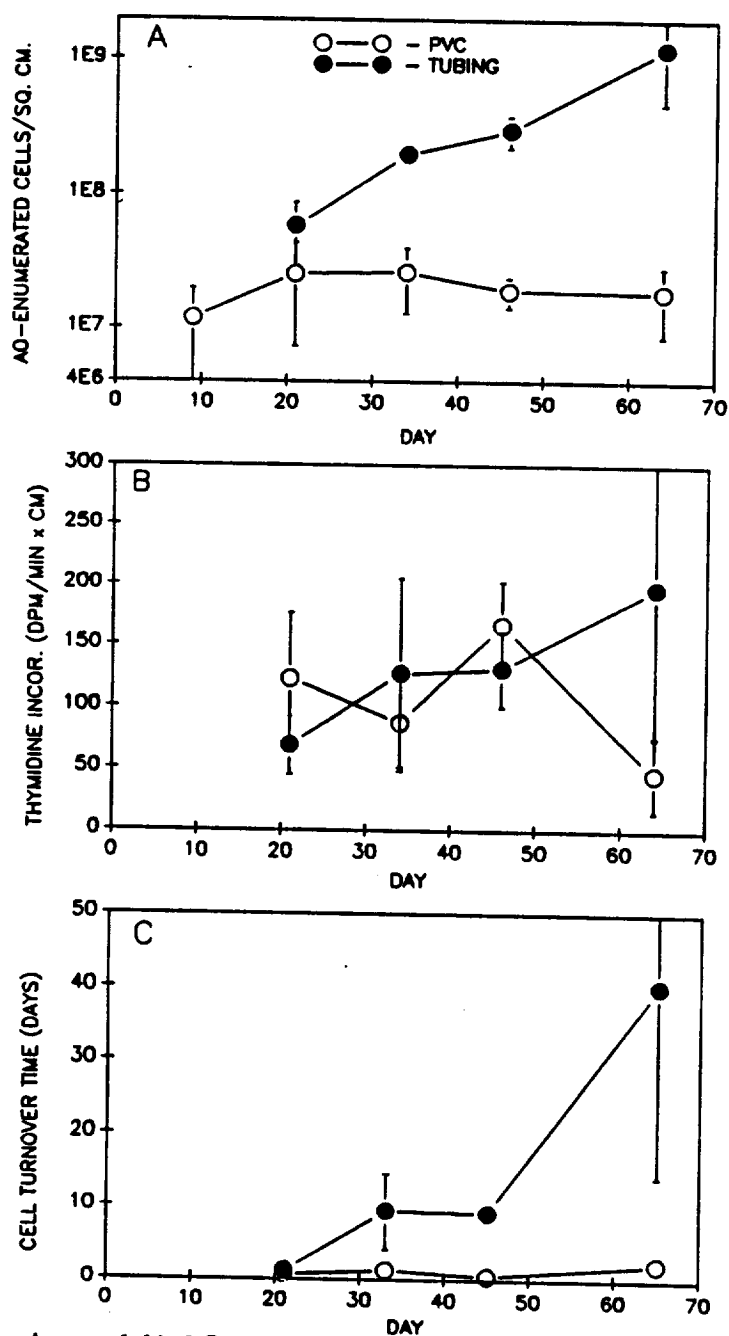
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FIGURE 10. Comparison of A) AO-enumerated cell density/cm2, B)3H-TdR
incorporation into cold TCA insoluble macromolecules, and C) cell turnover time
between PVC and tubing surfaces in inoculated leachate-based systems. Values for
days 34 and 63 represent means and std. dev. of samples from all three growouts,
