Quantification of root nitrification capacity of bibb lettuce plants for use in a recirculating aquaculture system (RAS)

Louis T Goldstein, Dr. Michael B Timmons, Pamela Schwartz, Erica Cartusciello, Jasmine Williams, Kendra Waddel

(towards fulfillment of MEng degree for Louis T. Goldstein)

Abstract

This research examined the feasibility of Bibb lettuce roots to provide a surface for nitrifying microbes to colonize, removing ammonia from wastewater. The work is geared towards sizing a rotational plant system to act as a natural biofilter to treat wastewater from the fish farming industry, making a recirculating natural system possible. It was observed that these natural, biotic surfaces provide a more suitable area for microbes to grow compared to inert materials. Nitrification rates were quantified, and were similar in magnitude to pre-experiment estimates. In addition, a correlation between Bibb lettuce leaf mass and root surface area was calculated, which greatly simplifies the calculation necessary to size a plant filtration unit.

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Table of Contents

1. Introduction

Recirculating Aquaculture Systems (RAS) and hydroponic plant systems have been operated successfully as independent processes over recent decades. In the current world, marriages between aquaculture and hydroponic systems are not uncommon, but it is clear that they have not realized their full potential. A common application involves large-scale hydroponic companies making use of the biological filtering capacity of plants to raise relatively small quantities of fish.

One of the major issues in any aquaculture system is the nitrogenous waste generated by the rearing of fish. An especially worrisome form of this waste is ammonia, un-ionized ammonia more specifically, as well as nitrite. (Greiner & Timmons, 1998) Even at very dilute concentrations, un-ionized ammonia is quite toxic to fish species. Concentrations as low as 0.08 mg/L for an especially sensitive species such as pink salmon will normally cause a 50% die-off in population over a 96-hour period (Timmons & Ebeling, 2007).

A successful RAS will need to be carefully monitored for removal of both ammonia and nitrite as necessary, via the biological filtration process of nitrification (Greiner & Timmons, 1998). Two major groups that are commonly used for this function include Ammonia Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB), both chemosynthetic autotrophs. They effectively work in conjunction to oxidize ammonia to nitrite, and nitrite to nitrate, respectively. The step contributed by the AOB has a much lower kinetic rate than the respective NOB reaction, so nitrite rarely has the opportunity to accumulate. Nitrate is the major end product of the two coupled reactions, and is the least toxic of all the nitrogen compounds (Timmons & Ebeling, 2007).

In many systems, water that leaves the tank as part of a recirculation process is treated via an attached, external filter before being returned to the tank in a cleaner form. Other aquaculturists may simply pump fresh influent water in on a consistent basis to maintain operations (Timmons & Ebeling, 2007). Yet, from an environmental and economic standpoint, it is clearly more favorable to recycle the tank volume, as long as the recirculating system works effectively and is robust.

2. Motivation for Research

Traditional aerobic biofiltration systems contain some type of inert media which provide surface area for biofilm growth with the surface area being proportional to the ammonia load on the system. One common biofilter example that is often employed in the world of wastewater treatment are trickling filters. In a trickling filter, wastewater is spread across a bed containing solid media such as plastic rings, and makes its way downward due to the force of gravity. As the bacteria migrates through the bed, a biofilm is formed where nitrifying bacteria are able to thrive, converting ammonia into nitrite and eventually nitrate. Fluidized bed reactors are also commonly used as biofilters. These systems are sometimes preferred to trickling filters, as smaller, lower-cost media with higher specific surface areas can be used. Alternatively, use of "floating beads" as growth surfaces allows for high hydraulic loadings without complex media retaining systems (Greiner and Timmons, 1998).

This research on using Bibb lettuce roots as natural surfaces to support biofilm growth is motivated by two major questions:

1) Can plant root surfaces effectively support biofilm growth; if so, what is the specific nitrification rate attainable with respect to root surface area? Also, do the biotic root surfaces provide an advantage over abiotic surfaces such as rocks or plastic beads?

