

**VEGGIE Pillow Testing: Microbial Load Analysis of Cut-
and-Come-Again Species Testing**

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VEGGIE Pillow Testing: Microbial Load Analysis of Cut-and-Come-Again Species Testing

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With NASA focused on researching and developing technology for deep space missions, the need for a reliable supplementary food source must also be considered. For the ISS, resupplying the food source is more practical and cost effect since the facility is in low Earth orbit. However, as NASA attempts to push the frontier in space, the costs and distance for resupply will surely increase. Plants would contribute to the proportion of food and reduce the dependency on food from Earth. In addition, plants would provide oxygen production, carbon dioxide removal, and psychological benefits. As a result, a vegetable production system, VEGGIE, was developed for NASA to produce salad crops with minimal resources and study the beneficial effects. The VEGGIE pillow is a single use bag for growing crops that is used with the VEGGIE hardware. The VEGGIE pillow was tested with four different species of plants with the cut-and-come-again harvest method to determine the greatest yield. Instead of harvesting the entire plant, the harvest consisted of cutting leaves to allow the plant to regrow leaves. The harvest methods included cutting the plants weekly, bi-weekly, and monthly. A fifth plant species, radishes, was also harvested and replanted. Microbial load analysis and an ANOVA significance test were utilized. The data suggest that the two Brassica plants have the greatest yields; however, the microbial load is also greatest for the two plants per gram of fresh weight. Furthermore, the results support the reuse of pillows for multiple harvests as shown by the replanted radishes.

Nomenclature

AODC = Acridine Orange Direct Counts
APC = Aerobic Plate Count
BPS_e = Biomass Production System for Education

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<i>BW</i>	= Biweekly
<i>CB</i>	= ‘Cherry Bomb II’ Radish
<i>CEC</i>	= Controlled Environment Chamber
<i>CFU</i>	= Colony Forming Unit
<i>FW</i>	= Fresh Weight
<i>DW</i>	= Dry Weight
<i>IMA</i>	= Inhibitory Mold Agar
<i>ISS</i>	= International Space Station
<i>KSC</i>	= Kennedy Space Center
<i>M</i>	= Mizuna
<i>M</i>	= Monthly
<i>OL</i>	= ‘Outredgeous’ Lettuce
<i>PCA</i>	= Plate Count Agar
<i>SL</i>	= ‘Sierra’ Lettuce
<i>TB</i>	= ‘Tokyo Bekana’ Chinese Cabbage
<i>W</i>	= Weekly
<i>Y+M</i>	= Yeast and Mold

I. Introduction

In order to achieve NASA’s goal of deep space missions, it is necessary to develop a food source that is independent from Earth. The possibility of producing salad crops in insulated habitats could provide a safe source of fresh food and a means of psychological release through recreation. In addition, once crops replace the need for resupply, oxygen needs and carbon dioxide removal will also be provided by plants. At NASA-KSC, VEGGIE units are being utilized in crop research to evaluate methods and growth patterns for future missions. The VEGGIE is a vegetable production unit currently being developed by ORBITEC and used by NASA. The products of salad crops for early lunar missions require use of limited area, mass, and electrical power (Kliss et al., 2000). The pillow is to be used in conjunction with the VEGGIE space flight hardware. As a low maintenance, low weight, and single use bags, the pillows are cost effective to make and require little volume. Using pillows with media instead of growing plants hydroponically decreases the need for precise overview of the media’s nutrients and contributes to the low maintenance (Stutte et al., 2009). The pillows are made of lightweight, electrostatic zip lock bags with a porous membrane made of Nitex (Figure 1). Since the VEGGIE unit has a water reservoir that is in contact with a capillary mat, the water wicks from the mat through the pillow’s porous membrane via capillary action.