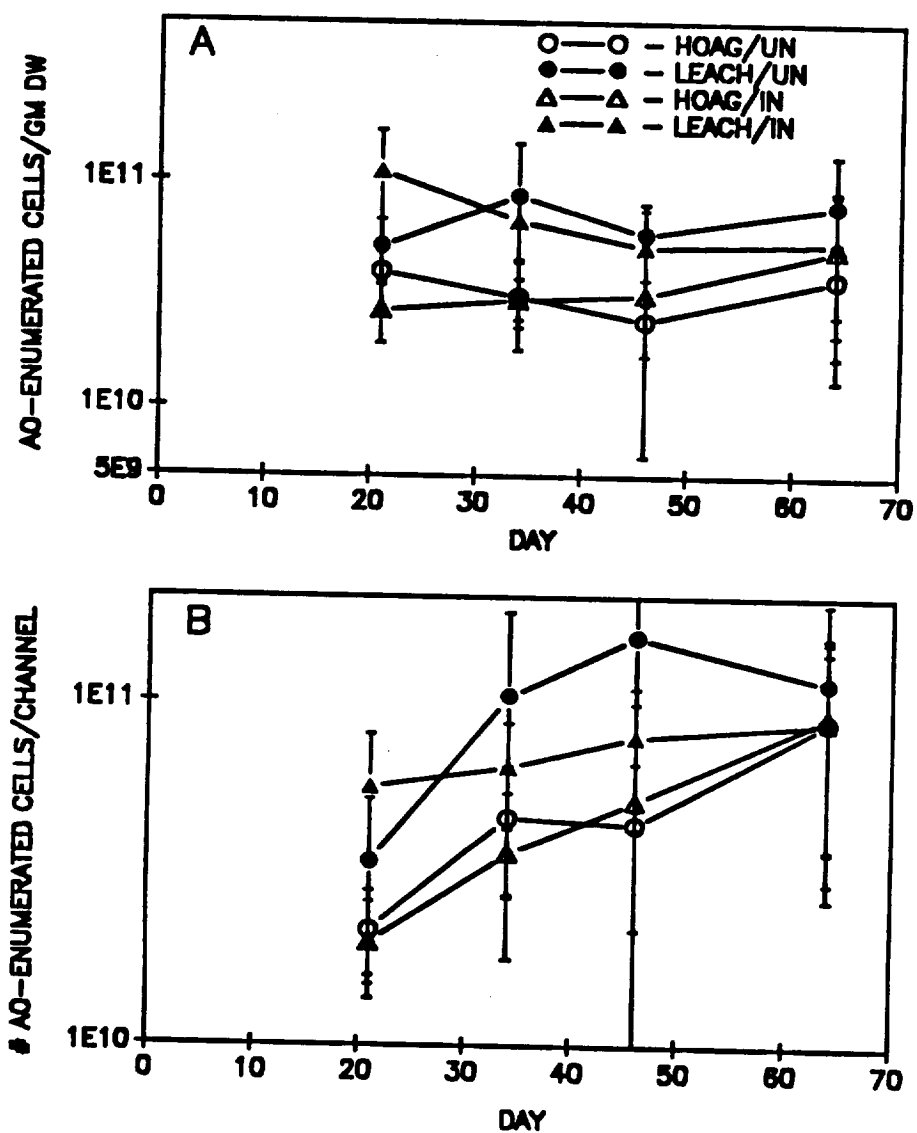values for day 21 and 46 represent means and std. dev. of values for growouts 2 and 3
only.

FIGURE 11. Acridine orange-enumerated cell counts in the rhizsophere expressed on a A) mg dry weight root, and B) whole channel basis. Means and std. dev. based on data from three replicate growouts.