2) In a complementary experiment, we seek to determine if a correlation between overall plant biomass and total root surface area is plausible. Being able to relate these two parameters will be useful when sizing a system for commercial or even personal use. Weighing a plant and applying a conversion factor is simpler than attempting to estimate the root surface area for each individual plant before placing them into a tank.

3. Materials and Methods

3a. Creation of Environment for Nitrifiers

The most crucial parameter to consider when designing a biological nitrification system is the maximum specific growth rate of the nitrifying bacteria. Since the oxidation step from nitrite to nitrate (performed by genus *Nitrobacter)* occurs much more quickly than the conversion of ammonia to nitrite (performed by genus *Nitrosomonas*), we will consider the growth rate of *Nitrosomonas* to be the limiting step. It is important that we considered pH and Temperature carefully while designing an optimum environment for *both* plant viability and nitrifier performance (Antoniou, 1990). Temperature and subsequently growth was controlled via use of a shade curtain to limit direct sun exposure; the average temperature between tubs over the experiment was 26.3 degrees Celsius with a standard deviation of 0.4. Alkalinity of the system, since it has a direct effect on pH, will be checked periodically. As ammonia is removed, total alkalinity should decrease; addition of ammonia nitrate should be enough to counteract this decease. These nitrifying bacteria are obligate aerobes, therefore it is of utmost importance that the plant roots are provided with adequate oxygen. Air stones will be used in each tank to ensure constant oxygen flow.

3b. System design

The system contained six individual polyethylene plastic tubs of dimensions 71.5" x 36" x 12" (1.82 m x 0.91 m x 0.30 m), which each sat inside its own hardwood container. These tubs were previously used to raise lettuce under similar conditions, making them an appropriate choice. The tanks were supported with 2" black steel angle irons to provide support and retain the tank shape against water pressure. The tanks were each risen to a height of 42" using concrete blocks. Each tank was filled with water to 90% capacity. Plant nutrient solution was added to each tank in accordance with Cornell University's "Sonneveld Solution" Recipe, adjusted for the tank volume. This solution was prepared as an "A-B" solution with a 1:1 ratio of each stock. Stock A and B were mixed directly before addition to plant tubs, and kept separate otherwise. Please see the table below for the exact composition of each stock solution:

Table 1. Cornell "Sonneveld Solution" recipe.

Compound	Stock A dissolved in 30 liters H2O grams	Stock B dissolved in 30 liters H2O grams
calcium nitrate	2916	
chelated iron, Sprint FeDTPA	67	
ammonium nitrate	84	
potassium nitrate	613	2038
potassium phospate monobasic		816
Epsom salts		738
manganese sulfate		2.56
boric acid		5.58
sodium molybdate		0.36
zinc sulfate		3.44
copper sulfate		0.56
potassium sulfate		65.5

The surface of each tank was covered with Styrofoam plant rafts, complete with 8" off-center spaced 1" holes; this gave each tank a maximum capacity of 55 plants. For this experiment, four of the tanks were filled with plants, and two were left without plants to serve as control tanks. 1 phase, 115 V, 50/60 Hz pumps supplemented with PVC pipe manifolds were used to promote circulation throughout the tanks. In addition, 8 W, 60 H air pumps with stones provided oxygen to the plant root surfaces. When filled with water, each tub had a liquid volume of 350 L.

3c. Experimental Process

I. Quantification of root nitrification capacity

The Bibb lettuce plants were obtained from a hydroponic facility, Fingerlakes Fresh, located just a few miles from the Cornell University campus. Originally, this greenhouse was used as a research center by Cornell University Controlled Environment Agriculture (CEA), and has since transformed into a commercial business. It has been in operation for over ten years to this current date. This facility operates year round, constantly producing several types of lettuce on a rotational basis.

Before data collection began, the plant nutrient solution was created, and this solution was added to each tank, along with ~1 gallon of "seed water" which was collected from the hydroponic system at Fingerlakes Fresh. This "seed water" is a broth containing a slew of microbes and micronutrients that have accumulated over many cycles of lettuce rotation.

