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TITLE: Are Microbial Community Inherently Unstable?

ABSTRACT:

A well-mixed microbial community fed with different food supply rates was analyzed over a 24 day period in 3 L chemostats by changing the dilution rate in increments from 0.1 d^{-1} (for 15.5 days) to 1.0 d^{-1} (for 5.2 days) to 10 d^{-1} (for 3.1 days). Amplicon sequencing of 16s rRNA was used to determine the composition of a microbial community at approximately four time points at each dilution rate. By comparing the composition within a dilution rate using beta diversity, we determine the stability of the community under the given dilution rate. We found that the microbial community was more stable under high food supply versus low food supply. Therefore, this experiment supports the hypothesis that food supply rate is one of the factors controlling microbial community dynamics.

INTRODUCTION:

Understanding the factors that determine ecosystem stability has been one of the main challenges for ecologists from many years. Knowledge of the conditions that affect stability is needed to determine the effects of external parameters on habitats and is valuable for environmental management and biotechnological applications. Many studies have looked at the microbial community dynamics under different conditions¹². In this experiment, the variable was the food supply rate. To determine the microbial composition, operation taxonomic units (OTUs) were determined by V4 plus V5 amplicon sequencing of 16s rRNA. We hypothesized that food supply rate is one of the elements that drive microbial community dynamics. For example, under low food supply, the community will be unstable and under replete food supply, the community will be more stable. This experiment is designed to use chemostats as a reactor to control the food supply rate.

¹ Atlas, R. M., A. Horowitz, M. Krichevsky, and A. K. Bej. 1991. Response of microbial populations to environmental disturbance. *Microbiol. Ecol.* 22: 249–256.

² Borneman, J., and E. C. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* 63:2647–2653.

METHODS:

Siders Pond sampling:

The water sample for this experiment was collected from Siders Pond on Oct. 30th, 2018. The water was collected 2.5 m below the surface and was passed through a 300 μM filter. At 2.5m, the condition of the water were: temperature = 12.88°C, specific conductivity = 9.744 mS/cm, salinity = 5.48, LDO (mg/L) = 9.93, LDO (%) = 96.5, pH = 7.09 and PAR = 19 $\mu\text{moles m}^{-2} \text{ s}^{-1}$.

Chemostat setup:

After water sample were collected from Siders Pond, experiment was conducted in duplicate 3 L chemostats; henceforth MC1 and MC2 will refer to chemostat 1 and chemostat 2, respectfully. The water in the chemostats was kept well-mixed using a paddle impeller spinning at 10 RPM. Chemostats were run in a growth chamber set to 25 °C and in the dark. The chemostats were fed from two reservoirs with one containing just inorganic nutrients and the other only carbon substrates. The concentrations in the nutrient feed consisted of 15 μM KNO_3 , 2 μM HK_2PO_4 , 100 μM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and was raised to 3 ppt salinity with Instant Ocean™. The carbon feed was composed of 5 mM glucose, 5.97 mM xylose, 20.61 mM methanol, 10.83 mM ethanol and 16.75 mM acetate. Medium was fed at three different dilution rates (D): $D = 0.1 \text{ d}^{-1}$, 1 d^{-1} and 10 d^{-1} , where dilution rate is volumetric flow rate divided by chemostat volume. At $D = 1 \text{ d}^{-1}$, the carbon to nutrient ratio was 1: 100. Due to an bug in the pump software at $D = 1 \text{ d}^{-1}$ and $D = 10 \text{ d}^{-1}$, the carbon to nutrient ratio was 1:10.

Chemostat Sampling:

Four samples were collected when the dilution rate was at 0.1 and 1 d^{-1} , and 5 samples were taken at the 10 d^{-1} dilution rate. Additionally, 1 sample was taken from the media feed. $D = 0.1 \text{ d}^{-1}$ was run for 15.5 days; $D = 1 \text{ d}^{-1}$ was run for 5.3 days; and $D = 10 \text{ d}^{-1}$ was run for 3 days.

Nutrient sampling for NO_3^- , PO_4^{3-} and NH_4^+ :

Samples were collected from chemostats by 50 mL syringes and were passed through Whatman GF/F filters and frozen at -20 °C until analysis.

Dissolved organic carbon (DOC) sampling:

Samples were GF/F filtered and is preserved by adding 100 uL of 43% phosphoric acid into 24 mL of water sample.

Bacteria cell counts sampling: Sample water was collected and preserved at a final concentration of 3.6% Formaldehyde. Samples were stored under 4°C.

DNA sequencing sampling: Samples were filtered onto a 0.2 um polyethersulfone (PES) filter. To collect enough microbial biomass for DNA sequencing, we filtered as much sample water as we could until the filter clogged. The filters were stored at -80°C prior to amplification and sequencing.

Gas measurements:

pH, CO₂, O₂ and dissolved oxygen were measured by on-line for each chemostat and recorded once every hour.

Water/Nutrient analyses:

Nitrate and phosphate concentrations were analyzed on a Lachat (QuickChem 8500)³. Ammonia concentration was analyzed by a modification of the phenol-hypochlorite method on a spectrometer⁴. Concentration of DOC was analyzed by DOC analyzer. CHN was analyzed on an elemental analyzer.