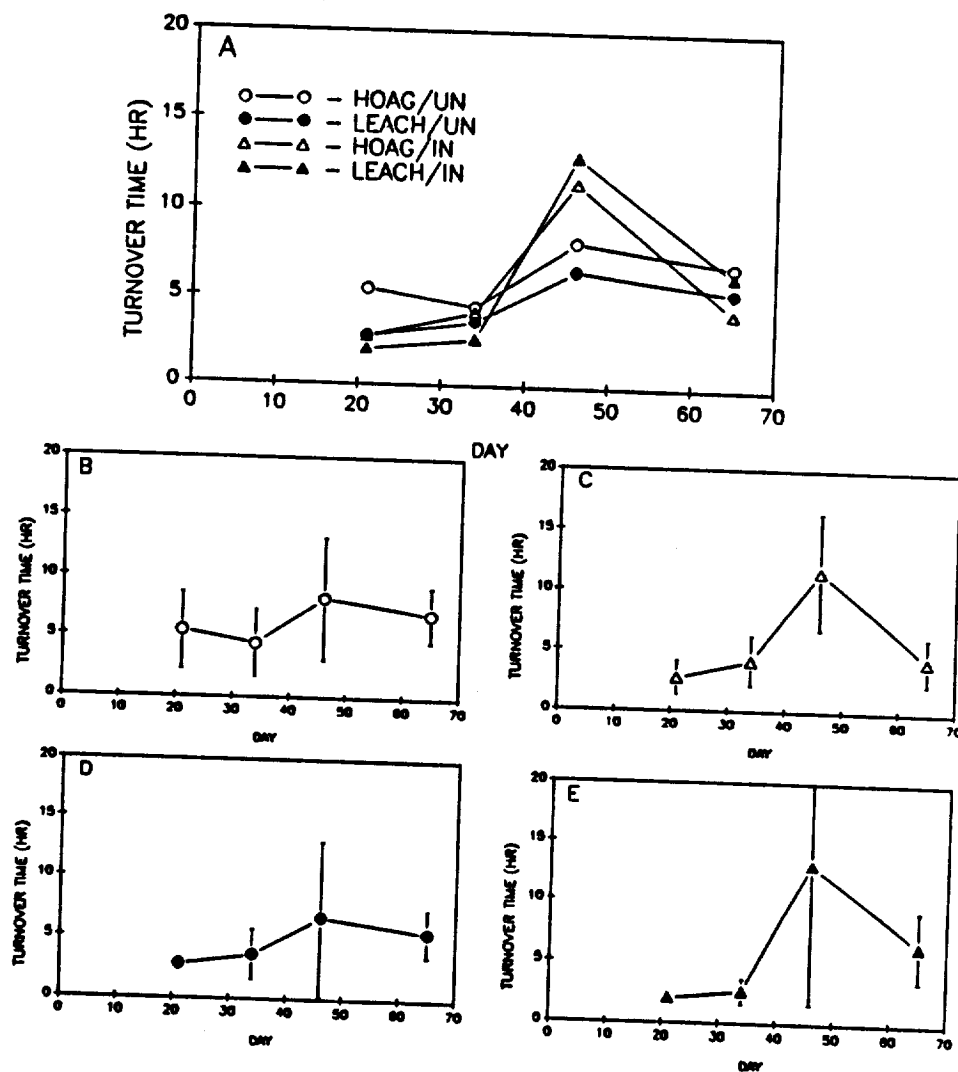FIGURE 12. Incorporation of 3H-TdR into cold TCA insoluble macromolecules for rhizosphere samples based on a A) mg dry weight root and B) total channel basis. Values for days 34, 46, and 63 represent means and std. dev. of growouts 2 and 3, values for day 21 represent data fromn growout 3 only.