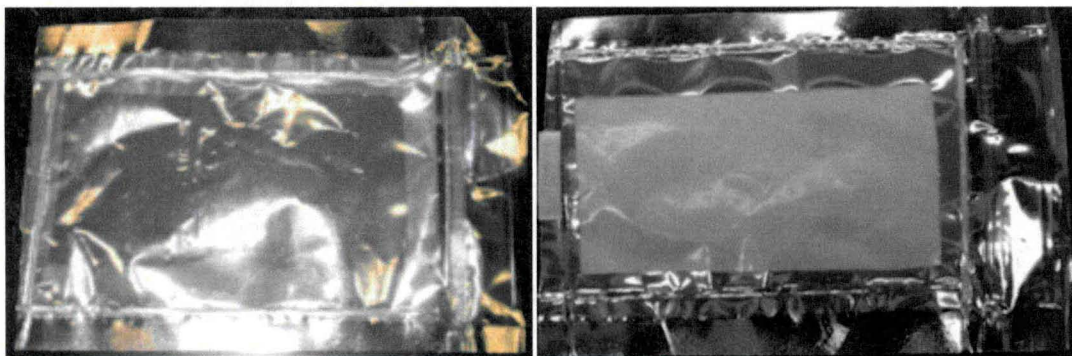


Figure 1. The VEGGIE Pillow made of a light weight, electrostatic material. The pillow was heat sealed with a Nitex material to act as a porous membrane (right)

In the experiment, the effects of different harvest techniques on changes in microbial populations and yield were analyzed. Based on previous studies, microbial magnitude was expected to increase because in each harvest, cutting the plant would wound and expose the plant tissues to the environment, providing resources for microbial growth.

II. M e t h o d s

A. Plant Material

Five species of plants were studied: 'Outredegous' lettuce (*Lactuca sativa* cv. 'Outredegous'), Mizuna (*Brassica rapa* cv. nipposinica), 'Sierra' lettuce (*Lactuca sativa* cv. 'Sierra'), 'Tokyo Bekana' Chinese cabbage (*Brassica rapa* var. chinensis 'Tokyo Bekana'), and 'Cherry Bomb II' radish (*Raphanus sativus* L. cv. Cherry Bomb II). These cultivars were selected based on previous research done at NASA's Kennedy Space Center. Each pillow had 2 slits for seeds. Seeds were planted at a density of 2 seeds per slit and then thinned to 1 seed per slit after 7 days. Each species grew in 12 pillows and within the 12 pillow for the species, each set of 4 pillows was assigned 1 of the 3 harvest methods. Therefore, each sample size was 4 pillows.

B. Growth Conditions

The pillows were designed for the VEGGIE unit, but were grown in controlled environment chambers (CEC). The plants were grown in NASA's CEC to mimic the condition in the VEGGIE unit. The temperature was set for 28°C during the day and 24°C at night. The relative humidity was 70% during the day and 75% during the night. The photoperiod was 16 hours of light and 8 hours of darkness. The light was kept at approximately 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ with CO₂ kept at 1200 ppm continuous. These environmental conditions were maintained using computerized control and checked daily.

C. Nutrient Management

Media was contained in pillows which are constructed from 15 cm x 10 cm electrostatic bags inset on one side with a 5.3 cm x 10.5 cm Nitex nylon mesh surface to allow capillary wicking. Each pillow contained 100mL media of mixed 7:3 Fafards #2 to 1-2mm of calcined montmorillonite clay (arcillite) that contained 7.5g of time active fertilizer (Nutricote 18-6-8, type 180, Florikan, Sarasota, FL) per liter of media. The tub consisted of an inserted foam mat covered with Nomax to act as a water reservoir for the pillows. 2 strips of Nitex in each slit were imbedded in the media to allow the water to wick from the reservoir. The pillows were placed in the tub with the Nitex side in contact with the Nomax and acted as reservoir analogs. Water was kept at a constant level of 2 liters per tub and checked daily. There were 12 pillows per tub for a total of 60 pillows (Figure 2). Tubs 1-4 were assigned pillows randomly with 4 plants in each column while tub 5 only contained radish pillows. Tub 5 had a separate harvest method because of the radishes.