Microbial samples:

Bacteria cell counts were determined using DAPI count⁵ method by using black 0.2 uM filters. A epifluorescence Axio Imager 2 Zeiss microscope with 100x objective and configured with DAPI filter sets was used for cell counts. For DNA samples, DNA was extracted using the PowerSoil Kit following the manufacture's methods. Amplicon sequences of the V4V5 regions of the 16S gene were generated and sequenced at the Bay Paul Center, MBL. Sequence data were assigned taxonomic identification via VAMPS2 (vamp2.mbl.edu) pipeline.

Statistical analyses:

The nutrient uptake rate, U, was calculated by equation:

³ Griess, P., 179. Bemerkungen zu der Abandlung er H. N. Weselsky und Benedikt 'Über einige Azoverbindungen'. *Ber. Dt. Chem. Ges.*, Bd. I2, pp. 426-8 (J. Murphy, 1962)

⁴ Solarzano, L. (1969). Determination of ammonium in natural waters by phenol hypochlorite method. *Limnol. Oceanogr.*, 14:799-800

⁵ Porter, K., Feig, Y. (1980), The use of DAPI for indentifying and counting aquatic microflora. *Limnol, Oceanogr.*, 15(5), 1980, 943-948

$$U = D * (C_{in} - C_{out}) / V,$$

Where D is dilution rate, C_{in} is the concentration of nutrient in nutrient feed, C_{out} is the concentration of nutrient in collected sample and V is chemostat volume.

For DNA sequence analysis, the alpha diversity was calculated from Shannon method. The beta diversity was calculated from Bray-Curtis method. We calculated the bray-curtis dissimilarity within each dilution rate across sample points. Then we compared this dissimilarity value across dilution rates using a one-way ANOVA. For alpha and beta diversity, alpha diversity is the diversity within the community itself, so it tells how many different species are within that community; beta diversity compared across different communities, so it tells how one microbial composition is compares to another. We use beta diversity as the indicator of the stability of the microbial communities in chemostats, because we want to compare the communities within each dilution rate and get the similarity of them. At a given dilution rate, the more similar communities are from one time point to the next, the more stable the community is. The larger the beta diversity, the more dissimilar between microbial communities in each dilution.

RESULTS:

The online system collected data from the Oxigraf O₂ and CO₂ gas analyzer and the DO and pH probes to monitor the CO₂, O₂, dissolved oxygen (DO) and pH in both chemostats (Figure 1). In both chemostats, CO₂ production and O₂ consumption increased when dilution rate increased and at dilution rate = 10 d⁻¹ (Figure 2 and 3), the O₂ and CO₂ concentrations oscillated and did not reach to a steady state in MC2 (Figure 3). pH also decreased as dilution rate increased (Figure 2,3). DO remained stable at 0.1 and 1 d⁻¹ dilution rates and decreased at dilution rate of 10 d⁻¹ (Figure 2, 3).

For nutrient uptake (nitrate and phosphate), both of them increased as dilution rate increased (Figure 4, 5). Oxygen uptake also exhibits the same trend that it increased with the increasing dilution rate (Figure 6). The bacteria production rate also increased as dilution rate increased (Figure 7). Concentration of ammonia, which is an indication of bacterial grazer activity, increased as the dilution rate increased except the first sample (Figure 9). The particular organic carbon and nitrogen was decreasing as dilution rate went up (Figure 10, 11).

We were unable to assess DOC uptake rate because the feed concentration was lower than the sampled DOC from the chemostats for several time points; consequently, we only show the DOC concentration (Figure 8).

For DNA sequencing result, things are little different in the two replicates. At dilution rate of 0.1 d^{-1} , both chemostats had very variable microbial composition, and at dilution rate of 1 d^{-1} , a few species were dominating the community (Figure 13, 14, 15, 16). At the dilution rate of 10 d^{-1} , in chemostat 1, one species (oceanspirillales) dominated, but in chemostat 2, several species exhibited oscillatory dynamics (Figure 13, 14, 15, 16). Alpha diversity decreased as dilution rate increased (Table 1). Beta diversity decreased as dilution rate increased (Figure 17) which means that the similarity of microbial community increased as dilution rate increased. ANOVA test showed that the three groups of beta diversity are significantly different (Table 2).

