The Efficacy of Reclaimed Water on Spinach Growth in Lunar Regolith

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Abstract

For extended missions and lunar colonization efforts, establishing sustainable extraterrestrial food growth is necessary. Extraterrestrial soils such as lunar regolith lack the nutrients needed to support crop growth, but supplementing dietary needs with packaged food is too costly. Recycling wastewater may provide a potential nutrient source for crops cultivated in regolith-based systems while dramatically reducing auxiliary costs and resources. Wastewater effluent has differing chemical and microbial compositions from purified water because it has been in contact with more influential factors and goes through a standardized treatment process, in turn contributing microorganisms (the microbiome) that can make nutrients more available, prevent plant diseases, and improve soil quality. This experiment investigated how reclaimed water influences plant growth and soil microbial communities in regolith amended substrates.

Water samples representing different levels of effluent were collected from the Platte River at an upstream location and downstream location, before and after urban contact respectively. Spinach was selected as the experimental crop for its measurable growth characteristics and representation of plants relevant to food production. Plant height, qualitative development such as color, and water chemistry of each condition were continuously measured to characterize nutrient differences between samples. After a nine-week period, final growth characteristics such as plant weight, chlorophyll concentration, and root qualities were measured. Water and substrate were analyzed to evaluate the microbiome of each group. Treatment with high effluent water is expected to have a positive impact on plant growth by providing essential nutrients and supporting beneficial microbial growth in regolith. This research supports the future of space travel by identifying conditions for optimal growth in regolith and reliable food infrastructure on the moon.

Introduction

In light of projects like Artemis and the success of the first manned mission to the moon in over fifty years, further exploration is closer than ever before. As development toward human inhabitation on the lunar surface is underway, we must consider renewable food systems in outer space. To meet nutritional needs while cutting the shipping cost of food items, agriculture outposts seem to be the best path forward. However, there remain three significant challenges in tending crops in space: plants require soil, water, and nutrients.

The first problem with creating sustainable agriculture on the moon lies in the substrate itself. Greek for “blanket rock,” the substrate regolith is a dust-like loose rock that sits on top of bedrock; on the Moon, regolith includes powdery dust and broken rock from the impact of meteorites on the lunar surface·[1] Regolith is sterile and devoid of nutrients essential for plant growth on its own, but when combined with a medium like soil and supplemented with nutrient-rich effluent, there may be potential for sustainable plant anchoring and growth as well as wastewater management in space.

The second issue—water in space—is not just limited to plant growth, as waste management systems are integral to human health. Current Supercritical Water Oxidation (SCWO) technology is used on missions to recycle wastewater in space but rarely used on earth. Research for agricultural use, drinking, and cooking in lunar colonization are underway.[2] Wastewater effluent on earth is generated from rainwater, human activity (e.g., business/domestic plumbing, infrastructure, irrigation), etc. aka sewage.[3] Various urban and rural areas differ in debris, chemicals, nitrogen, phosphorus, and potassium (NPKs), pathogens such as bacteria and viruses, plastics, metals, and other substances that contaminate water runoff which alters the chemical and microbial composition of effluent compared to ocean water or potable water. Wastewater effluent is also called reclaimed water. In short, wastewater effluent would have different chemical and microbial compositions from tap water because it has been in contact with more influential factors and goes through a different standardized treatment process. To model the conditions of low and high wastewater effluent, samples were collected from the South Platte River upstream before urban contact, and downstream just after exiting a processing plant. Wastewater effluent from recycled sources is expected to be higher in NPKs supporting plant growth and contain different microbial compositions from non-reclaimed water.

Third comes the challenge of adequate nutrients in extraterrestrial substrates and their microbiomes. A microbiome can be defined as a community of microorganisms (such as bacteria, fungi, and viruses) that inhabit a particular environment.[4] This in particular is important to understand because the microbial composition (microorganisms) present in soil can make nutrients more available by breaking down organic matter present in the soil into resources a plant is able to uptake, cycle existing nutrients for longevity, prevent plant diseases by outcompeting harmful bacteria, and improve soil quality by interacting with the plants directly.[5] Though it may seem counterintuitive, the best method for providing renewable nutrients in soil is a healthy microbiome filled with bacteria. Lunar regolith is sterile, which means starting from nothing in terms of microbiome.[6] However, exposure to non-sterile water could potentially introduce a soil microbiome. This could help us identify a compound of beneficial microbes ideal for supplemental treatment via reclaimed water in substrates like regolith on the moon.

In the case of lunar colonization, we expect increasing demand for food crops without abundantly available terrestrial soil or nutrients combined with wastewater accumulation from humans. To assess these issues, we grew Bloomsdale spinach (Spinacia oleracea) to observe the growing capacity of food crops in soil as well as a lunar regolith simulant blend with the support of wastewater effluent/reclaimed water. Spinach itself is an asset in a food system because of its ability to be consumed raw or cooked at nearly every stage of maturation, great nutrient density, and yield of roughly 1.5 tons per acre.[7] Spinach conveniently demonstrates readily measurable growth characteristics such as plant height, weight, and chlorophyll concentration. It also models other desirable crops such as chard or companion crops like legumes.

