THE IMPLICATIONS OF THE SPATIAL DISTRIBUTION OF NITRIFIERS IN ADVANCED LIFE SUPPORT SYSTEMS

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Abstract:

Human urine represents a major problem with respect to ammonia vapors in the crew cabin and the amount of water necessary for human hydration (2 kg/person/day). Bacterial nitrification represents an important element of the solution to water resource limitations in extended space flight missions. Nitrifiers convert potentially volatile NH$_3$ to NO$_3^-$ eliminating the problem of volatile ammonia in the cabin, and the NO$_3^-$ is potentially removable by another biological process, denitrification, leaving water that can be easily finished by physical-chemical means for reuse by the crew. Nitrifiers form biofilms on reactor surfaces, and the distribution of nitrifiers in biofilms (which can affect the efficiency of the process) is controlled by gradients of O$_2$ and NH$_3$. Changes in organism distribution can impact the rates at which the two genera of nitrifiers (i.e. ammonia oxidizing and nitrite oxidizing bacteria, AOB and NOB, respectively) function. Fluorescent in situ Hybridization (FISH) and microscale electrodes are used to study the distribution of these organisms, precursors, and products within the biofilms. Current results in planktonic cultures show growth and oxidation rates where uniform distribution of microorganisms and nutrient resources are maintained. Cell doubling time for AOB and NOB combined is 1.4 days. Data from these studies will be compared to rates found in biofilms to determine how these biofilms will benefit advanced life support systems.

Introduction:

Water resources are highly limited on extended space flight missions due to cost and space restrictions. Water costs $5,000 per kilogram to launch into Earth’s orbit and the cost increases with distance. An astronaut requires 2 liters or $10,000 of water d$^{-1}$ just to maintain hydration. The overall water requirement per astronaut, including activities such as laundry and bathing, is 11.5 L d$^{-1}$. On an extended space flight mission the cost of shipping the entire water supply would quickly become cost prohibitive. Currently on board the International Space Station (ISS) urine waste is ejected from the cabin or returned to earth for disposal. Urine is approximately 95% water. Recovering this water, in addition to water lost through perspiration will greatly reduce the need to store water on extended space flight missions. In September 2008 the ISS will begin recycling its water through physical and chemical means. However, studies are under way to provide a biological means for waste water recycling.

Biological reactors are an important part of Advanced Life Support Systems (ALSS) since they require little space and are energy efficient. However, there are special challenges associated with engineering biological systems in microgravity environments. In the absence of gravity density based mixing processes, such as convection (Figure 1), cease to occur.

Figure 1: A candle flame in outer space (right) has a distinctly different shape without the updraft created by convection.

In these instances diffusion is extremely important as a means of resource distribution in non-mixed, and even some mixed, systems. Diffusion becomes still more important when delivering a gas to a liquid. Because of the behavior of bubbles in microgravity (see, for example, http://microgravity.grc.nasa.gov/balloon/blob.htm), bubbleless aeration is required to oxygenate a liquid. Systems such as in the Aerobic Rotating Membrane System (ARMS) have been developed to provide aerobic conditions for advanced life support systems (ALSS) that can be used for water purification (Figure 2).
In particular, the ARMS reactor was intended to remove NH$_3$ from urine collected aboard ISS or Mars transit vehicles. ARMS uses a series of Silastic® hollow-fiber membranes to deliver oxygen to an attached biofilm of nitrifying microorganisms (Figure 3).

Biofilms are aggregates of microorganisms that develop on the surface of solid materials and that interact with one another in a variety of ways and for various reasons. The applications of biofilms in science and technology are amazing, however understanding the ecology of the microorganisms which compose the biofilm is important for maximizing the functionality of biofilms. Although the growth of films of organisms on surfaces has been recognized for many decades, scientists have only begun to study the microbial communities that form biofilms within the past 15 to 20 years. Biofilm studies represent a major shift in the way microorganisms are perceived, from independent planktonic cells to organisms co-existing in an aggregate and participating in synergistic interactions such that the biofilm functions at the gross scale like a single organism\textsuperscript{10, 11}.