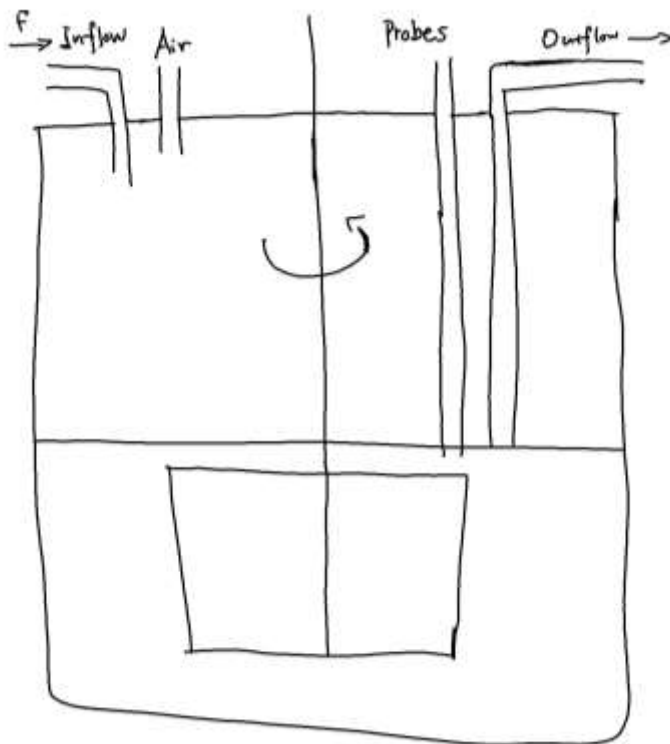


Figure 1. Concept map of a chemostat

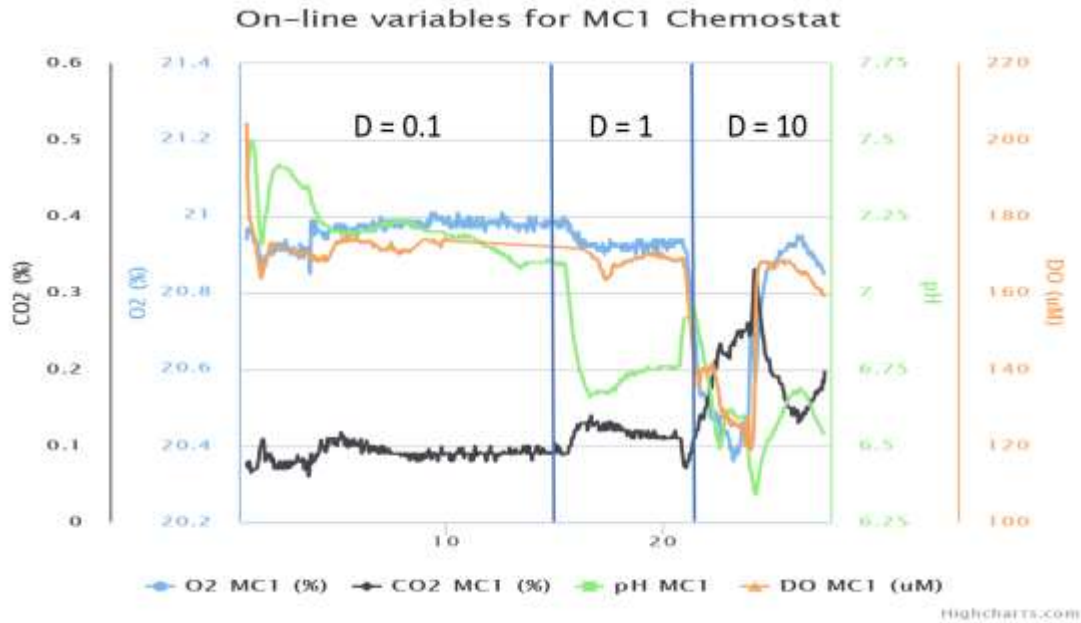


Figure 2. Online dataset of chemostat 1. CO₂ and O₂ percentage of chemostat air, pH of chemostat water, and concentration of dissolved oxygen (uM) vs. experiment days.

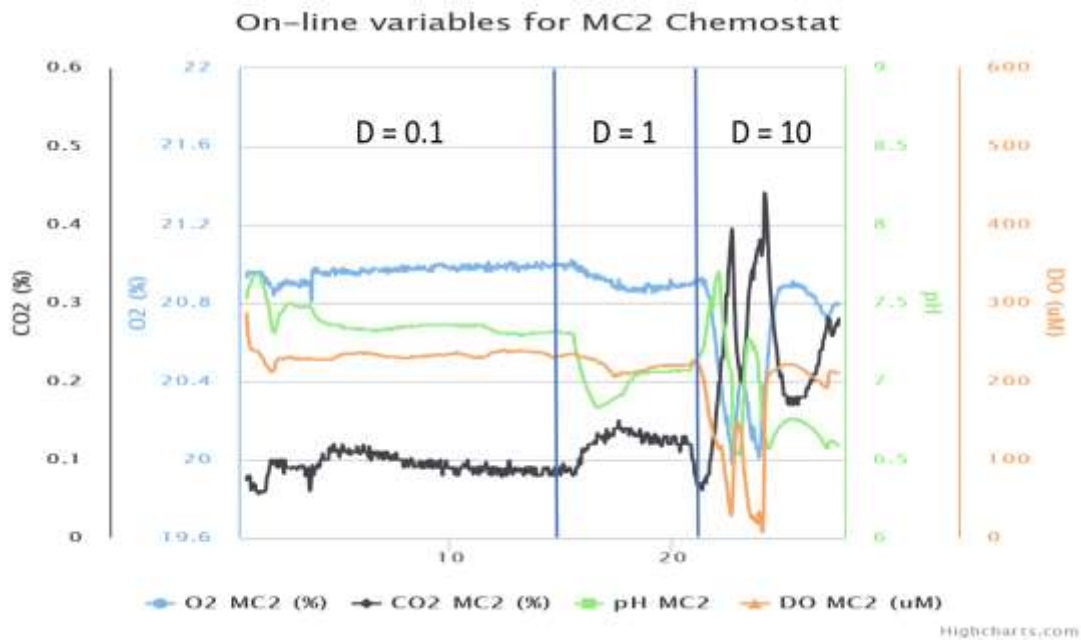


Figure 3. Online dataset of chemostat 1. CO₂ and O₂ percentage of chemostat air, pH of chemostat water, and concentration of dissolved oxygen (uM) vs. experiment days.