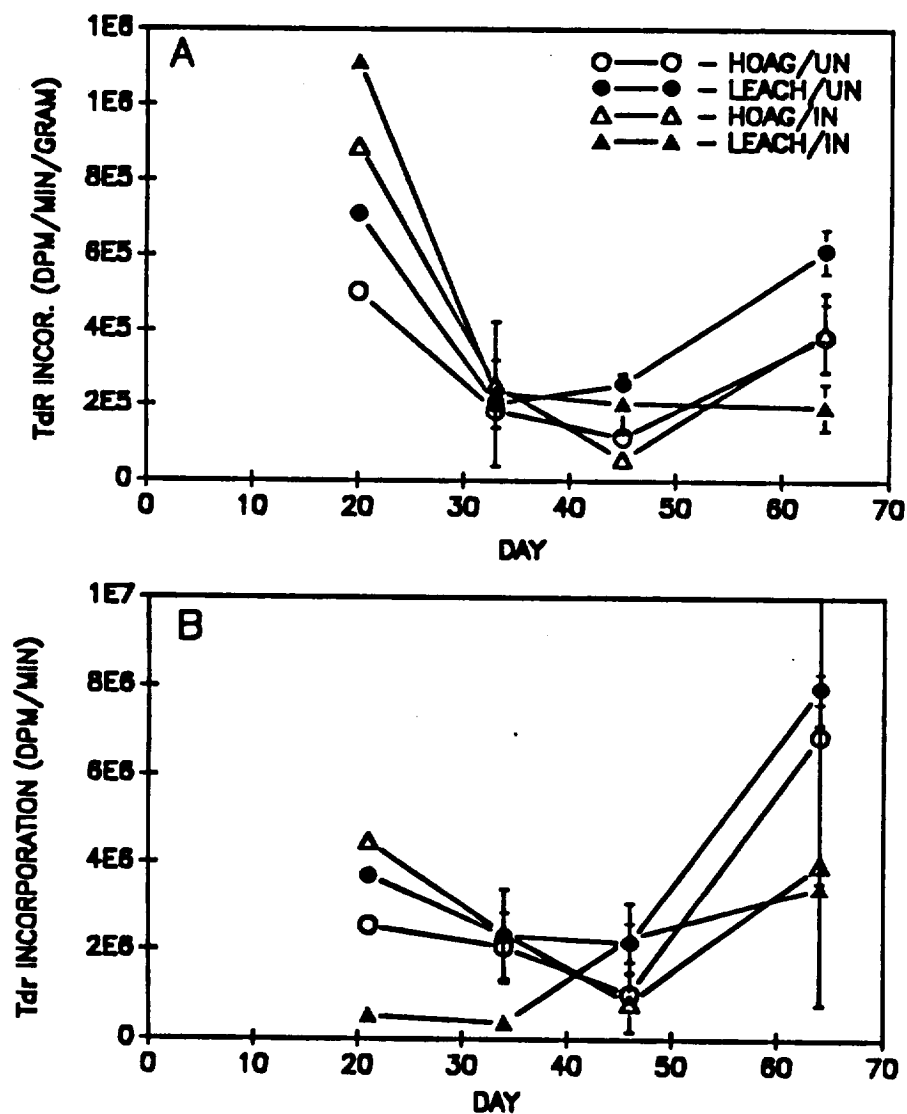
FIGURE 13. Turnover time of amino acids in rhizosphere samples based on combined respiration and assimilation of ^{14}C-labelled amino acid mixture in A) all, B) uninoculated Hoagland, C) inoculated Hoagland, D) ininoculated leachate, and E) inoculated leachate treatments. Values in all graphs represent means of three replicate growouts, std. dev. presented in graphs B-E only.
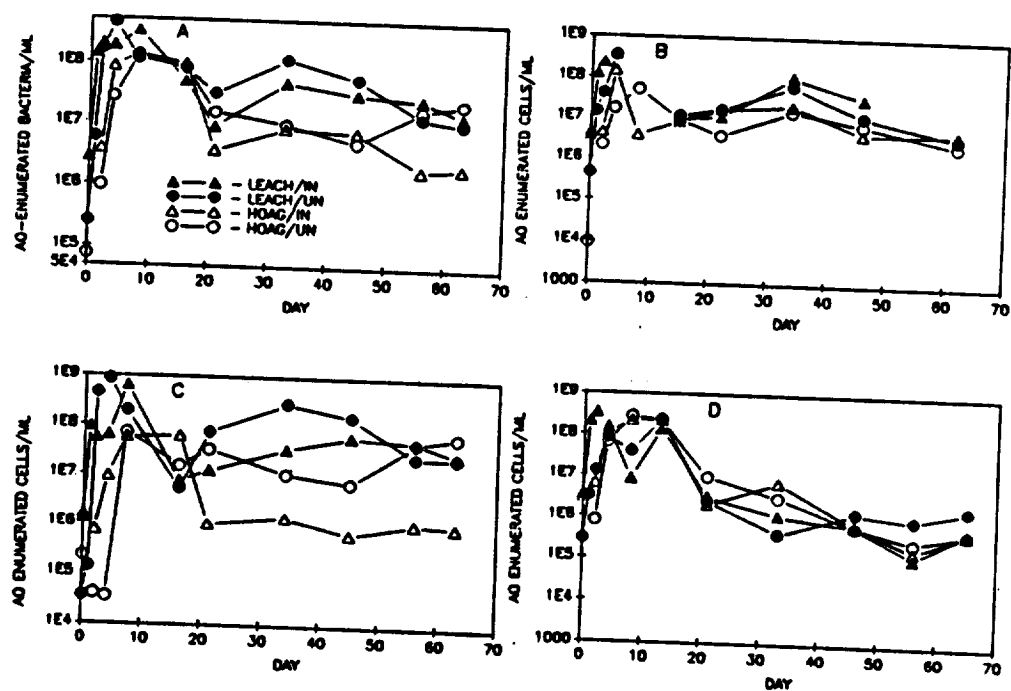
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FIGURE 14. The number of acridine- orange-enumerated cells/ml of nutrient solution. Numbers represent A) mean values of all three growouts, and B-D) values for growouts 1-3, respectively.