Plant rafts were then added to each tank, and Bibb lettuce plants were added to non-control tanks 2-5. Tanks 1 and 6 served as negative controls; all aspects were alike except for the presence of the lettuce. The lettuce was placed into each tank at an age of 14 days, and a desired leafy mass of 2 g (wet-weight); this is referred to as *Nursery* size in the hydroponics industry. Plants were allowed to

grow for 10 days to 20 g (wet-weight); this is known as *Stage 1* size. At this point, we began to take measurements. See figure 1 below for a plan view of the greenhouse:
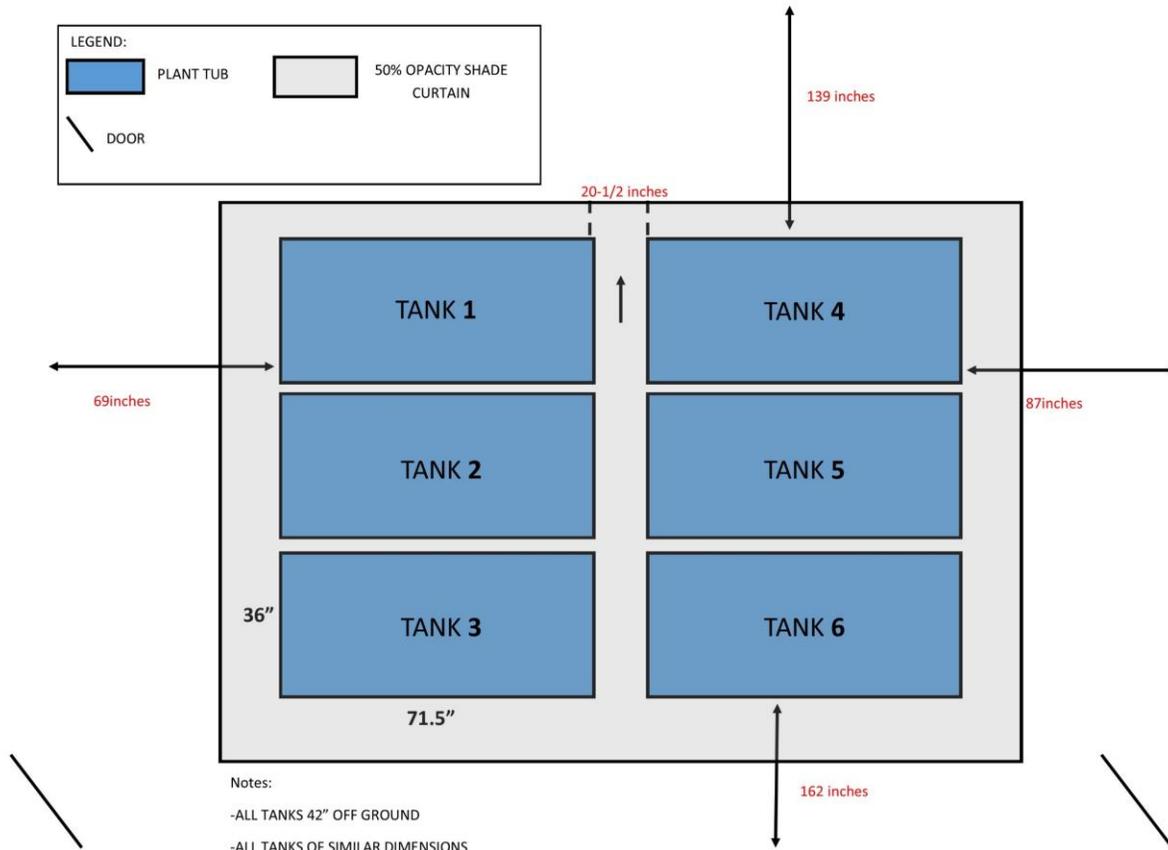

Figure 1. Plan view of Greenhouse setup.