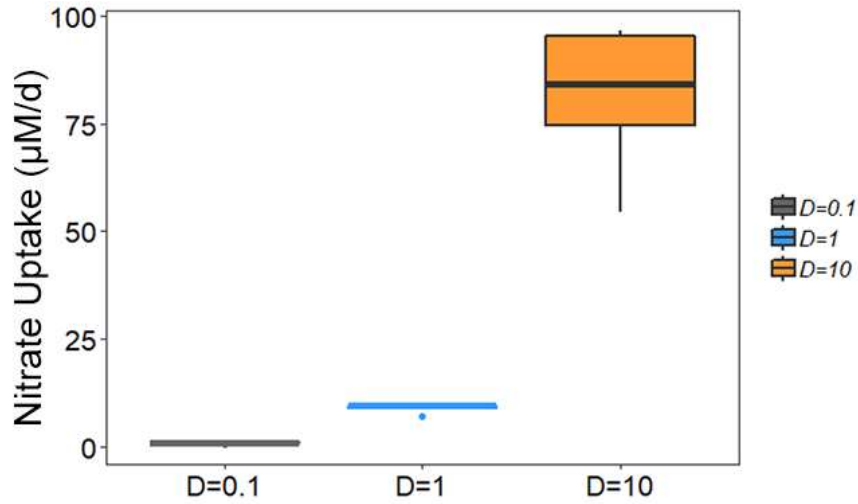


Figure 4. Nitrate uptake (µM/d) in each dilution rate

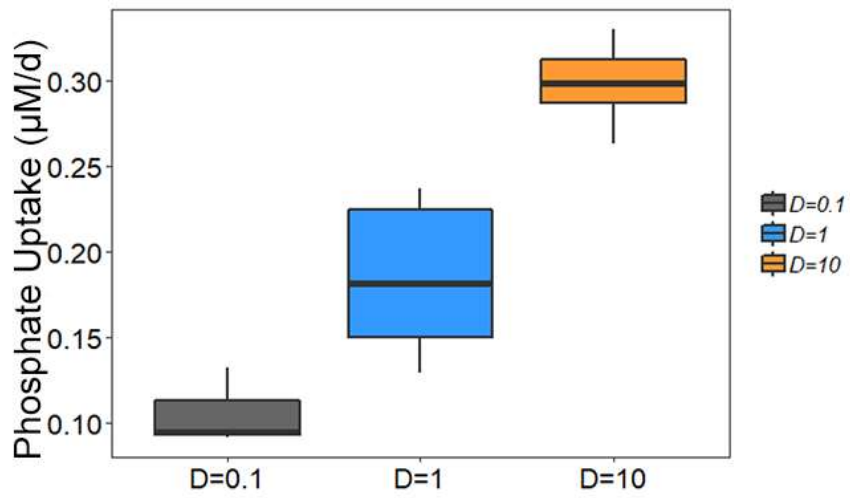


Figure 5. Phosphate uptake (µM/d) in each dilution rate

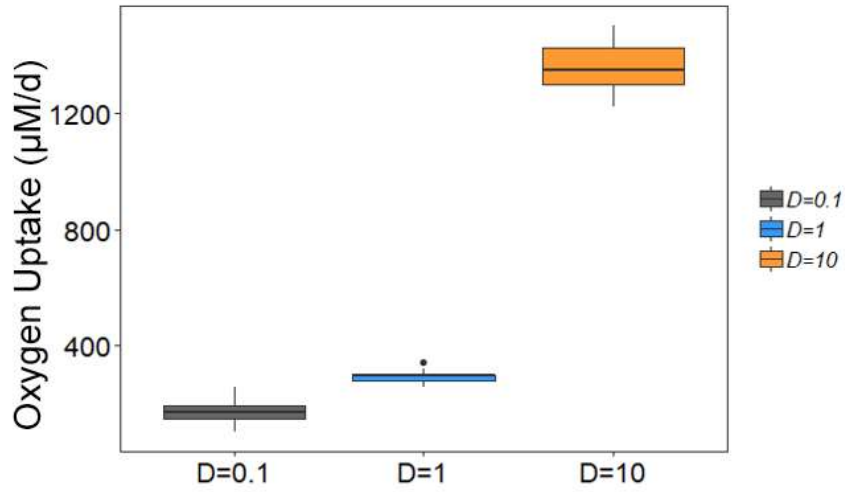


Figure 6. Oxygen uptake ($\mu\text{M/d}$) in each dilution rate

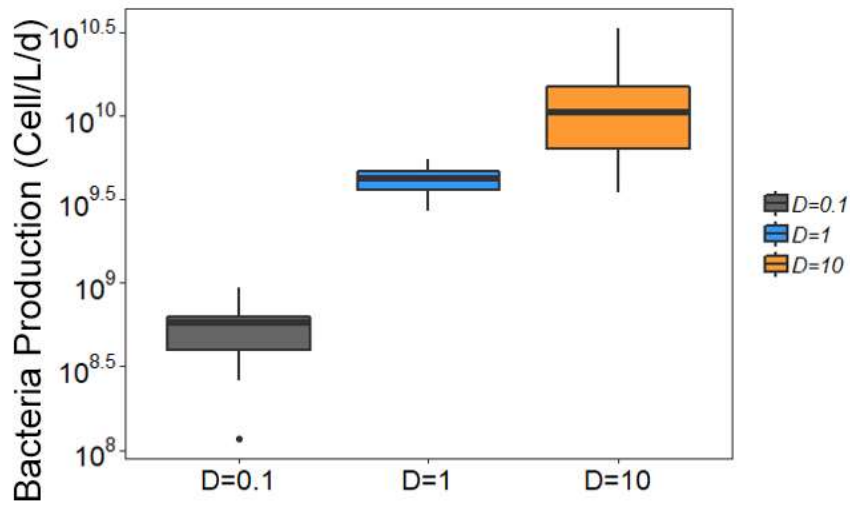


Figure 7. Bacteria production (cell/L/d) in each dilution rate calculated from bacterial concentration and dilution rate of medium feed.