D. Harvest Measurements

The 3 harvest methods consisted of a weekly, bi-weekly, and monthly harvest. The weekly harvest began a week after the pillows were thinned to 1 seed per slit. For each harvest, fresh mass for the leaves was determined. The plant tissue was dried at 70°C in paper bags for at least 48 hours after microbial techniques were performed. After drying, the bags were left at room temperature for at least 2 hours to return to room humidity. For the weekly harvests, the 2 largest and oldest leaves were cut from each plant for a total of 4 leaves from each pillow. For the bi-weekly harvests, the 4 oldest leaves were cut from each plant for a total of 8 leaves from each pillow. For the monthly harvest, the entire plant was cut. Since the entire radish has to be harvested, the radish pillows were harvested and replanted during the bi-weekly harvest. Separate measurements were taken for the leaves and storage roots.

E. Microbial Measurements

At the first weekly harvest, the plant tissues were shaken in centrifugal tubes with sterile H₂O and sterile glass



Figure 2. Pillow arrangement for tubs. Pillows were randomly assigned to position using a random number generator.

beads to remove microbes from the plant surface. In the subsequent harvests, the plants were placed in stomacher bags with sterile H₂O and stomached for 2 minutes. The amount of water added to the centrifugal tubes and stomacher bags was dependent on the general fresh weight of the samples. With the solution, serial dilutions were utilized and plated on plate count agar plates (PCA) and inhibitory mold agar plates (IMA) made from DIFCO products. PCA plates were incubated for 2-3 days at 30 °C while IMA plates were stored for ~5 days at room temperature. After incubation, bacteria colonies and yeast/mold colonies were counted.

Acridine orange direct counts (AODC) were also used to determine microbial population changes after harvests. For the AODC, 0.5 mL of formalin was added to 9.5 mL of the sample solution for preservation purposes. The solution was sonicated for 15 seconds to separate microbes from plant matter. 1 mL of solution was dyed with 100 µL of 0.1% of AO. The dyed solution was vortexed and allowed to sit for approximately 5 minutes. After, the solution was vortexed momentarily again and filtered through Whatman’s nuclepore track-etch membrane. The filter was allowed to dry for at least 10 minutes. The dried sample was placed on a drop of oil that was present on a microscope slide. A second drop of oil was placed on the sample and was covered with a slip. Before using the Zeiss Epi-Fluorescent Axioskop Microscope to observe the samples, another drop of oil was placed on the cover slip to reduce unevenness in the images. The images were taken and saved using DP Controller and DP Manger. The microbes were counted manually using Image-Pro Express 7.0 software.

F. Statistical Analysis

Data were analyzed using One-way and Two-way ANOVA statistical tests with GraphPad Prism statistical software.