The following parameters were measured daily: pH, temperature, and electrical conductivity (EC). It was crucial to carefully monitor the aforementioned parameters to ensure a suitable environment both for the plants and the nitrifying bacteria. pH was measured using a pH meter with a glass composite electrode , and EC measured with a similar probe. Temperature readings were displayed on each of the two probes, and the temperature of a given tank was assumed to be the average of the two respective readings. Alkalinity was measured using a direct titration method. Methyl orange was used as a pH indicator, and titrated with 0.02M H_2SO_4.

The central parameter related to the objective of this study was ammonia-nitrogen concentration, since TAN removal capacity of the root systems was the major focus. A standard curve for ammonia-nitrogen was created using an ammonium chloride compound, and a combination of Hach® Mineral Stabilizer (potassium sodium tartrate, sodium citrate), Hach® Polyvinyl Alcohol Dispersing Agent, and Nessler's Reagent were added to samples to induce a color change proportional to concentration. Both standard curve and tank samples were run through a "Spectronic 20 Genesys" spectrophotometer for analysis at 425 nm, where ammonia-nitrogen concentration was assumed to

increase linearly proportional to absorbance. Control concentrations of ammonia were used to verify calibration and accuracy.

Once it became apparent that the nitrifiers were fully present in each tank as evidenced by reduction in TAN concentration (TAN removal confirmed via colorimetric methods), ammonia-nitrogen was added to each tank in the form of ammonium nitrate ("spiked") to raise the TAN to 6 mg/L, and then TAN was measured once daily. Once TAN concentration fell below 2 mg/L, each tank was re-spiked up to a target TAN level of 6 mg/L. From this "steady-state" data, a nitrification rate for each tank could be calculated.

Nitrite-nitrogen and nitrate-nitrogen concentrations were also measured using Lamotte brand test kits, although not as frequently as ammonia-nitrogen. These tests were both based on a colorimetric reduction method.

II. Correlation of Plant Biomass to Root Surface area

The first step in this process was to collect plants from four representative plant size groups: size A (leaf weight < 1 g), size B (1 g < leaf weight < 25 g), size C (25 g < leaf weight < 50 g), and size D (leaf weight > 50 g).   Each plant was carefully washed to remove excess dirt. WinRHIZO software was used, which is an image analysis program specifically geared towards root measurement and analysis. The software is coupled with a scanner; roots are carefully separated and placed on a specialized tray with a shallow layer of water. After carefully choosing settings to account for parameters such as desired resolution and scan area size, the image is processed by the software. Quantifiable outputs of the image analysis include total root length, total surface area, and total volume occupied by the roots. From this data, we were able to calculate a root surface area (cm^2) to plant leaf mass (g) ratio for each plant. Plotting the results over a range of plant sizes allowed us to calculate a correlation factor using a linear regression model.