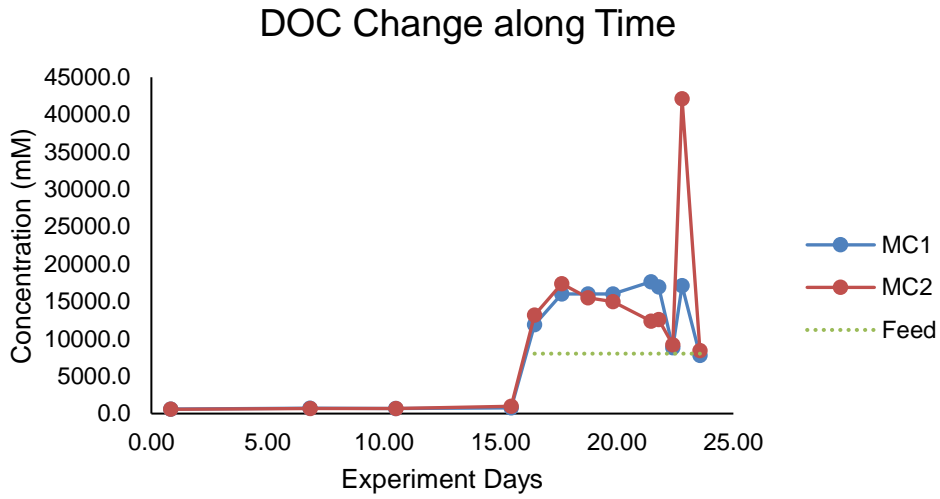


Figure 8. Concentration of dissolved organic carbon (mM) versus experiment time.