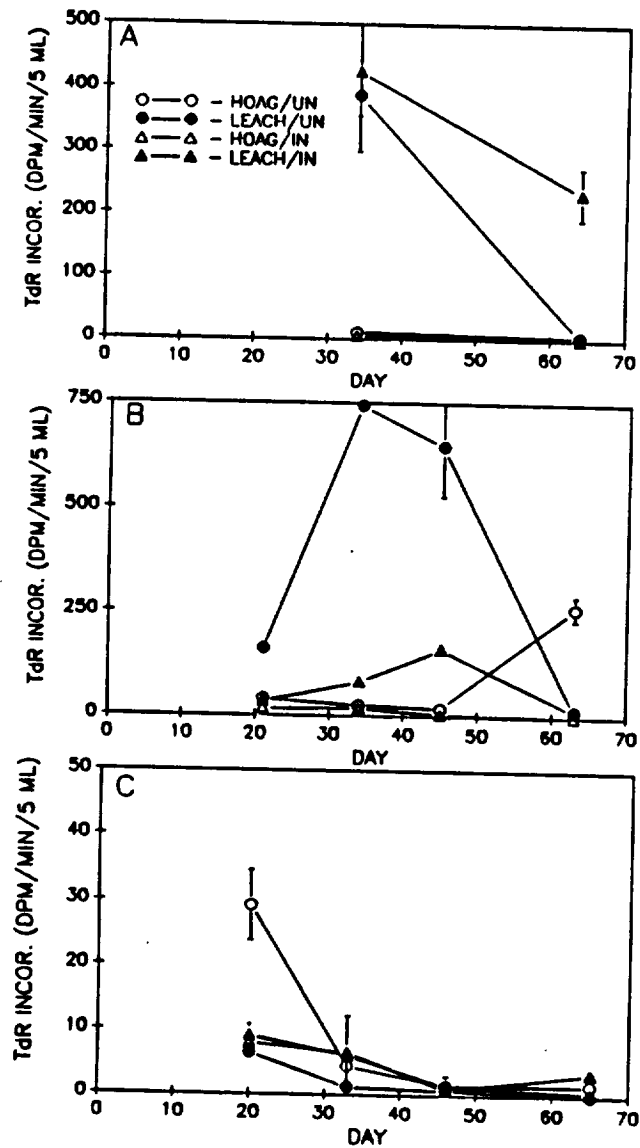
FIGURE 15. Incorporation of 3H-TdR into cold TCA insoluble macromolecules for nutrient solution samples. Values represent means and std. dev. of replicate samples at specific sampling days during growouts A) 1, B) 2, and C) 3.
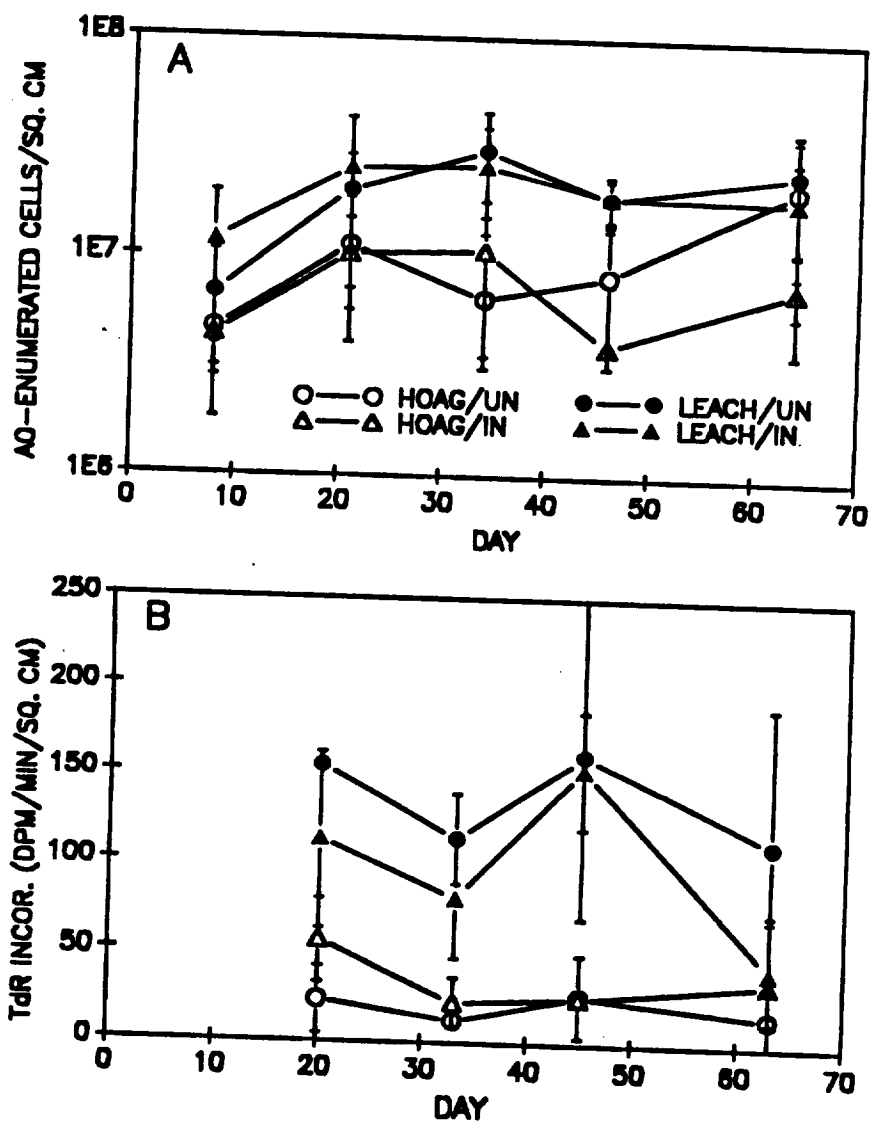
FIGURE 16. Number of acridine orange-enumerated cells and B)^{3}H-TdR incorporation into cold TCA insoluble macromolecules per cm^2 of PVC sampling chip. Values represent means and std. dev. of three growouts.