4. Results and Discussion

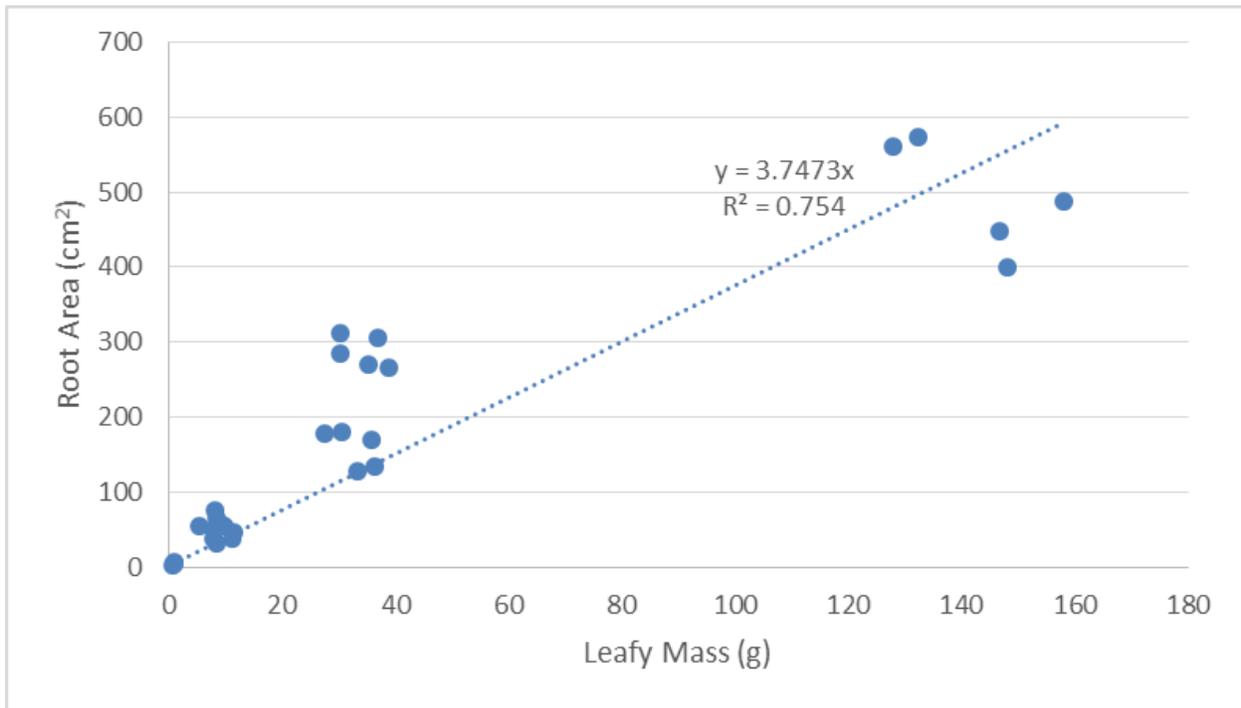### 4a. Plant Biomass to Root Area correlation

Figure 2. Root Area vs. Leafy Mass of 28 Bibb lettuce Samples

As shown in figure 2, there was a strong positive correlation between root area and leafy mass. Therefore, given the equation of the trendline, we can predict the root area of a plant easily after weighing (assuming the roots have a negligible contribution to overall plant mass). This is a valuable tool when it comes to sizing biological nitrification systems. Given a nitrification rate, we can quickly calculate the total plant surface area necessary to remove a given ammonia loading. Ammonia loading can be directly calculated based on fish feed; 30 g TAN production are assumed per kg of fish feed.

In the aquaculture industry, the size of a plant system is commonly expressed in surface area based on a standard spacing scheme. For Bibb lettuce, plants are arranged in staggered rows with spacing according to size; this corresponds to approximately 36 plants per m^2 of raft area. Combining this ratio with an observed nitrification rate, the calculated leafy mass:root area correlation, and the average weight of a Bibb lettuce plant, we can determine the exact footprint of lettuce required for TAN removal.