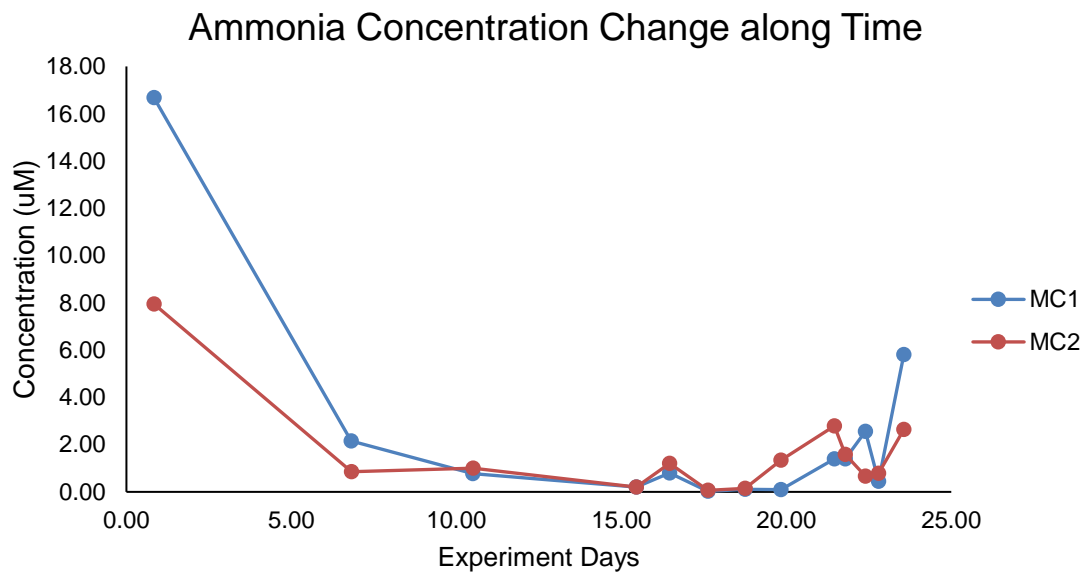


Figure 9. Concentration of ammonia (uM) change among experiment days

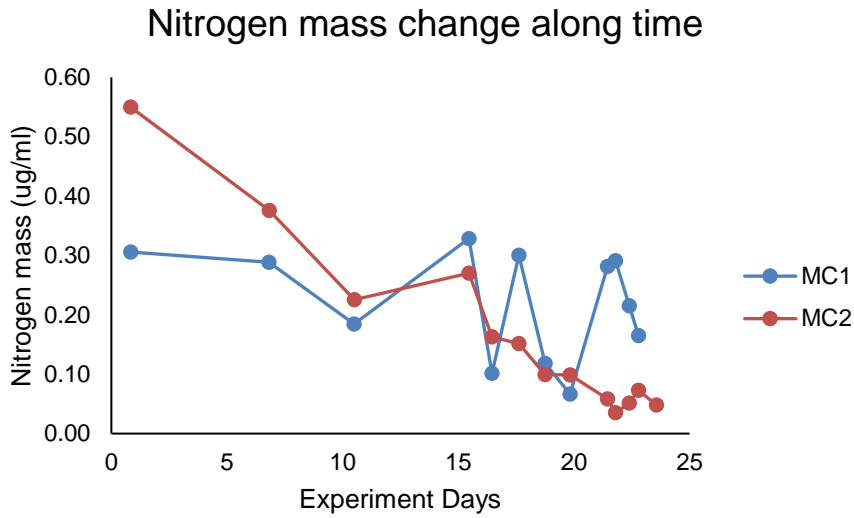


Figure 10. Nitrogen mass (ug/ml) change among experiment days

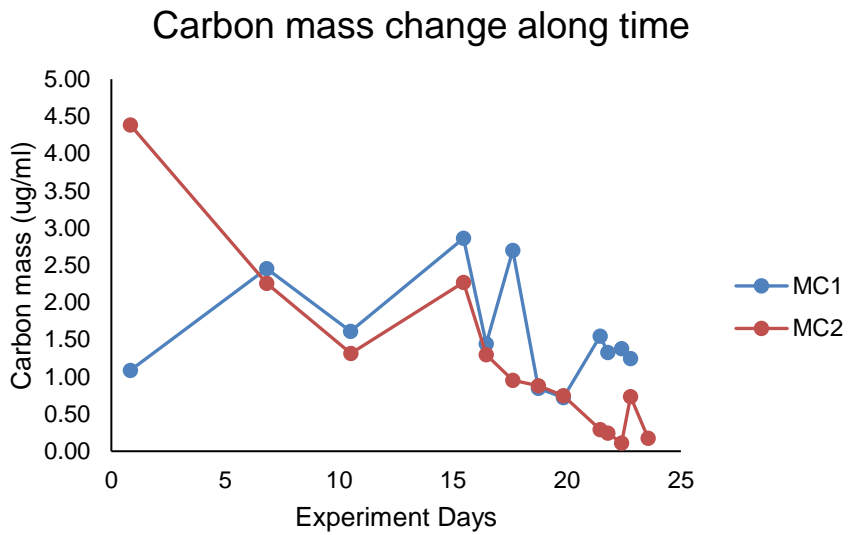


Figure 11. Carbon mass (ug/ml) change among experiment days

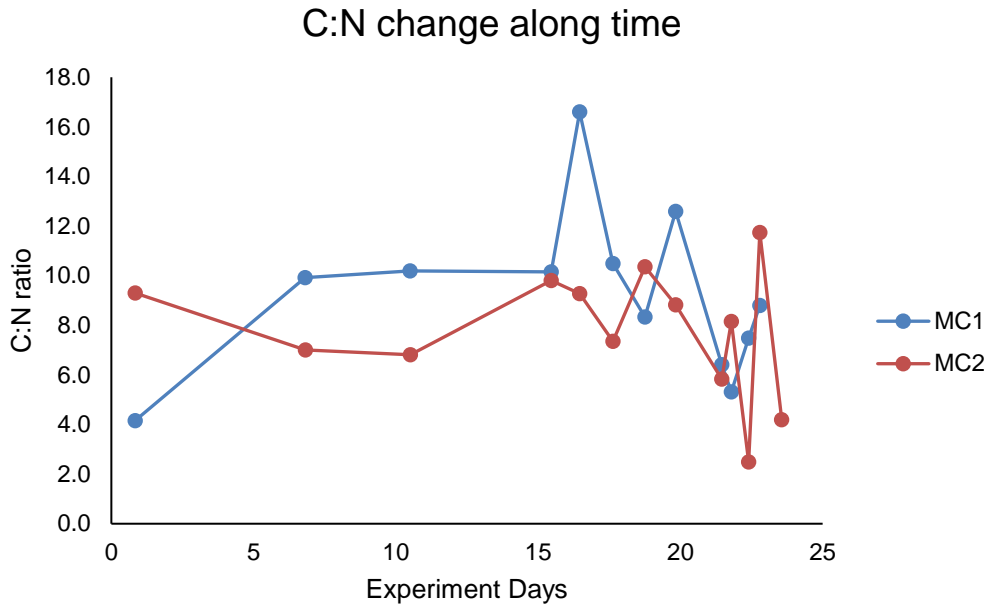


Figure 12. C:N ratio change among experiment days

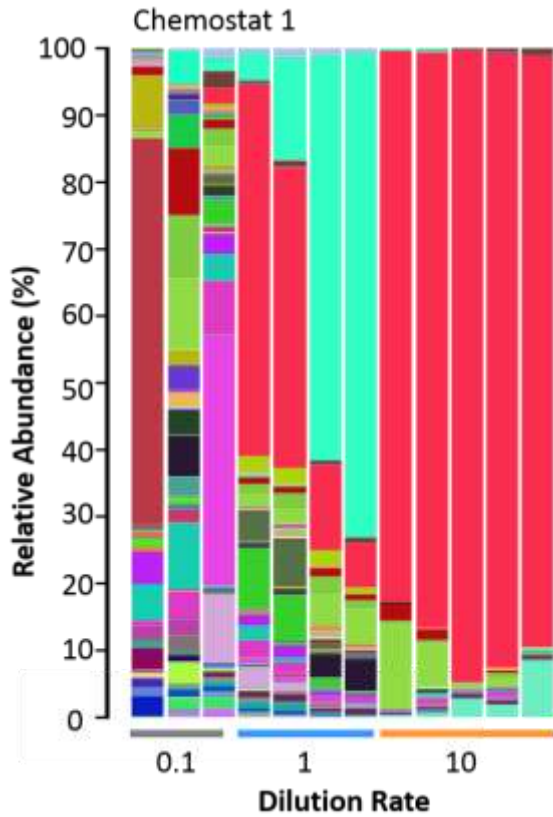


Figure 13. Relative abundance of each operational taxonomic unit in chemostat 1 in samples