To understand how wastewater effluent impacts the growth of spinach plants in regolith, two factors were manipulated: substrate and water. Twelve pots were divided into two groups, either 100% soil or a soil-regolith mix to study the effects regolith has on the plants' growth. Those two groups were further divided into three categories: low-effluent (LE) water, high-effluent (HE) water, or regular tap water, which all gradually reintroduced microorganisms over the course of nine weeks (see Figure A below). The plants were continuously monitored during the growth period with height and qualitative observations until they were finally measured for weight and chlorophyll concentration. After the full nine weeks, DNA from the soil samples as well as the collected water were extracted, then the 16S gene was amplified and sequenced to identify the bacterial composition. We expected the spinach grown with high-effluent water to result in the most robust community of microorganisms and greater plant growth and health compared to plants given low-effluent water.

Figure 1

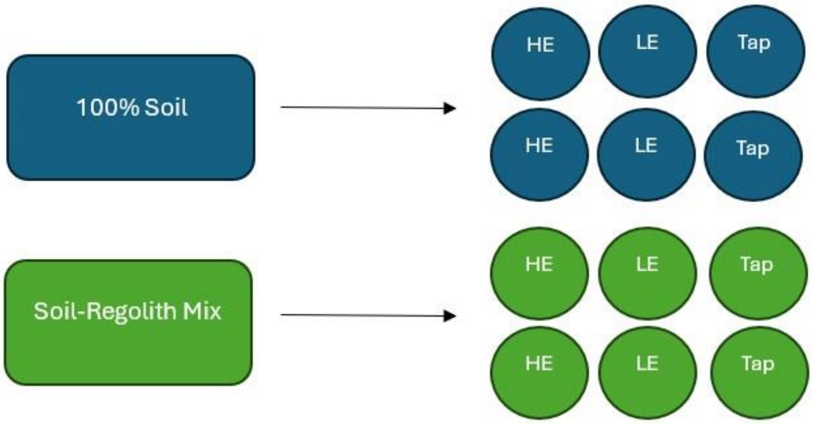


Illustration of plant pot organization adapted from Table 1

Methods

Initial Water Collection and Set-Up

To model high and low effluent conditions, water samples were collected before and after passing through Denver in two areas of the Platte River; the first near Waterton Canyon Trailhead, and the second near Pelican Ponds Open Space. A water pump was fully submerged in each of the respective creeks to rinse the pump, then used to fill 3 disinfected 5-gallon buckets from each location, totaling 6 buckets. The buckets were labelled with their condition, high effluent or low effluent (shortened to HE and LE) as well as the order in which they were filled. Tap water was collected from CCA, room C303.

In preparation for standardized watering, the collected buckets were tested for base levels of ammonia, nitrate, phosphorus, nitrite, and pH. Six disinfected 6-quart bins were labelled either

LE, HE, or Tap and Soil or Soil-Regolith Mix based on their condition. A 1-inch line was drawn on each bin and filled from its respective 5-gallon bucket as to not overwater each pot while bottom watering.

For plant set-up, paper towels were first placed at the bottom of 12 pots, given ID numbers corresponding to their water and substrate conditions (see in Table 1 below). Sterile potting soil filled six of the pots while the other six were filled with a mixture of 76.79% sterile soil and 23.20% regolith. The mass of soil in the pots was within <5% difference of the other pots with the same soil condition. Two spinach seeds were then planted in each pot, about 1.5 inches from each other and approximately 0.5 inches deep.

Table 1

	High Effluent	High Effluent	Low Effluent	Low Effluent	Tap	Tap
100% Soil	7	8	11	12	3	4
Mix	5	6	9	10	1	2

Pot ID numbers by soil and water condition. Trays consisted of pairs (e.g., IDs 1 and 2, 7 and 8).

Plant and Water Quality Monitoring During Growth

On watering days, each pot was placed into the bin with their water condition until soil was saturated. Throughout the duration of the experiment, the 6-quart bins were cleaned out and refilled from collected water buckets as water was depleted or showed change in color, odor, particulate, etc. During germination and in cases where the soil was particularly dry, plants were top watered until the upper layer of soil was moistened. For the first week after planting seeds, pots were watered three times per week, then twice per week. Plants went back to being watered three times per week for the last four weeks of the experiment, after supplemental lights were installed. Water quality of the collected buckets was monitored weekly to account for potential differences in pH, ammonia, nitrate, nitrite, and phosphorus over time, which did not end up having significant changes from the base levels taken at set-up. All water was stored away from light and covered with lids.

Plants were inspected daily to evaluate seed germination, soil quality, room temperature, leaf count, color, plant height (from soil level of pot to the tallest leaf) and any abnormalities or patterns in plant growth. Humidity covers were removed once each tray had a germinated seedling. Pots that did not germinate within the first month of the experiment were re-seeded. The right-most tray of pots was rotated to the left-most position to equalize the sunlight each plant was receiving. Heating mats were placed under each tray, drying out oversaturated soil. A full spectrum lamp was placed over 5 out of the 6 trays. The right-most tray position had an individual lamp. Competition between the two seedlings in each pot was controlled when the second sprout was cut off.

Final Plant Growth and Health Measurements

Several measurements were taken at the end of plant growth to reflect which plants were most successful. When gathering weight measurements, only the green growth was used, achieved through cutting at the line where the green stem transitioned into the beginnings of the root. This was done to account for differences in root weight that weren't a byproduct of plant growth, such as dirt clumps or pebbles trapped within larger roots that would skew data if the whole plant were weighed.

Plant height was gathered through taking measurements both from the surface level of the soil as well as along the stem from the start of the first true leaf sprout. Height measurements were then further standardized using five reads (each team member providing their own data) to account for differences in method of data collection between members.