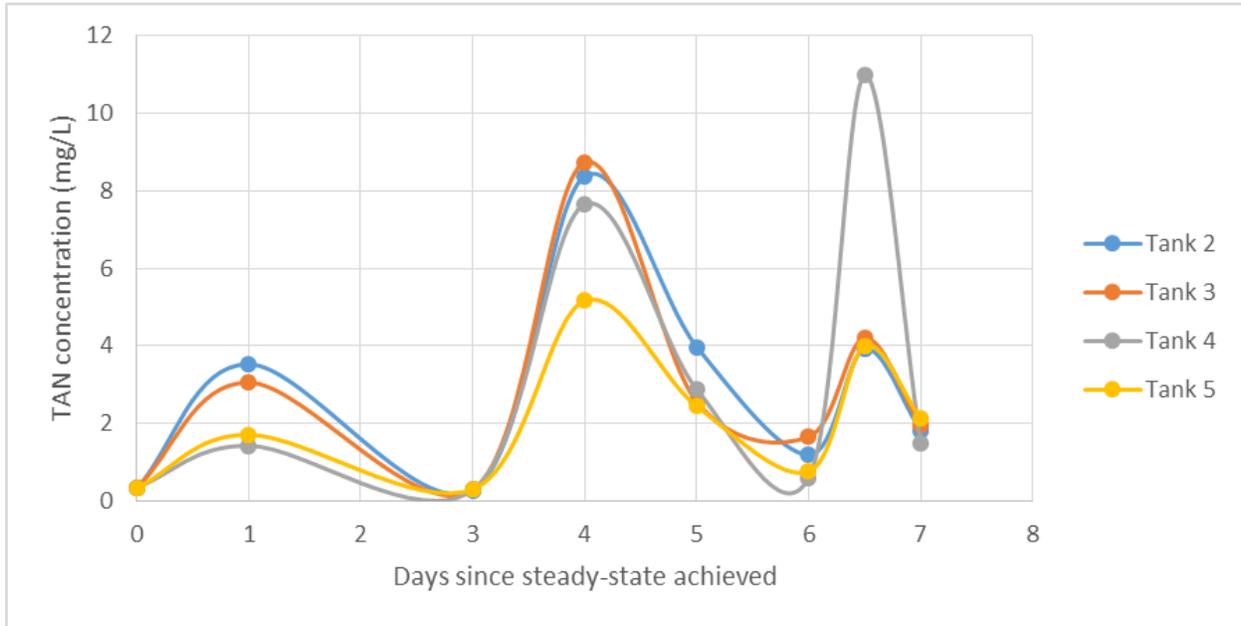**4b. Root Nitrification Capacity**

Figure 3. TAN concentration in non-control tanks once steady-state with respect to TAN removal was observed.

There was a notable lag period once plants and the nutrient solution was added. This is almost certainly due to the time it takes for a mature microbial community to develop. After 22 days, TAN levels dropped significantly, indicating a strong presence of nitrifying bacteria. After each ammonia-nitrate spike (evident in peaks shown on figure 3), each tank displayed effective nitrification capacity. In addition, the nitrification rate was similar across each tank between successive spikes. One unexpected data point occurred in Tank 4 (day 6.5); possible explanations include contamination of sample vials and/or measurement errors when performing ammonia-nitrogen assays.
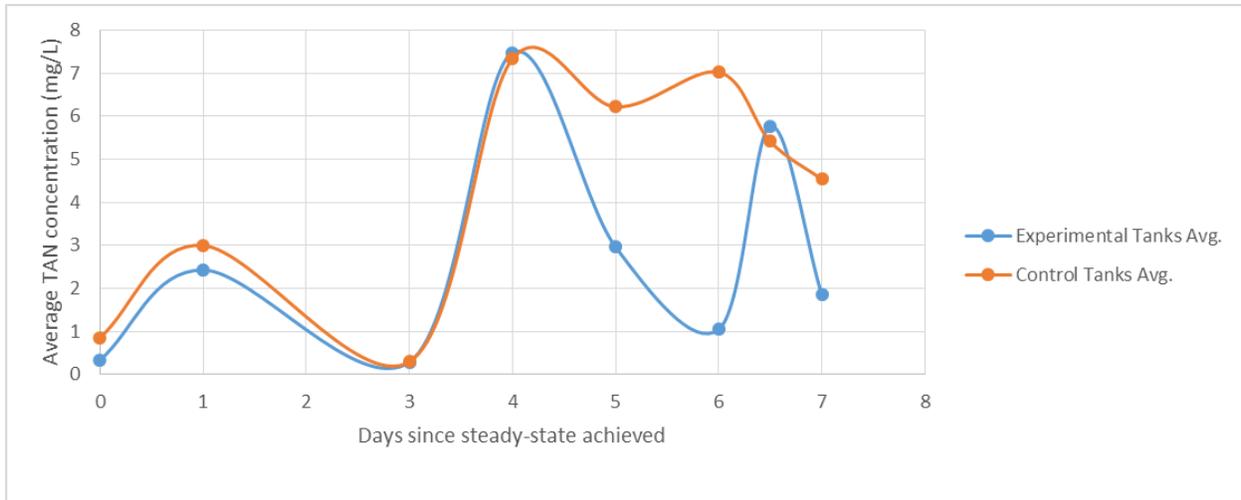
Figure 4. TAN concentration in control tanks once steady-state with respect to TAN removal was observed.