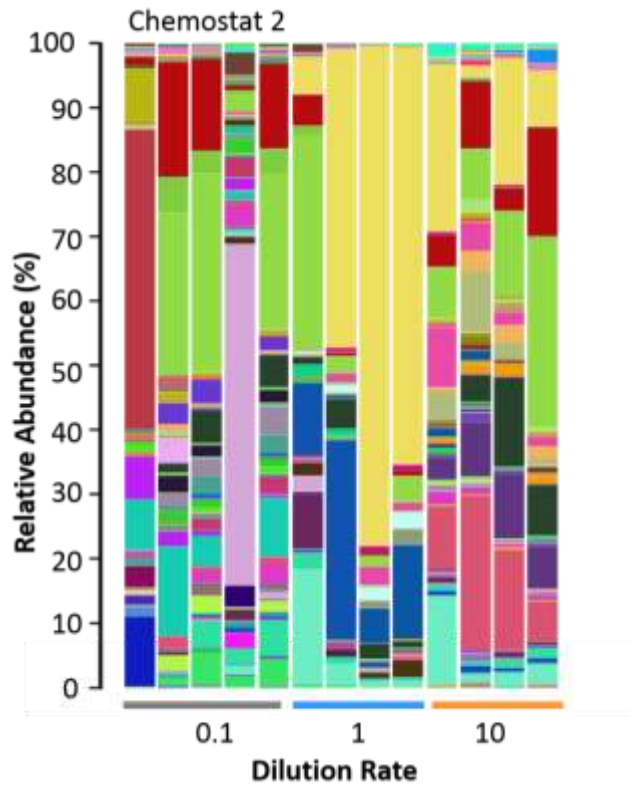


Figure 14. Relative abundance of each operational taxonomic unit in chemostat 2 in samples

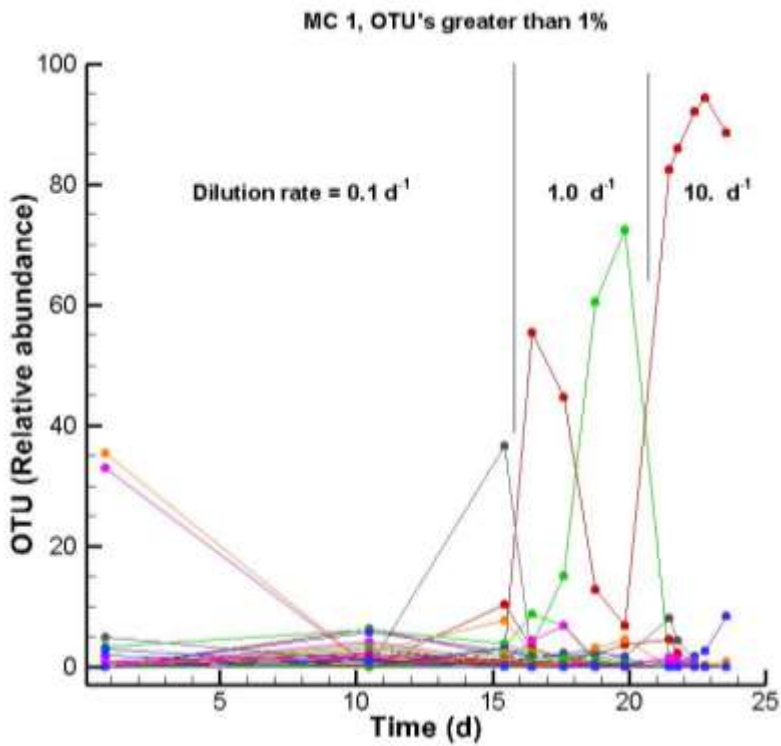


Figure 15. Relative a abundance of operational taxonomic unit greater than 1% in chemostat 1

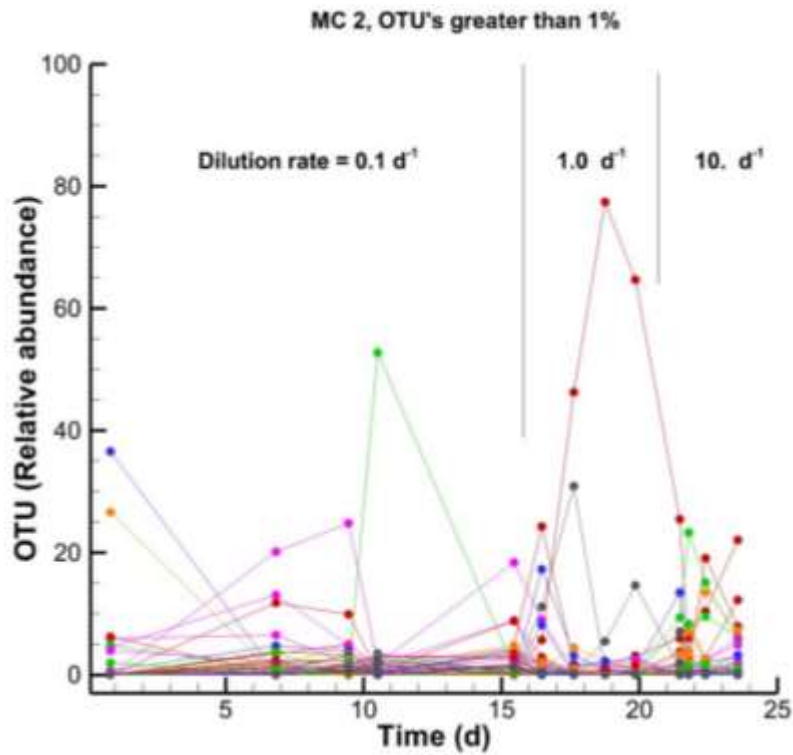


Figure 16. Relative a abundance of operational taxonomic unit greater than 1% in chemostat 2