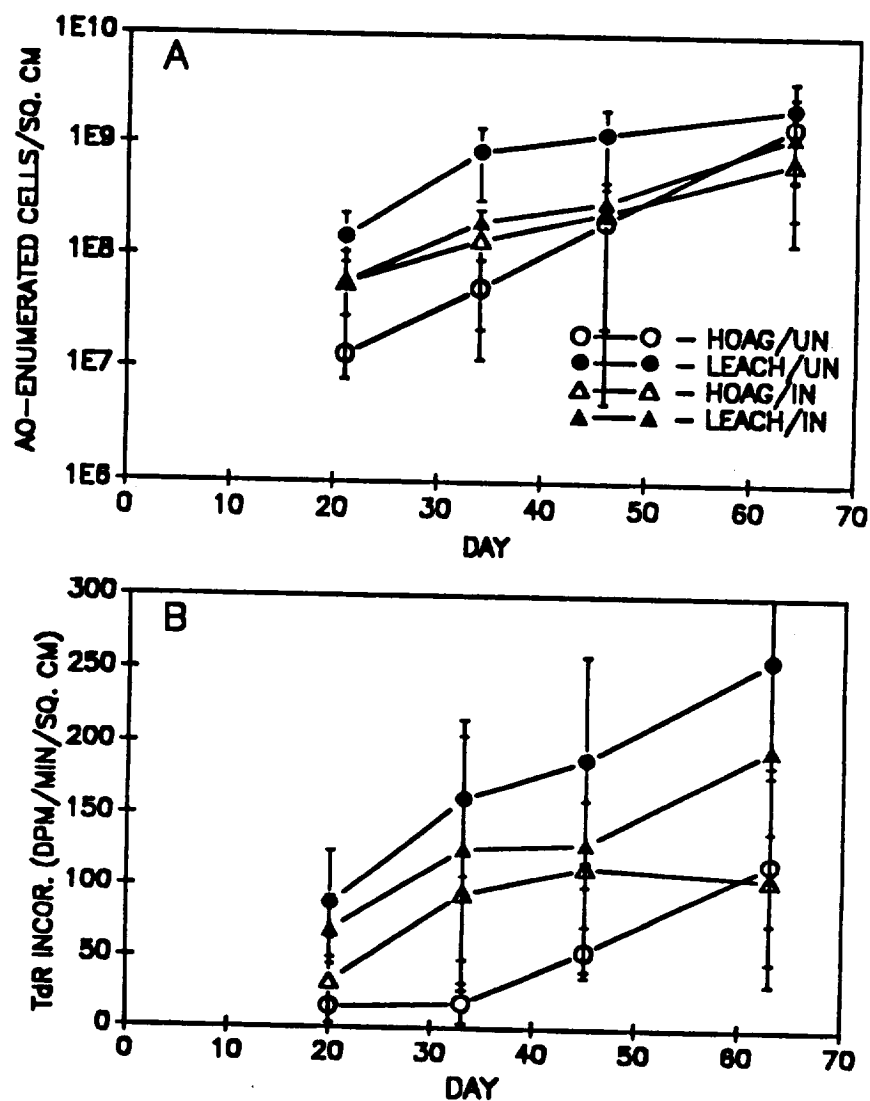
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FIGURE 17. Number of acridine orange-enumerated cells and B) 3H-TdR
incorporation into cold TCA insoluble macromolecules per cm2 of tubing section.
Values for days 34 and 63 represent means and std. dev. of all three growouts, values
for day 21 and 46 for growouts 2 and 3 only.

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REPORT DOCUMENTATION PAGE

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13. ABSTRACT (Maximum 200 words)

The development of bioregenerative systems as part of the Controlled Ecological Life Support System (CELSS) program depends, in large part, on the ability to recycle inorganic nutrients contained in waste material into plant growth systems. One significant waste (resource) stream is inedible plant material. This research compared wheat growth in hydroponic solutions based on inorganic salts (modified Hoagland's) with solutions based on the soluble fraction of inedible wheat biomass (leachate). Recycled nutrients in leachate solutions provided the majority of mineral nutrients for plant growth, although additions of inorganic nutrients to leachate solutions were neccessary. Results indicate that plant growth and waste recyling systems can be effectively coupled within CELSS based on 1) equivalent wheat yield in leachate and Hoagland solutions, and 2) the rapid mineralization of waste organic material in the hydroponic systems. Selective enrichment for microbial communities able to mineralize organic material within the leachate was neccessary to prevent accumulation of dissolved organic matter in leachate-based solutions. Extensive analysis of microbial abundance, growth, and activity in the hydroponic systems indicated that addition of soluble organic material from plants does not cause excessive microbial growth or "biofouling", and helped define the microbially-mediated flux of carbon in hydroponic solutions.

14. SUBJECT TERMS
Life Support, CELSS, and Hydroponic solution

15. NUMBER OF PAGES
54

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
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