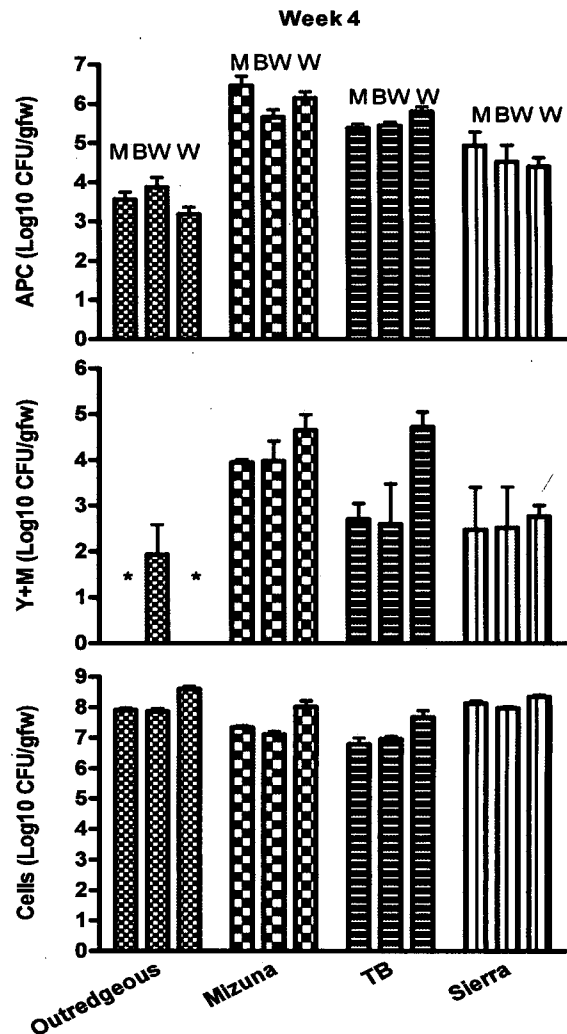


Figure 3. Aerobic plate counts, yeast + mold counts, and cell counts of plant species and harvest methods. Bars represent mean + 1 standard deviation. * represents counts that were below detection limit for that harvest method.

III. Results and Discussion

Significant differences in aerobic plate counts (APC), yeast and mold (Y+M) counts, and AODC were found between the cultivars with $P < 0.0001$ (Figure 3). The results suggest that the plant species may affect the plant's susceptibility to the microbial environment. The two lettuces, 'Outregeous' and 'Sierra', had less average bacterial colonization and Y+M counts than the two Mizuna and Chinese Cabbage, both in the Brassica family. Mizuna had the highest bacterial and fungal counts at all three harvest methods. However, this may be caused by the impact of its more open outward leaf growth on microbial populations than the close growth of lettuce leaves (Hunter et al., 2010). It should also be noted that the pillows were grown in tubs instead of the enclosed VEGGIE unit. The tubs were exposed to the open environment, making the plant more susceptible to microbes.

No significant differences between weekly, bi-weekly, and monthly harvests for APC were observed except in one instance between Chinese Cabbage harvested weekly and monthly (TBM and TBW) at $P < 0.05$. The data does not support the original hypothesis that multiple harvests would cause increased microbial loads due to the plant tissue being exposed to the environment with each cutting. Monthly harvest pillows were wounded and exposed to the environment momentarily before being placed in a stomacher bag, while weekly harvest pillows were handled and wounded more frequently. However, the bacterial load of the weekly pillows was still lower than the monthly for three of the four species.

There were no significant variations between harvest methods for Y+M counts. The monthly and weekly fungal counts for 'Outregeous' lettuce (OL) were below detection limit. As a result, the data cannot be used to support that harvest methods affected counts. However, for the other three species, fungal counts were higher for the weekly harvest than the monthly, which was opposite the observation seen in the microbial counts. Since fungal growth can have antibiotic properties, the increased fungal counts in weekly harvests may have influenced the microbial counts.

Cell counts were also found to differ significantly between harvest methods and cultivars. However, the images used for the counts were affected by large amounts of plant matter. Despite the efforts to sonicate and filter the matter, the images still contained significant amounts of matter. It is possible that the matter covered the microbes and affected the AODC. Ideally, AODC are used for pure cultures that are not affected by other particles in the sample.

All species showed excellent growth and re-growth in pillows (Fig. 4). Fresh weight yield was significantly different between species at $P < 0.05$. However, there were no significant differences between the harvest methods. TB had the highest fresh and dry mass yield in each of the harvest methods when compared to the other species. However, there was variability in the yield for the pillows as seen in Figure 4. The growth of other species of plants produces leaf canopies which shade other adjacent plants (Stutte et al., 2009). Since different species were used and harvest frequency differed, heights of the plants varied greatly at different times and affected neighboring plant growth.