Rotation of the membrane bundle that the biofilm grows on provides radial mixing of the bulk solution, and thereby maintains a strong positive gradient of NH$_4^+$ to the developing film of microbes. Silastic® is a gas-permeable silicone rubber which is used to provide bubbleless aeration via diffusion. Gas-permeable rubbers with higher diffusivities than water provide minimal resistance to the diffusion of a gas into the aqueous phase\textsuperscript{1-7}. In microgravity environments, bubble-free aeration is essential as bubbles tend to coalesce in the liquid preventing uniform distribution throughout the liquid. In addition, bubble-free aeration of this kind creates opposing gradients within the biofilm where O$_2$ diffuses from the membrane surface into the biofilm, while nutrients and NH$_4^+$ diffuse into the biofilm from the bulk fluid\textsuperscript{14}. It is on the effect of this kind of gradient opposition on the spatial distribution of microorganisms that my research questions focus.

Microbial communities like those of other organisms, tend to organize themselves spatially in response to the distribution of resources in the environment. Communities may organize at landscape scales that have correlation lengths of 10s of meters if the resources are distributed similarly. Alternatively, they may have correlation length scales of meters or centimeters if that is the scale of the distribution of the necessary resources and environmental conditions. Within biofilms we expect to see organisms align themselves along microscale gradients, in particular DO. It is expected that AOB will grow on the surface of the Silastic® and NOB will grow on top of the AOB as depicted in Figure 4.

There are many instances where microorganisms are oriented along gradients in the environment on a large scale. A vertical profile of a lake, for example, has different guilds of microorganisms oriented in space based upon available electron acceptors, light intensity and organic carbon content (Figure 5). Changes in guild type follow changes in reducing potential because the different

![Figure 2](image1.png)

**Figure 2:** The Aerobic Rotating Membrane System (ARMS), a pilot scale project to examine the capability of biological systems to purify water

In particular, the ARMS reactor was intended to remove NH$_3$ from urine collected aboard ISS or Mars transit vehicles. ARMS uses a series of Silastic® hollow-fiber membranes to deliver oxygen to an attached biofilm of nitrifying microorganisms (Figure 3).

![Figure 3](image2.png)

**Figure 3:** At left, a schematic diagram of an ARMS reactor vessel depicts the rotation of the membranes as well as fluid and gas inflows. At right, a picture of biofilms formed on the Silastic® membranes during biofilm harvesting.

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guilds are respiring on the different available electron acceptors, or photosynthesizing under different light conditions.

![Image](image-url)

**Figure 5:** A cross section of Lake Plusse showing the change in organism distribution along a vertical gradient.

The stratification of microorganisms along environmental gradients is also seen in mesocosms such as Winogradsky columns (Figure 6).

![Image](image-url)

**Figure 6:** A schematic diagram taken from Atlas and Bartha depicting the spatial distribution of microorganisms in a Winogradsky column. The organisms are aligned along resource gradients within the column.

Microbes in these columns are strongly stratified based on resource availability. Algae, cyanobacteria, and aerobic heterotrophs grow in the water at the surface of the column. Below this, microaerophilic sulfide oxidizers such as Beggiatoa grow. The uppermost portion of the sand beneath the water takes on a reddish brown hue from the growth of purple, non-sulfur, anaerobic heterotrophs such as the Rhodospirillaceae. Purple sulfur bacteria create a red violet region and beneath this a greenish region composed of green sulfur bacteria forms. At the bottom of the column a black zone forms due to the formation of iron sulfides from the products of sulfidogenic microorganisms.

These studies demonstrate the effect environmental gradients have on the distribution of communities, guilds, and populations of bacteria at visible scales. However, to fully understand the microbial ecology of individual organisms and interactions among organisms at a scale relevant to individual microbes, studies on a microscale level are required. Because liquid cultures tend to mix the microbes whenever the fluid in the container moves, a situation in which the microbes are fixed in place is more amenable to studying the organization of populations and communities in microscale gradients.