Examining figure 4, we can see that the control tanks (CT) performed similarly to the experimental tanks (ET) for lower ammonia loadings. However, the CT demonstrated highly diminished performance when ammonia loading was increased on day 3. Averaging the nitrification rates for all tubs, the CT removed 1.1 g TAN/day compared to 4.0 g TAN/day removed for ET.

We can use the aforementioned value for the ET tanks to calculate TAN removed per unit root surface area:

$$\frac{4.0 \ g \ TAN \ removed}{tub - day} \times \frac{tub}{55 \ plants} \times \frac{plant}{140 \ g \ leaves} \times \frac{g \ leaves}{3.5 \ sq. \ cm \ root \ surface \ area}$$
$$\times \frac{10000 \ sq. \ cm}{sq. \ m.} = \boldsymbol{\frac{1.5 \ g \ TAN \ removed}{sq. \ m. \ root \ SA - day}}$$

It is important to note that the *tubs containing the plants* had a significant contribution to the total surface area available for nitrifiers to colonize. The tubs contributed approximately twice as much surface area as the plants they contained (in the ET). Although the ET only contained 50% more surface area compared to the CT, they performed almost 4 times as well with respect to TAN removal. We may conclude from this data that natural, biotic surfaces provide a more suitable environment for nitrifiers when compared to the standard inert alternative.

4c. Example calculation for a full-scale hydroponic facility

As mentioned previously, Bibb lettuce is usually grown on a rotational basis. The approximate time elapsed during a single rotation is 25 days. This can be broken down into three stages of growth: 1) *Nursery*, where plants are first placed into the raft system, 2) *Stage 1* (after 10 days of growth) and 3) *Harvest*, where plants are fully sized and ready for removal and subsequent sale. See table below for information on plant stages and spacing:

Stage	Age at end, days	Mass/plant, g	Spacing, plants/m^2
Nursery	10	2	n/a
Stage 1	20	20	78
Harvest	35	150	24

Our previous correlation of plant leafy mass to root area will allow us to size a plant system based on fish feed input. One of the major assumptions going forward is that the plants at nursery stage comprise a negligible amount of the total surface area (since plants are so tightly packed), and their roots promote an insignificant amount of nitrification when compared to the more mature plants.

Example calculation:

Initial Assumptions:

1) We would like to support 100 kg of fish.

2) Required daily fish feed is 1% of total fish weight, or 1 kg/day

3) As a "rule of thumb" in the aquaculture industry, 1 kg of fish feed produces 0.03 kg or 30 g of TAN

4) Assume the Nursery level plants are negligible for sake of footprint calculations

5) Assume complete removal of TAN from system as a conservative estimate

I. We can use the standard spacing scheme, combined with assumption 4) from above to determine the plan surface area this number of plants corresponds to.

How many plants are contained in 1 m^2 plan area of Bibb lettuce?

Comparing spacing, (78/24) = 3.25 Stage 1 plants take up the space of 1 Harvest size plant.

Let x = area contained by Harvest plants

Let 3.5 x = area contained by Stage 1 Plants

$$x + 3.5x = 1 \, m^2$$

$$x = 0.22 \, m^2$$

$$3.5\ x = 0.78\ m^2$$

Finally,

$$0.22\ m^2\ *\frac{78\ Harvest\ Plants}{m^2} + 0.78\ m^2 * \frac{24\ Stage\ 1\ Plants}{m^2} = \textbf{36 total plants}\ \text{in 1 m}^2\ \text{of lettuce footprint}$$

II. We can calculate a "weighted average plant size" in the system by considering the spacing of both the Stage 1 and Harvest Size plants. This will allow us to combine the calculations in sections **4a.** and **4b.** to determine a specific nitrification rate.