Root growth data was exclusively collected via qualitative measurement. This choice was made due to the inability to reliably keep all the fine roots and root hairs intact for proper weighing and further manipulation. Despite this, clear visual differences were still observed as longer, thicker, and more developed roots were apparent in some plants (see Figure 6).

The final metric used to evaluate the quality of growth between plants was a measure of the concentration of chlorophyll present within 0.28 grams of leaf tissue. Once collected, the leaves were ground in mortar and pestle for pigment extraction and diluted using 70% ethanol. Prepared chlorophyll extractions were loaded into a spectrophotometer, testing at both the wavelengths 663 nm and 645 nm for a rate of absorbance. The chlorophyll concentration (mg/mL) was determined using the formulas:

$$\textbf{Chlorophyll a} = (12.7 * x - 2.69 * y) * 5$$

$$\textbf{Chlorophyll b} = (22.9 * x - 4.68 * y) * 5$$

Where x is the spectrophotometry reading at 663 nm, y is the reading at 645 nm, and the multiplication factor of 5 accounts for the ethanol dilution of samples. Chlorophyll extraction samples all contained 15mL volume. Both chlorophyll a and b values were combined to determine the sum of total chlorophyll present at the end of the growth period. Tap water and low-effluent plants were measured in replicate pairs (n=2) due to lack of leafy tissue necessary for grinding and extraction, whereas high-effluent plants had plenty of growth and were all measured as individuals (n=1). In figures below, plants with shortened growth periods are separated, when possible, to prevent underdevelopment of chlorophyll from skewing results.

Soil Microbial Diversity Analysis

To test the microbiome of our pots, genetic samples were obtained from 200-250mg of soil from each pot. Collected water samples were filtered, then the filter discs were cut into quarters to be tested. A total of 16 samples from all 12 pots and replicates of the 2 collected waters had DNA extracted using the Qiagen DNeasy PowerSoil Pro Kit. The 16S gene was amplified in PCR following the Oxford Nanopore 16S Barcoding Kit 24 V14 protocol, then run in a 1.5% agarose gel electrophoresis against a 100 base-pair ladder and a Lambda DNA HindIII Digest ladder to evaluate the size of the DNA present, confirming 16S amplicons (bacteria). If the soil were sterile, the gel electrophoresis would not show PCR product for the 16S gene.

Once PCR was confirmed, the product was pooled to create a DNA library and loaded onto a MinION flow cell using a Flongle adapter, following the Oxford Nanopore Rapid Sequencing DNA protocol from 16S Barcoding Kit 24 V14. This process reads the genetic codes of the samples loaded into the flow cell so that the raw data can be read and analyzed to identify taxonomic classifications. In this case, EPI2ME wf-16s software was used to identify taxonomic classifications of 16S rDNA. However, one barcode (the identifier used by the software to read sequences) was corrupted during processing and could not be run alongside the other 15. This omission is reflected in Figures 8 and 10 where one replicate of HE water is missing.

Because sequencing and metabarcoding can identify classifications of organisms, we are also able to use this information for calculations of diversity from the number of species (also called richness) and frequency of each species (also called relative abundance). The Shannon Diversity Index is commonly used to quantify species diversity in ecology and studies on human interference using these metrics in the formula:

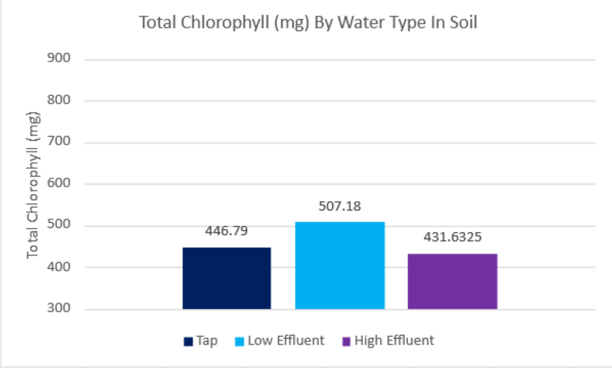
$$H = -\Sigma p_i * ln(p_i)$$

Where H is the Shannon Diversity Index and p_i is the proportion of the entire community made up of species *i.* A value of $H = 0$ indicates a community that only has one species. In the Shannon Diversity Index figures below, all values are equal to $n = 1$, meaning they correspond to a single barcode. Figures 7a and 9a exclude one 100% soil sample, and Figures 7b and 9b exclude two soil-regolith samples due to shortened growth periods which substantially impact the capacity for bacterial growth in an environment.

Results

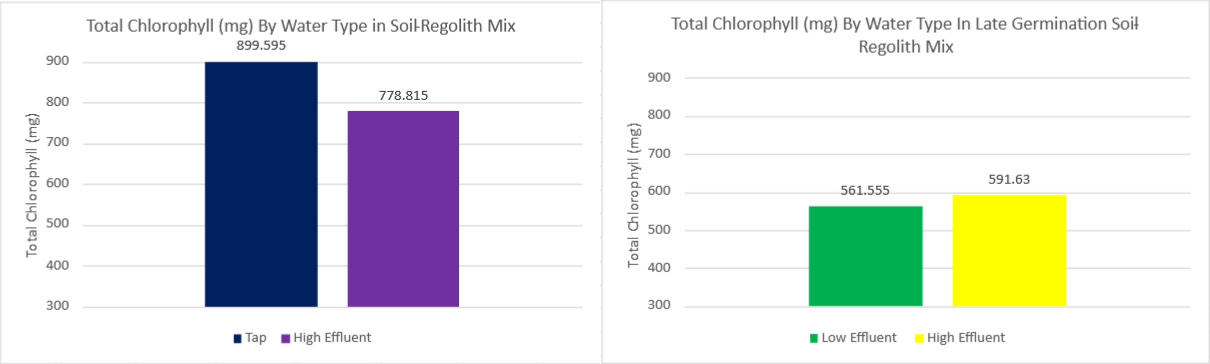
Final Plant Growth and Health Measurements

Figure 2



Low effluent plants had higher total chlorophyll than tap or high effluent plants.

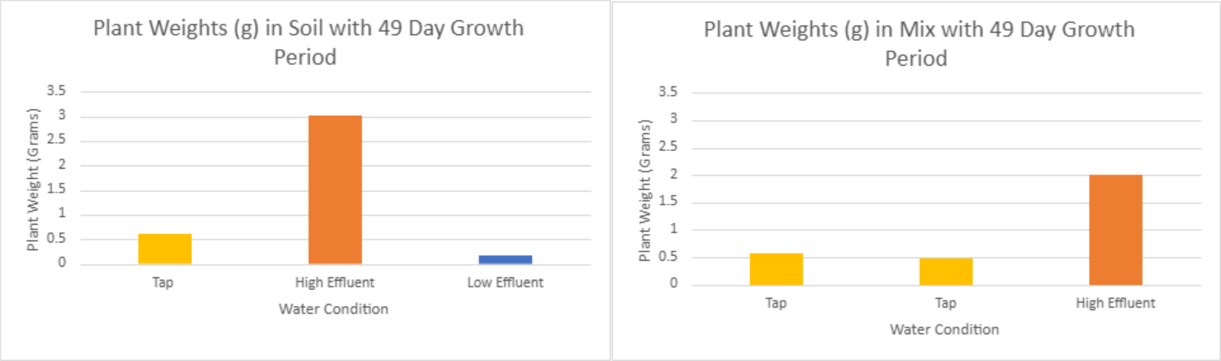
Figure 3a & 3b



a. Tap watered plants had higher total chlorophyll than high effluent plants with the same growth period.

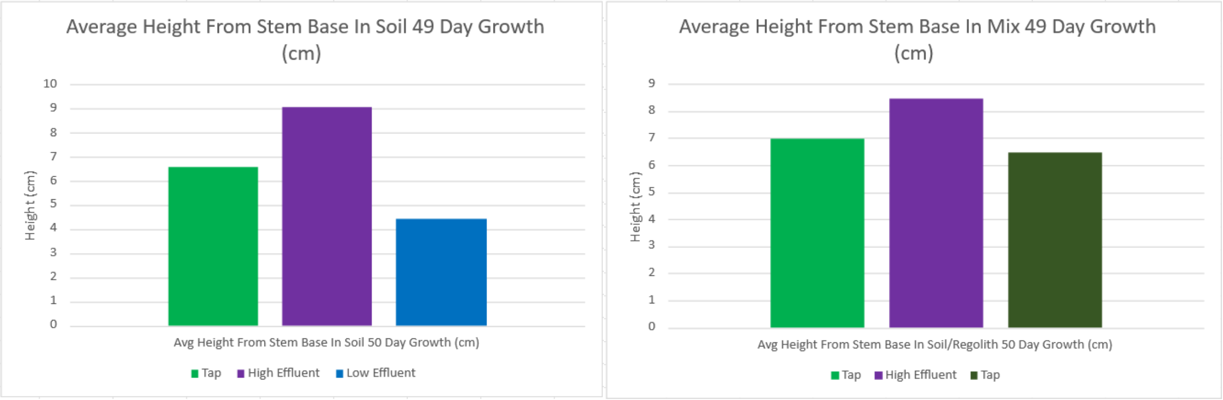
b. High effluent plants showed slightly elevated levels of total chlorophyll compared to low effluent plants with the same growth period, but they remain similar.

Figures 4a & 4b



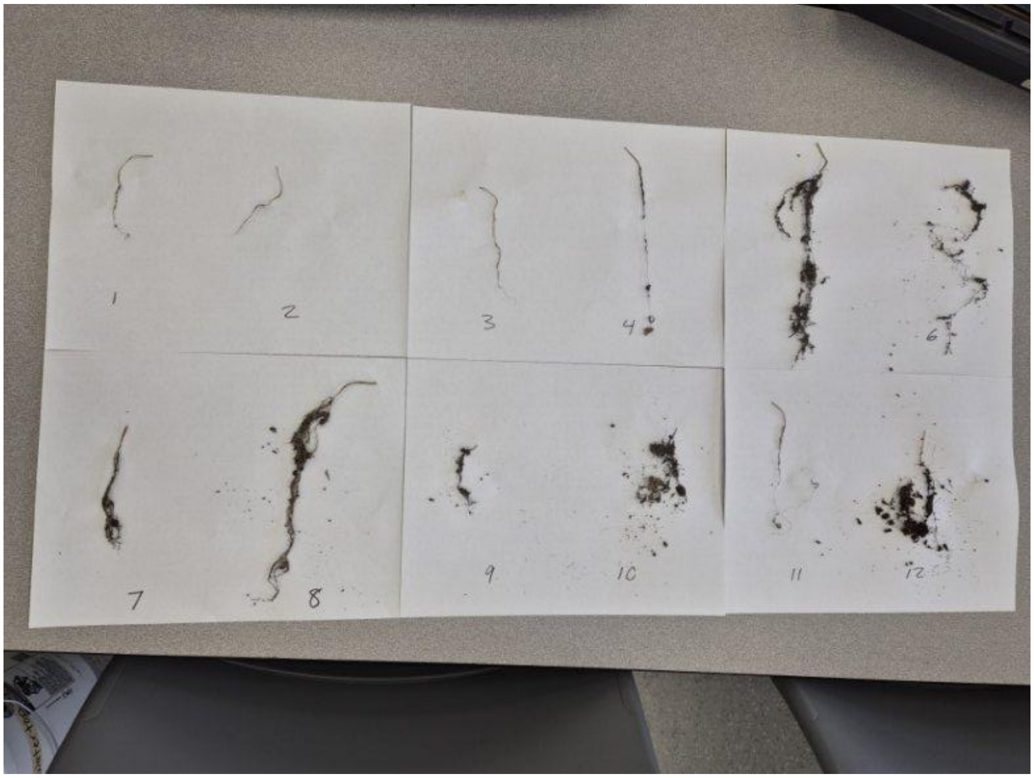
High effluent plants weighed more than four times the amount as other tap and low effluent plants grown over the same time, regardless of substrate.

Figures 5a & 5b



a. Average Height from the determined start of the stem, defined as the growth starting at and above the first true leaves sprouted. Comparing conditions present within the 100% soil and only in plants that achieved a growth period of 49 days. HE plants are consistently taller than tap and LE plants by multiple centimeters.
b. Average Height from the determined start of the stem. High effluent plants are consistently taller than tap and low effluent plants by multiple centimeters.

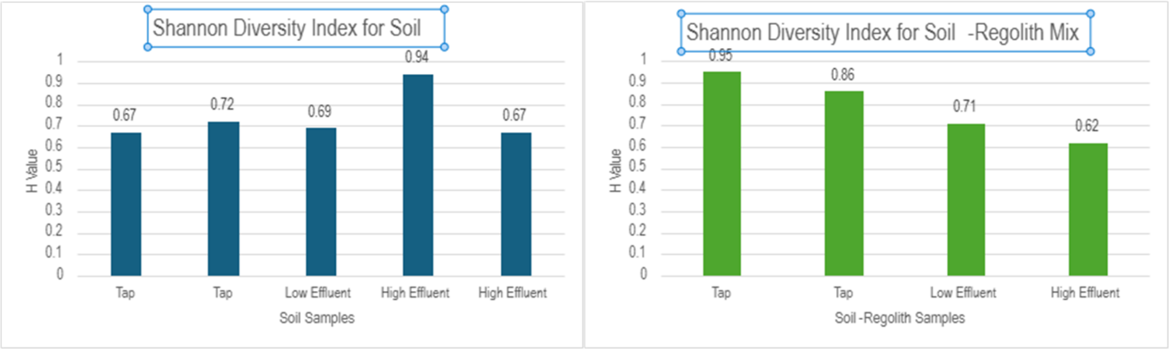
Figure 6



Photographed roots collected from all 12 plants after the growth period, placed side by side for qualitative analysis. Samples labelled 5, 6, 7, and 8 have the longest, thickest roots and are from plants grown with high-effluent water. Tap (1-4) and low-effluent (9-12) roots are similar in lengths and thinness.

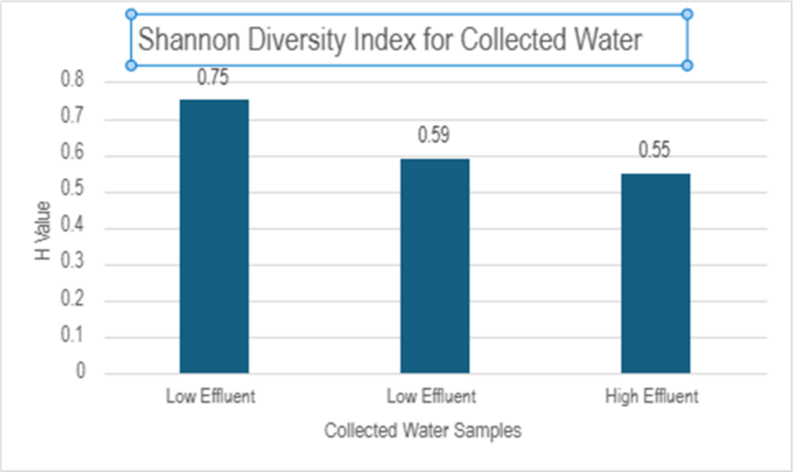
Soil Microbial Diversity Analysis

Figures 7a & 7b



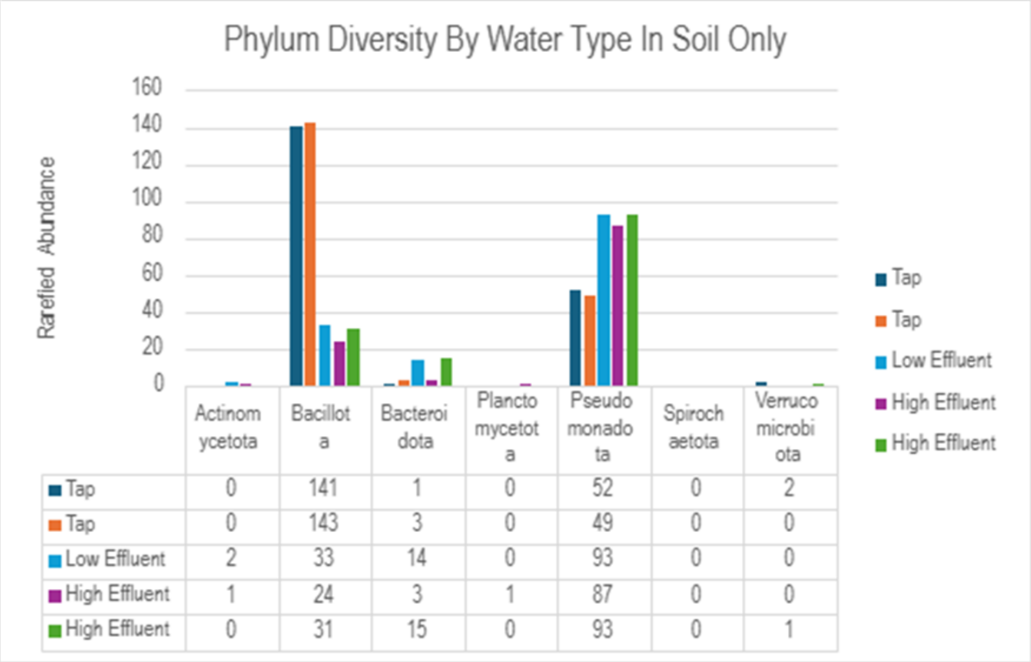
a. Shannon Diversity Index for soil samples, excluding one pot with a shortened growth period. One high-effluent sample is higher than the rest, but the majority of samples trend around 0.7.
b. Shannon Diversity Index for soil-regolith mix samples, excluding two pots with a shortened growth period. Tap water samples in soil-regolith mix have higher H-values than in soil only while collected water samples remain stable.

Figure 8



Shannon Diversity Index for collected water samples, excluding one replicate due to a corrupted file in metabarcoding. One replicate of low effluent water is higher in diversity than the other, which is closer to the high effluent measurement. In general, the collected water samples have similar levels of diversity.

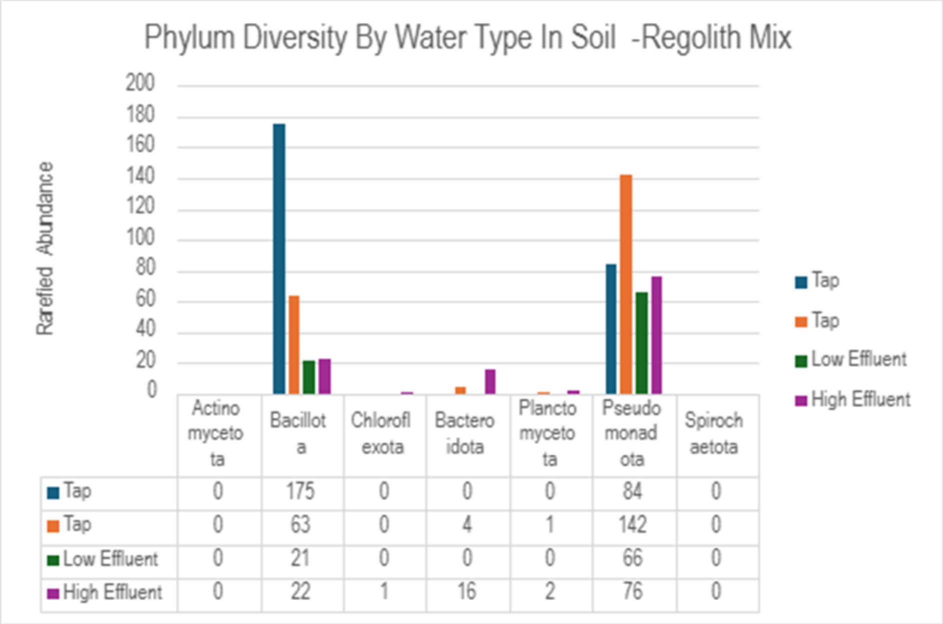
Figure 9a



	Actinomycetota	Bacillota	Bacteroidota	Planctomycetota	Pseudomonadota	Spirochaetota	Verrucomicrobiota
Tap	0	141	1	0	52	0	2
Tap	0	143	3	0	49	0	0
Low Effluent	2	33	14	0	93	0	0
High Effluent	1	24	3	1	87	0	0
High Effluent	0	31	15	0	93	0	1

Bacteria phylum diversity by water type in 100% soil samples showing higher amounts of Bacillota in tap water and higher amounts of Pseudomonadota in collected water samples, excluding one pot with shortened growth period.

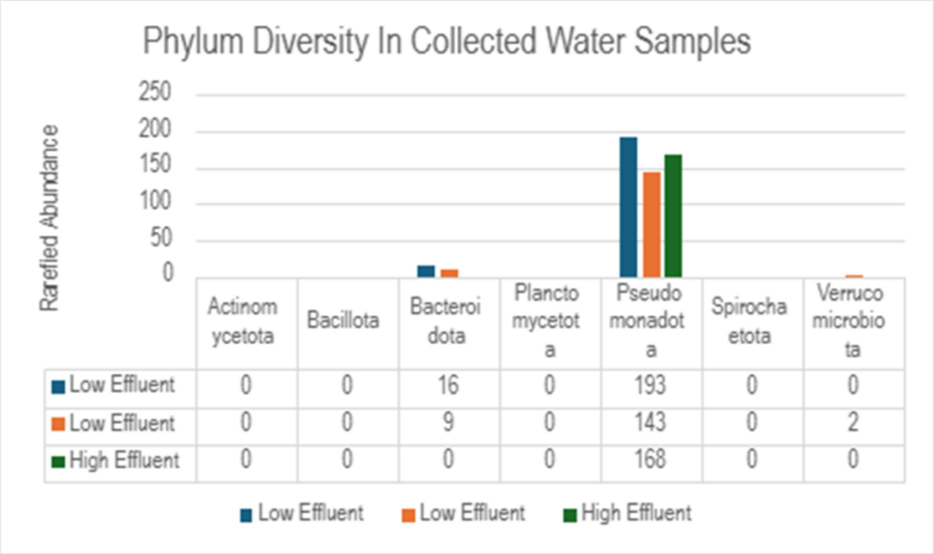
Figure 9b



	Actinomyceta	Bacillota	Chloroflexota	Bacteroidota	Planctomyceta	Pseudomonadota	Spirochaetota
Tap	0	175	0	0	0	84	0
Tap	0	63	0	4	1	142	0
Low Effluent	0	21	0	0	0	66	0
High Effluent	0	22	1	16	2	76	0

Bacteria phylum diversity by water type in soil-regolith mix samples showing higher amounts of Bacillota in tap water and higher amounts of Pseudomonadota in tap water, with similar trends across collected water samples, excluding two pots with shortened growth period.

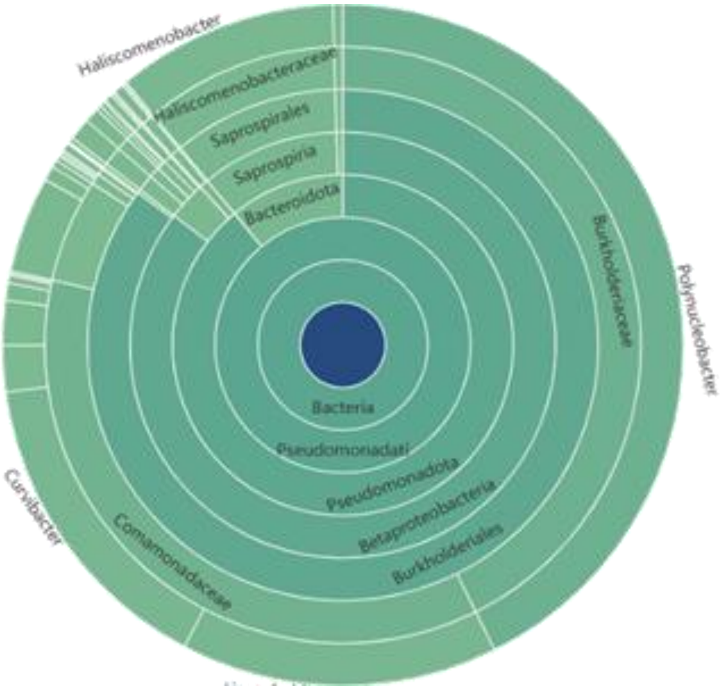
Figure 10



	Actinomycetota	Bacillota	Bacteroidota	Planctomycetota	Pseudomonadota	Spirochaetota	Verrucomicrobiota
🟦 Low Effluent	0	0	16	0	193	0	0
🟧 Low Effluent	0	0	9	0	143	0	2
🟩 High Effluent	0	0	0	0	168	0	0

Bacteria phylum diversity in collected water samples showing higher levels of Bacteroidota in low-effluent water and similar amounts of Pseudomonadota across collected water, excluding one replicate due to a corrupted file in metabarcoding.

Figure 11a & 11b



a. Sunburst graph depicting the 856 identified bacteria present in one replicate of low-effluent collected water. Most bacteria are Polynucleobacter (6.84%), Limnohabitans (2.42%), and Curvibacter (2.42%). 4,483 counts are unidentified.
b. Sunburst graph depicting the 162 identified bacteria in the second replicate of low-effluent collected water. The abundance per bacterium is consistent with Figure 11a, with a slight increase in Curvibacter. 1,245 counts are unidentified.

Figure 11c

Sunburst graph depicting 371 identified bacteria present in high-effluent collected water. There is a large increase in Liminohabitans (7.58%) composition compared to low-effluent water. 2,755 counts are unidentified.

Discussion

Among the data collected, there was a clear trend in growth favoring high effluent plants. The high effluent plants followed expected trends with higher height, weight, and longer roots, but defied our expectations with less chlorophyll than low effluent plants. The high and low effluent plants were surprisingly similar in microbiome diversity and abundance, whereas tap water showed unexpected, elevated levels of Bacillota.

Final Plant Growth and Health Measurements

When compared within the same growth medium in addition to growth period, plants given high-effluent water were found to be outperforming in height. Every high-effluent plant exceeded the height of their counterparts. Coinciding with this, plants treated with high-effluent water were observed to be substantially heavier than plants treated with either low-effluent water or tap, resulting in the largest crop produced by volume and by mass, independent of growth period or substrate.