The ARMS reactor is an ideal environment for studying the arrangement of organisms within a biofilm. Not only does ARMS support fixed colonies of microorganisms, but these biofilms are composed of nitrifiers. Nitrification involves the sequential biochemical oxidation of ammonia to nitrate, and requires two distinct kinds of bacteria, AOB and NOB, to achieve complete oxidation to nitrate. AOB oxidize NH$_4^+$ to NO$_2^-$:

$$\text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + 2\text{H}^+ + \text{H}_2\text{O}$$  \(\Delta G = 64.7 \text{ kcal/mol NH}_4^+ \text{ oxidized} \)  \(1\)

and NOB oxidize NO$_2^-$ to NO$_3^-$:

$$\text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^-$$  \(\Delta G = 18.5 \text{ kcal/mol NO}_2^- \text{ oxidized} \)  \(2\)

These two kinds of microorganisms, collectively known as nitrifiers, are responsible for the complete oxidation of ammonium from wastewater. DO is a determinant in the distribution of AOB and NOB because both organisms are competing for dissolved oxygen availability is a known stressor for both groups.

The objective of my research is to compare the organization of cells along DO profiles within biofilms under varying circumstances and observed reaction rates with reaction rates observed in planktonic cultures grown in flasks.
Methods:

Section 1, A Breadboard scale bioreactor, flasks and DO gradients:

To examine biofilm characteristics in an environment dominated by opposing gradients, a simplified breadboard-scale reactor that mimics the biochemical functions of the ARMS will be used (Figure 7).

Figure 7: A depiction of the breadboard scale bioreactor showing the two chambers separated by a Silastic® membrane. Oxygen diffuses from the gas chamber into the biofilm, while ammonia diffuses from the bulk fluid.

This reactor is smaller than the ARMS and instead of the hollow tubes uses a flat Silastic® membrane for oxygen delivery. A fluid containing NH$_4^+$ at the concentration seen in urine waste streams (up to 600 mg L$^{-1}$ NH$_4^-$-N) will be passed through the upper chamber, while air (or pure O$_2$) is used to fill the chamber in the bottom. A nitrifying biofilm develops on the submerged side of the membrane. At the scale of the microorganisms, this membrane mimics the opposing gradients experienced by biofilms grown in ARMS (Figure 4).

Membranes are prepared in the laboratory by curing the Silastic® mix between two panes of glass. The thickness of the membrane is controlled by spacers placed between the panes to minimize the distance between them. Once the rubber has cured completely, a section is cut to fit the reactor body and it is cleaned and placed into the reactor. Unlike the hollow fiber membranes in ARMS, the Silastic® sheet provides a larger surface area for sampling without disturbing the organization of the biofilm.

The Silastic® membrane provides oxygen to the biofilm. This results in a DO gradient originating from the opposite direction of the NH$_4^+$ which will be diffusing from the bulk fluid (Figure 4). The DO gradient can be monitored using a microscale DO probe $^{13}$. Reactors were inoculated and then allowed to stagnate for three days to allow the organisms to adhere to the surface of the Silastic® membrane. This is to prevent the organisms from being flushed out of the reactor before they have an opportunity to colonize the membrane. After the three day period fluid flow through the reactor was maintained at 0.001 mL s$^{-1}$. The retention time within the reactor at this flow rate is approximately 13.89 hours. This flow was increased if flow rates did not provide enough NH$_4^+$ for the biofilm to develop across the entire Silastic® membrane. The composition of the fluid passing through the reactor is given in table 1 and is a modified version of the medium used in the ARMS reactor $^{14}$. The phosphate buffer was increased to prevent pH fluctuation and the NH$_4^+$ concentration reduced to 100 ppm NH$_4^+$-N to encourage growth.

For this experiment three flasks were inoculated with nitrifiers harvested from the ARMS reactor at Kennedy Space Center. Flasks were kept on a shaker table to maintain aeration in a darkened room. Flasks were sampled every two to three days. Samples stored for ammonia analysis were frozen and samples stored for acridine orange direct counts were preserved with 4% formaldehyde. Acridine orange was performed according to Hobbie $^{12}$.