There were extremely significant differences in dry weight between the species and harvest methods with $P < 0.01$. It is important to note that there was a greater loss of water mass in the OL and SL than the two

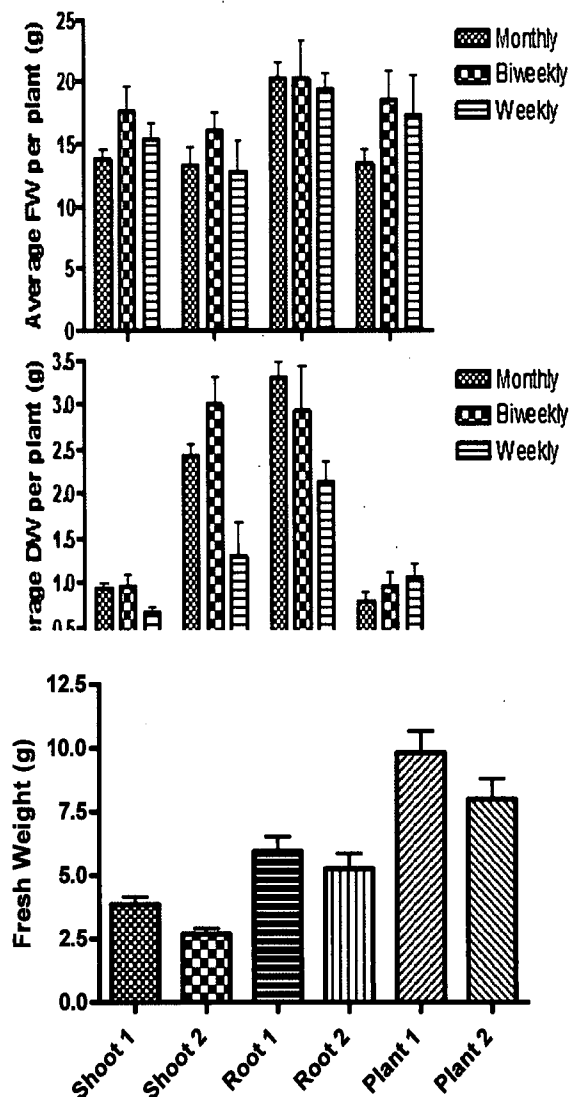


Figure 5. Fresh weight of initial and replanted radish plants. Initial radishes are labeled as 1 and replanted are 2. Bars represent mean + 1 standard deviation.

Brassica plants. TB and Mizuna had the two highest dry yields while Mizuna was one of the species with the lowest fresh weights. In addition, Mizuna weekly significantly differed from the biweekly and monthly yields; yet, the Mizuna biweekly and monthly did not differ significantly. OL and SL showed no differences in dry yield with different harvest methods. Only TB weekly and monthly differed significantly for that particular species.

No significant differences between the initial radishes and the replanted radishes were observed for the APC, Y+M, and AODC measurements (data not shown). The harvest method did not have a significant effect on microbial load, which was similar to the levels seen in the leafy greens used. Conversely, differences in fresh yields were observed for the entire radish plant at $P < 0.05$ and shoots at $P < 0.01$, but not the storage roots. There were no significant differences in dry weights for the plants, roots, and shoots (data not shown). The initial radishes had higher fresh weights than the replanted ones. Since the same VEGGIE pillow was reused for the replanted, the media had no mean of regenerating the nutrients used by the initial plant. Consequently, the replanted radishes were grown in a more-deficient media and had less space for growth because the roots of the initial radish were still present.

This experiment will continue with ongoing evaluation of the issues associated the VEGGIE pillows and VEGGIE hardware. Sample size needs to be increased from the four pillows used for the associated species and treatment. A focused experiment on a single crop instead of five species will improve the ability to determine the effects of harvest methods on total yield and microbial loads.

III. Conclusion

Significant differences were seen between cultivars for the microbial load analysis and yields. Mizuna and TB had the highest microbial and fungal counts as well as the highest dry weight yields when compared to the two lettuce plants that were also utilized. Although the microbial counts were normalized with fresh weights, the Brassicas had the highest microbial and fungal counts per gram of fresh weight. These results suggest that Brassicas provide the highest yield for the VEGGIE pillow, but may raise food safety concerns with their microbial load. The results for radishes support the possibility of reusing bags for multiple harvests without an increase in microbial levels. This experiment stimulates further study in the utilization of the VEGGIE pillow for future deep space missions.

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