The impact HE water had on the plants is even apparent when observing differences in the roots collected, with the most robust and furthest reaching belonging to the downstream high-effluent condition. This suggests that for a more desirable harvest, water that is high in effluent might serve as the best option as opposed to heavily treated water, since it would promote the greatest yield of food crops.

The only metric tested that doesn't seem to support this idea is the chlorophyll content. If chlorophyll concentration were to follow the trend of the dependent variables previously mentioned, it would have also shown elevated levels in the high-effluent samples. However, counter to this expectation, HE samples were shown to have the lowest chlorophyll count in nearly all comparisons made. This can possibly be explained by the fact that HE samples were measured at a single value (n=1), while others were combined with their replicates (n=2) to produce adequate media for the extraction process. Further optimization of samples and plant variety is necessary to confirm.

Soil Microbial Diversity Analysis

Positive results for the 16S gene confirm the presence of bacteria, and upon further analysis we have identified two most prominent phyla: Bacillota and Pseudomonadota.

The Shannon Diversity Indices shown in Figures 7-8 indicate low variation between collected water conditions at the phylum level, yet plants grown with high-effluent water consistently demonstrated increased growth. High-effluent and low-effluent are similar in diversity rarified abundance while tap water is much higher in two phyla, supporting that differences in plant growth are not artifact. This could possibly be explained by differences in microbiomes at the genus level. Figure 11 illustrates the differences in composition from identified bacteria, where we can see one glaring deviation. High-effluent water, without a replicate, has nearly twice the amount of Limnohabitans (phylum Pseudomonadota) by percent composition compared to both low-effluent replicates combined.

Limnohabitans are a gram-negative bacterium, with thinner, wavier cell walls than their gram-positive counterparts. They are generally more resistant to antibiotics and have a structure called porins in the outer membrane which allow nutrients to flow through channels, allowing a broader range of enzymes with metabolic functions that help digest organic molecules for nutrient cycling in their environment.[8] Inversely, tap water was extremely abundant in Bacillota, a gram-positive bacterium commonly found in the human gut.[9] This disparity is likely due to chlorination in the tap water treatment process that kills other bacteria, allowing Bacillota to thrive in tap water and the soil it is applied to. Despite the majority of knowledge on Limnohabitans pertaining to aquatic environments, the increased range of Limnohabitans' function could make them key components in the development of early microbial communities in rock-like regolith systems.[10] Further research should be conducted to study the differential bacteria killing behaviors of chlorine, bacterial diversity of wastewater effluent in true closed systems such as SCWO, as well as the role of Limnohabitans in soil.

Conclusion

To be confident in this first round of results, our data should be replicated with consideration of the limitations in the initial experimental procedure. During experimental design, it is imperative to note the relatively short 6-to-10-day germination and, by comparison, long 2-year maturation of spinach. Because the experimental period was limited to nine weeks, plant growth was stopped prior to optimal growth. A longer growing period would show more definite results, either supporting a direct relationship between high effluent water and plant growth, or that trends become less apparent as growth continues.

Some plants developed a fungal disease through the process of damping off, where seedlings die early due to overwatering. The fungi may have affected those pots' microbiome compositions by facilitating growth of specific microorganisms. The reseeding of dying plants led to differences in germination periods, meaning some samples were incomparable to others, as they had different amounts of time to grow. North-facing windows did not provide as much light and warmth needed by the plants, causing some to wilt, impacting the observed height trends and chlorophyll concentrations. It is possible that the location of the plant set up being in a classroom influenced the soil microbiomes of our plants through human interferences such as foot traffic and activity or potential contamination from breath and saliva.

Germination could be optimized by planting more seeds and introducing heating mats from the onset of planting. Implementing heat mats resulted in a higher rate of successful germination and greater longevity for the second sprouts compared to the first plants, which failed during the 5th week. More seedlings grown over a longer period would rationally provide more replicates of each condition to support stronger data groups in the event of plant disease, poor genetics in the seeds, or other outliers.

Substrates were autoclaved before planting seeds, meaning that the soil was sterilized and microbiomes gradually increased to a maximum of 77 species. If the plants had started in non-sterile soil or were allowed to develop longer, it is reasonable to expect far more species richness. Further research should be conducted to determine exactly how much of a microbiome autoclaving removes from soil, and how many species of bacteria may be introduced to sterile soil via water. In future experiments regarding microbial diversity and abundance, a larger DNA library could be supplied during sequencing with diverse flow cell adaptors to potentially increase the number of identified reads and processing bandwidth.

To minimize some of the complications in future experimentation, reducing the watering frequency by using a wicking system—a self-watering technique transporting water to plant leaves via capillary action—could be beneficial. Better storage of the water in bins in a dedicated abiotic space could also preserve their initial microbial diversities, making the replication of high and low wastewater effluent more accurate.

Despite the challenges lunar agriculture faces, our data shows a promising path forward to supporting plant growth on the moon. After several weeks of growth, it can be determined that water high in effluent increasingly supports the health of spinach plants and soil. With even longer growth periods and consistent testing, we could even infer the reproductive rate of microorganisms introduced to regolith from water for sustainable soil colonization and food crop optimization. Using wastewater to our advantage, the dusty regolith of the moon may be turned into a viable substrate with a microbiome that allows plants to grow, and food to become accessible in outer space. With the life-sustaining support of farms on the moon, missions for lunar colonization and even further beyond earth become a possibility.

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