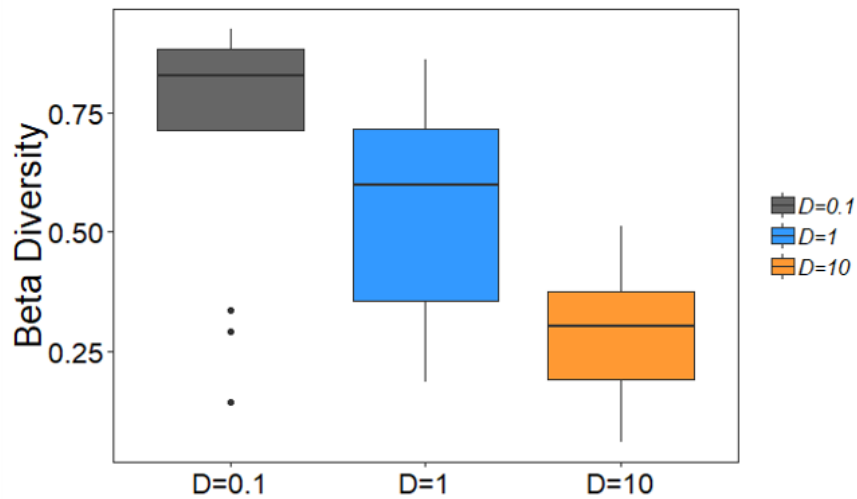


Figure 17. Beta diversity in each dilution rate.

Dilution Rate	D = 0.1 d ⁻¹	D = 1 d ⁻¹	D = 10 d ⁻¹
Alpha Diversity	3.85 ± 0.63	2.57 ± 0.63	2.25 ± 1.74

Table 1. Alpha diversity in each dilution rate

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
D = 0.1	13	9.14125	0.703173077	0.070328407
D = 1	12	6.46933	0.539110833	0.060974228
D = 10	16	4.69214	0.29325875	0.016676163

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	1.237908634	2	0.618954317	13.3274401	4.11589E-05	3.244818361
Within Groups	1.764799833	38	0.046442101			
Total	3.002708467	40				

Table 2. ANOVA test of beta diversity

DISCUSSION:

Examining Figures 2, 3 and 6, we can see that the consumption of O₂ increased with the increase of dilution rate. So, we know that the microbial community took more oxygen to grow as dilution rate raised. CO₂ production should show the same trend because microbes consume O₂ and produce CO₂. The production of CO₂ did increase as dilution rate increased (Figure 2, 3). But quantitatively, the O₂ consumption and CO₂ production cannot be reasonably explained by respiration. By looking at pH data, pH was decreasing as dilution rate increases. We realized that some CO₂ dissolved in water and form carbonate acid which caused the decrease of pH (Figure 2,3). Therefore, it is also hard for us to calculate carbon with the equation: particulate organic carbon (POC) + CO₂ production + dissolved organic carbon (DOC) = carbon from the media feed carbon.

Additionally, the nutrient uptake rate (nitrate and phosphate) also increased with the increase of dilution rate (Figure 4, 5). The bacteria production rate also increased as dilution rate increased. Therefore, we can see that the increase of dilution rate represents the increase of food resource supply and microbial uptake, which is our premise in the hypothesis. For more information, the increase of ammonia concentration with the increase of dilution rate also showed the increase of bacteria production because ammonia is produced by grazers (the high ammonia concentration in the first day should not count since the water is originally from Siders pond) (Figure 9).

As for stability of a community, we use beta diversity as an indicator. Because beta diversity decreased as dilution rate increased, it is more stable in higher dilution rate (Figure 17). Since higher dilution rate represents higher resource supply, the community is more stable when resource supply increases. The result of this experiment did not go against our hypothesis, it supported our hypothesis.

There is some improvement we can do for future experiments. First of all, we should collect more samples for nutrient analysis (nitrate, phosphate, ammonia, DOC, CHN) to monitor the microbial community more. Because in this experiment, we were able to pick up on some patterns but not at a high enough resolution. Also, we did not keep collecting media feed sample to make sure it is going at the concentration we wanted. Secondly, we should use more accurate machine to examine our samples. In this experiment, the DOC data seems inaccurate. Thirdly, instead of using the same media and increasing the flow rate, we can try to keep the flow rate same but increasing the concentration of the media. In that case, we can eliminate the probability of high flow rate flush out communities quickly.

In conclusion, our results show that food supply rate is one of the factors that drive the microbial community dynamics. The increase of nutrient and gas uptake showed when dilution rate went up, food uptake also went up. The beta diversity data showed that as dilution rate went up, the microbial community is more stable. Therefore, food supply rate is one of the factors that drive the microbial community dynamics; the community is more stable when food supply is replete.

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