$Weighted\ Average\ Plant\ Size$

$$= \frac{\left(\#\ of\ Stage\ 1\frac{plants}{m^2}\right)(Avg.\,Mass\ of\ Stage\ 1\ Plant) + (\#\ of\ Harvest\frac{plants}{m^2})(Avg.\,Mass\ of\ Harvest\ Plant)}{\left(\#\ of\ Stage\ 1\frac{plants}{m^2}\right) + (\#\ of\ Harvest\frac{plants}{m^2})}$$

$$= \frac{(78)(20\ g)\ +\ (24)(150\ g)}{(78 + 24)}$$

= 51 g

Based on our results from the root area to leafy biomass correlation from section 4a, a 51 g plant will provide $3.7473 * 51 = 191\ cm^2 = 0.0191\ m^2$ of root surface area.

We must remove **30 g of TAN** from the system daily. Using calculations from Part 4b, we can determine how many plants are necessary to process this TAN loading:

# of Plants Required:

$$\frac{30\ g\ TAN\ removed}{day}*\frac{1\ plant}{0.0191\ m^2\ root\ surface\ area}*\frac{m^2\ root\ surface\ area}{1.5\ g\ TAN\ removed} = \textbf{1050 plants}$$

Combining parts I and II above, we can calculate the amount of Bibb lettuce required in terms of area:

$$\frac{1050\ plants}{36\dfrac{plants}{m^2\ lettuce}} = \textbf{29 m}^2\textbf{lettuce}$$

4d. Tracing the path of ammonia-nitrogen

There were 2 major paths for the ammonia-nitrogen to travel: 1) oxidation into nitrite-N and finally to nitrate-N by the microbes or 2) assimilation into plant leaves for growth. Given the data collected during our experiment, we can quantitatively estimate (by percent) the fate of the added TAN.

Tank 3 had consistent and effective performance, therefore it is a good choice to examine for this estimate. In order to control the amount of variability in our analysis, we will only look at the system from day 0 until day 22, before additional ammonia spikes were performed. Furthermore, the plants did not grow significantly from this time period on, leaving the plant uptake route to be negligible over that interval.

Assumptions

1) All ammonia-N oxidized was completely transformed to nitrate-N

Part 1: Determine how much ammonia-N was processed between t = day 0 and t= day 22

Initial mass of ammonia-N in tank 3 (t = 0 days):

$$\frac{6.54\ mg}{L} * 350\ L = 2289\ mg\ ammonia-N$$

Mass of ammonia-N in tank 3 at t=22 days:

$$\frac{0.34\ mg}{L} * 350\ L = 117\ mg\ ammonia-N$$

Loss of mass of ammonia-N = (2289-117) mg = 2172 mg

Therefore, 2172 mg of ammonia-N were either transformed or uptaken over this time period.

Part 2: Estimate how much of the ammonia-N was oxidized to nitrate-N

Initial mass of nitrate-N in tank 3 (t = 0 days):

$$\frac{1.28\ mg}{L} * 350\ L = 448\ mg\ nitrate-N$$

Mass of nitrate-N in tank 3 at t=22 days:

$$\frac{3.96\ mg}{L} * 350\ L = 1386\ mg\ nitrate-N$$

Gain in mass of nitrate-N = (1386-448) mg = 938 mg

Therefore, 938 mg of ammonia-N were converted to nitrate-N or $\frac{938}{2172} * 100 = \mathbf{43}\%$**.**

Part 3: Estimate how much ammonia-N was uptaken by plants.

After oxidation, there are 1234 mg of ammonia-N remaining to account for; as stated above, it can be assumed that it was used for plant growth.

We can therefore estimate that **57% of the ammonia-N was assimilated into the plants.**

In summary, the ratio of ammonia-N assimilated by plants to that oxidized to nitrate-N is **~1.3:1**.

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