**Modified ARMS Feed**

<table>
<thead>
<tr>
<th>Component</th>
<th>g · L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.84</td>
</tr>
<tr>
<td>KHCO$_3$</td>
<td>3.72</td>
</tr>
<tr>
<td>KHSO$_4$</td>
<td>0.80</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2.72</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>5.22</td>
</tr>
<tr>
<td>NH$_3$CO$_3$</td>
<td>0.34</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.82 mL 1M stock solution</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>1 mL stock solution</td>
</tr>
</tbody>
</table>

**FeCl$_3$ Stock Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>g · 100 mL$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_3$</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Table 1: These are the components of the medium used in all bioreactor and flask experiments. This is a minimal medium designed to encourage the growth of autotrophic nitrifiers. The ammonium concentration is 100 ppm NH$_4^+$-N.

Section 2, Colorimetric Techniques:

Colorimetric techniques are used to monitor the production of nitrite and the oxidation of ammonia $^9$.

Section 3, Fluorescent in situ hybridization (FISH):

Biofilm samples were processed using commercially available Fluorescent in Situ Hybridization (FISH) kits, which label ammonia and nitrite oxidizing bacteria with fluorescent probes for identification when viewed under an epifluorescent microscope. FISH probes bind to specific regions of the microorganisms’ ribosomal RNA. The binding region is specific for AOB or NOB making it possible to distinguish between the two organisms when viewed under a microscope.
Results & Discussion:

Growth curve results from cell counts show a slow growth rate, which is expected for nitrifying microorganisms\(^2\) (Figure 8).

![Growth Curve](image)

**Figure 8:** The average number of cells mL\(^{-1}\) increases over time in all three flasks.

An overall decline in ammonium concentrations is observed (Figure 9).

![Ammonia Loss](image)

**Figure 9:** A decline in ammonia is seen among all three flasks being monitored in this experiment. This graph shows the average ammonia concentration over an 8 day period. Losses in ammonia are from ammonia oxidation and a negligible amount due to assimilation.

Cell doubling time can be determined for the flasks using a standard growth curve where \(g\) is the cell doubling time, \(m\) is the abundance of cells and \(t\) is the elapsed time in days.

\[
g = \frac{\ln 2(t_f - t_i)}{\ln m_f - m_i} \tag{3}
\]

Cell doubling times for each flask are depicted in Table 2. The change in ammonia concentration for on cell doubling is only reported for flask one because it is the only one that shows consistent reduction in ammonia.

<table>
<thead>
<tr>
<th>Flask</th>
<th>((g) (Days))</th>
<th>Average Ammonia Loss (ppm NH(_4^+) N d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.7</td>
<td>4.88</td>
</tr>
<tr>
<td>2</td>
<td>12.8</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>2.64</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 2: Cell doubling time for nitrifiers is very slow in planktonic cultures. Most of the ammonia removal from the bulk fluid should be due to ammonia oxidation because it is assumed that assimilation values are small.

The doubling times presented in Table 2 represent the cell doubling time for both AOB and NOB present in culture flasks. Since NOB garner even less energy from the oxidation of NO\(_2^-\) and have to wait for the production of NO\(_3^-\) by AOB it is unlikely that they are contributing to this value.

The ammonia oxidized per cell formed is a way to gauge the efficiency of cells in planktonic flasks. This is only available for two data points where we see a decline in ammonia concentration. The average value for ammonia oxidation efficiency is \(7.05 \times 10^{-5}\) ppm NH\(_4^+\) cell\(^{-1}\) mL\(^{-1}\).

While these bacteria are slow growing it is important to note that when a culture is fully established it is capable of oxidizing large quantities of ammonia. Several research facilities exist which have demonstrated biological reactors capable of ammonia removal from waste waters here on earth\(^3-5, 17, 20\).  

Conclusions:

Although nitrifiers are slow growing they are still a valid means of recycling water and are an important part of future water recycling efforts. Potential technological developments that this research can be applied to are exploratory vehicles used on the future mission to mars, and on lunar colonies to provide a portable potable water supply. Further work is needed to compare growth rates and ammonia oxidation rates of planktonic cells with cells grown in biofilms upon Silastic\textsuperscript{®} membranes.

Acknowledgements:

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Works Cited:


