

## Video Article

# A Novel Bioreactor for High Density Cultivation of Diverse Microbial Communities

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## Abstract

A novel reactor design, coined a high density bioreactor (HDBR), is presented for the cultivation and study of high density microbial communities. Past studies have evaluated the performance of the reactor for the removal of COD<sup>1</sup> and nitrogen species<sup>2-4</sup> by heterotrophic and chemoautotrophic bacteria, respectively. The HDBR design eliminates the requirement for external flocculation/sedimentation processes while still yielding effluent containing low suspended solids. In this study, the HDBR is applied as a photobioreactor (PBR) in order to characterize the nitrogen removal characteristics of an algae-based photosynthetic microbial community. As previously reported for this HDBR design, a stable biomass zone was established with a clear delineation between the biologically active portion of the reactor and the recycling reactor fluid, which resulted in a low suspended solid effluent. The algal community in the HDBR was observed to remove 18.4% of total nitrogen species in the influent. Varying NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations in the feed did not have an effect on NH<sub>4</sub><sup>+</sup> removal (n=44, p=0.993 and n=44, p=0.610 respectively) while NH<sub>4</sub><sup>+</sup> feed concentration was found to be negatively related with NO<sub>3</sub><sup>-</sup> removal (n=44, p=0.000) and NO<sub>3</sub><sup>-</sup> feed concentration was found to be positively correlated with NO<sub>3</sub><sup>-</sup> removal (n=44, p=0.000). Consistent removal of NH<sub>4</sub><sup>+</sup>, combined with the accumulation of oxidized nitrogen species at high NH<sub>4</sub><sup>+</sup> fluxes indicates the presence of ammonia- and nitrite-oxidizing bacteria within the microbial community.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/53443>

## Introduction

Municipal wastewater is commonly treated with activated sludge processes in order to reduce the suspended solids (SS), biological oxygen demand (BOD), organic and inorganic nitrogen, and phosphorous content<sup>5,6</sup>. The activated sludge process, a means of secondary wastewater treatment, entails the oxidation of organic carbon in an aeration tank filled with a mixed liquor of incoming wastewater and recycled heterotrophic microorganism (commonly referred to as activated sludge)<sup>5-7</sup>. The mixed liquor then enters a relatively large clarifier (settling tank) where the sludge settles for easier collection, to either be disposed or recycled back to the aeration tank, while the clarified, treated wastewater can continue to tertiary treatment or disinfection before being released into receiving waters<sup>5-7</sup>. Efficient separation of the treated wastewater and solids (sludge) in the secondary clarifier is essential for the proper function of a wastewater treatment system, as any activated sludge continuing beyond the clarifiers will increase the BOD and SS in the effluent<sup>5-8</sup>.

A number of alternative biological processes exist for secondary treatment of wastewater, which reduce or eliminate the need for large clarifying tanks, including attached-growth (biofilm) reactors, membrane bioreactors (MBRs), and granular sludge reactors. In biofilm reactors, the formation of biofilms, in which microorganisms naturally aggregate and attach as a layer on a solid surface, allows for biomass retention and accumulation without the need for a clarifying tank. Biofilm reactors can be classified into three types: packed bed reactors, fluidized bed reactors, and rotating biological contactors. Packed bed reactors, such as a trickling filters and biological towers, utilize a stationary solid growth surface<sup>5,6</sup>. Fluidized bed reactors (FBRs) depend on the attachment of microorganisms to particles, such as sand, granular activated carbon (GAC), or glass beads, which are kept in suspension by a high upward flow rate<sup>9,10</sup>. Rotating biological reactors depend on biofilms formed on media attached to a rotating shaft allowing the biofilm to be alternately exposed to air and the liquid being treated<sup>5,6</sup>. MBRs use membrane filtration units, either within the bioreactor (submerged configuration) or externally via recirculation (side-stream configuration)<sup>5,11</sup>. The membranes serve to achieve good separation of biomass and solid particles from the treated liquid<sup>11,12</sup>. Granular sludge reactors are upflow reactors in which the formation of extremely dense and well-settling granules of microorganisms occurs when they are exposed to high superficial air upflow velocities<sup>13</sup>.

As another alternative to the activated sludge process, a novel upflow reactor system, now called a high density bioreactor (HDBR), was designed and built by Sales and Shieh (2006) to study COD removal by activated sludge from synthetic waste streams in low F/M conditions that are known to cause the formation of poor settling sludge (*i.e.*, bulking sludge)<sup>1,7,14</sup>. The HDBR system utilized modified fluidized bed reactors that typically consist of an upflow reactor and an external recycle tank. Fluidized bed reactors are typically operated with recycle stream flow rates

high enough to keep the biofilm growth substratum suspended but low enough so that the biofilm-covered substrate is retained. Unlike fluidized bed reactors, the HDBR described in Sales and Shieh (2006) used relatively low recycle stream flow rates which, along with external aeration, prevented disruption of the biomass zone formed within the reactor<sup>1</sup>. Subsequent studies have demonstrated this reactor design's ability to successfully treat a range of nitrogen fluxes using nitrifying/denitrifying bacteria<sup>3,4</sup>. In all studies the formation of a stable, dense biomass zone within the HDBR eliminated the need for an external flocculation/sedimentation process<sup>1-4</sup>.

As we report here, the use of the HDBR to grow dense cultures has also been tested in a photobioreactor (PBR) configuration for the cultivation of algae. We discuss the benefits and drawbacks of this novel reactor system for algal cultivation and its potential for overcoming a large hurdle in the commercialization of algal biofuels associated with biomass harvesting (*i.e.*, good solid-liquid separation<sup>15,16</sup>). The following protocol outlines the steps needed to assemble, startup, sample from, and maintain an HDBR with algae as the microbial community of interest. Variations in the startup and operation protocol of heterotrophic and nitrifying/denitrifying cultures will also be mentioned. Lastly, general advantages, disadvantages, and unknowns of this novel reactor design will be highlighted.

## Protocol

### 1. Reactor Assembly

1. Arrange the reactor components according to the schematic in **Figure 1**.
  1. Place the reactor (R) on a mixing plate, add a stir bar to the reactor. Place the recycle tank (RT) beside the stir plate and reactor so that the effluent (top) port of the tank is directed towards the edge of the lab bench.
  2. Place the waste container (W) underneath the effluent (top) port of the recycle tank (RT). Place the feed tank (FT) next to the recycle tank (RT).  
Note: The feed tank has a total capacity of 5 L.
2. Secure the reactor (R) against tipping with an appropriately sized stand and clamp. Likewise, secure the recycle tank (RT) to prevent movement.
3. Insert neoprene peristaltic pump tubing in the recycle (Pump A) and feed (Pump B) pump heads. Refer to the Materials table for additional tubing specifications. Install the pump heads onto the pump drives with the screws provided with the pump drives.
4. Connect Pump A's tubing to the ports on the reactor and the recycle tank. Insert the end of Pump B's tubing into the feed tank and the recycle tank. Connect the top reactor port to the recycle tank with tubing. Apply clamps to the tubing at the reactor ports.  
Note: Photosynthetic communities may benefit from artificial illumination provided by lamps.

### 2. Preparation of Stock Solutions, Influent/Feed Solutions, and Algae Inoculant

1. Prepare the mineral stock solution. Add the following to a 1 L volumetric flask with 500 ml of deionized water: 200 g sodium bicarbonate, 40 g monobasic potassium phosphate, 4 g magnesium sulfate, 4 g ferric chloride, 4 g calcium chloride, 1 g copper chloride, 1 g cobalt chloride hexahydrate, 1 g nickel chloride hexahydrate, 1 g zinc sulfate heptahydrate. Add an additional 400 ml of deionized water. Swirl forcefully to encourage dissolution of salts. Following dissolution of salts, add deionized water to bring the total volume of solution to 1 L.
2. Prepare the ammonia stock solution. In a 1 L volumetric flask, dissolve 38.214 grams of ammonium chloride in approximately 900 ml of deionized water. After dissolution, add deionized water to bring the total volume up to 1,000 ml.  
Note: 1 ml of stock solution diluted to 1 L yields a 10 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N solution.
3. Prepare the nitrate stock solution. In a 1 L volumetric flask, dissolve 72.413 g of potassium nitrate in approximately 900 ml of deionized water. After dissolution, add deionized water to bring the total volume up to 1,000 ml.  
Note: 1 ml of stock solution diluted to 1 L yields a 10 mg L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N solution.
4. Prepare feed/influent solution. To make a feed solution containing 20 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N and 20 mg L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N, dilute 2 ml of ammonia stock solution and 2 ml of nitrate stock solution to 1 L total volume. Prior to dilution, add 0.5 ml mineral solution/L of solution being made. Prepare 5 L of influent in total to start up the reactor.
5. Prepare the algae inoculant.
  1. Collect a large volume (at least 10 L) of water from an algae-containing water body such as a stream or pond. Allow the algae to settle by leaving the water samples undisturbed for 24 hr.
  2. Decant and discard the clear (non-algae containing) water at the top of the samples, leaving a concentrated algae suspension within the sample bottles. Combine the algae suspension from all of the samples into one container and repeat the settling and decanting steps.
  3. Measure the biomass within the concentrated sample.
    1. Dry a paper vacuum filter (0.45 μm MCE vacuum filter) and aluminum weigh boat O/N in an oven which has been set to 103 °C. After cool-down in a desiccator for 30 min at RT measure the combined mass of the filter and weigh boat.
    2. Vacuum filter 20 ml of concentrated algae suspension and return the filter and weigh boat to oven to dry O/N.
    3. Measure the combined mass of the filter and weigh boat. Calculate the biomass density within the concentrated sample.  
Note: The total volume of water sample that investigators will need to collect will depend upon the source water body.

### 3. Seeding and Starting the Reactor

1. Add 750 ml of feed solution to the reactor. Fill the recycle tank with 500 ml of feed solution.
2. Use a long pipette to gently add an inoculate suspension containing 1.5 g of algae near the bottom of the reactor. Allow the inoculum to settle to the bottom of the reactor, ensure this by visual observation, before proceeding to the next step.

- Once the cells have settled, remove the tube clamps and turn on Pump A to a slow flow rate (10 revolutions  $\text{min}^{-1}$ /38 ml  $\text{min}^{-1}$ ). Air trapped in the tubing will be expelled into the reactor.  
Note: The addition of 750 ml to the reactor will prevent any biomass disturbed by the pump from leaving the reactor. Squeeze the tubing to ensure that all air has been expelled.
- Gradually add feed solution to the recycle tank as the solution is pumped into the reactor. Continue the addition until both the reactor and the recycle tank are at capacity and effluent starts to exit the recycle tank via the top port.  
Note: The volume of feed solution to be added to the recycle tank will vary with the volume of the inoculant added to the reactor.
- Pour the remaining feed solution into the feed tank.
- Set the recycle pump (Pump A) to 19 revolutions  $\text{min}^{-1}$ , establishing a recycle flow rate of 72.5 ml  $\text{min}^{-1}$ . Observe the algae begin to loft from the bottom of the reactor. Using the gradations on the reactor, determine the algae biomass zone height. Ensure that the height is constant before proceeding to the next step.
- Turn on the mixing plate at very low speed; a setting of 1 or 2 is appropriate to start. The mixing bar will assist in lofting biomass further, but aggressive mixing will cause algae to leave the reactor, enter the recycle tank, and leave in the effluent. Set mixing speed at a setting needed to establish a clear algae boundary within the reactor (**Figure 2A**); the algal biomass zone should be approximately 10-15 cm in height.
- Start the feed pump after observing a clear boundary between the algal plug and the reactor fluid. Set the pump to 25 revolutions  $\text{min}^{-1}$ , establishing a flow rate of 1.5 ml  $\text{min}^{-1}$ . Observe the reactor fluid exit the effluent port due to gravity and displacement caused by the incoming influent stream.

## 4. Sample Collection and Analysis

- Carry out sample collection activities prior to performing maintenance on the reactor system. Collect 20 ml of effluent and influent samples daily. Collect effluent samples from within the recycle tank. Collect influent samples directly from the feed tank.
- Vacuum filter samples to remove suspended solids prior to storage and analysis.
- Store the influent and effluent samples at -20 °C until further analysis. Limit the number of freeze thaw cycles samples are subjected to. If needed, samples can be split into aliquots to maintain sample integrity.
- Carry out sample analysis for nitrate, nitrite, and ammonia using standard techniques<sup>17</sup>.  
Note: The authors utilized Ion Chromatography (IC) to produce the results presented herein. Refer to the Materials table for specification.

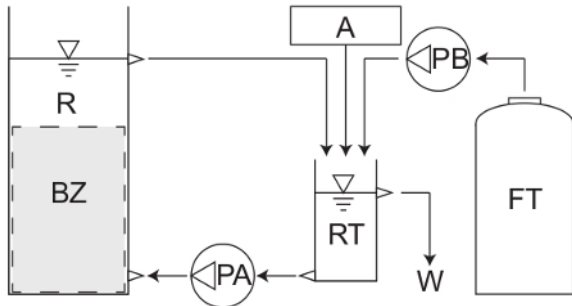
## Representative Results

The HDBR was used to cultivate algae over several ratios of influent ammonia and nitrate concentrations, while maintaining a total nitrogen content in the feed at 40 mg  $-\text{N L}^{-1}$ . Influent and effluent samples were taken daily; biomass density samples were taken at the beginning and end of each condition. The reactor took on average 3-5 days to reach steady state equilibrium after conditions were changed. Over a wide range of influent conditions a distinct biomass zone was established, as observed previous studies (**Figure 2**). The algal culture in the HDBR was found to remove an average of 18.4% of total nitrogen species in the feed (n=44). Within the biomass zone, total algal biomass and biomass density were consistent over the course of this study.

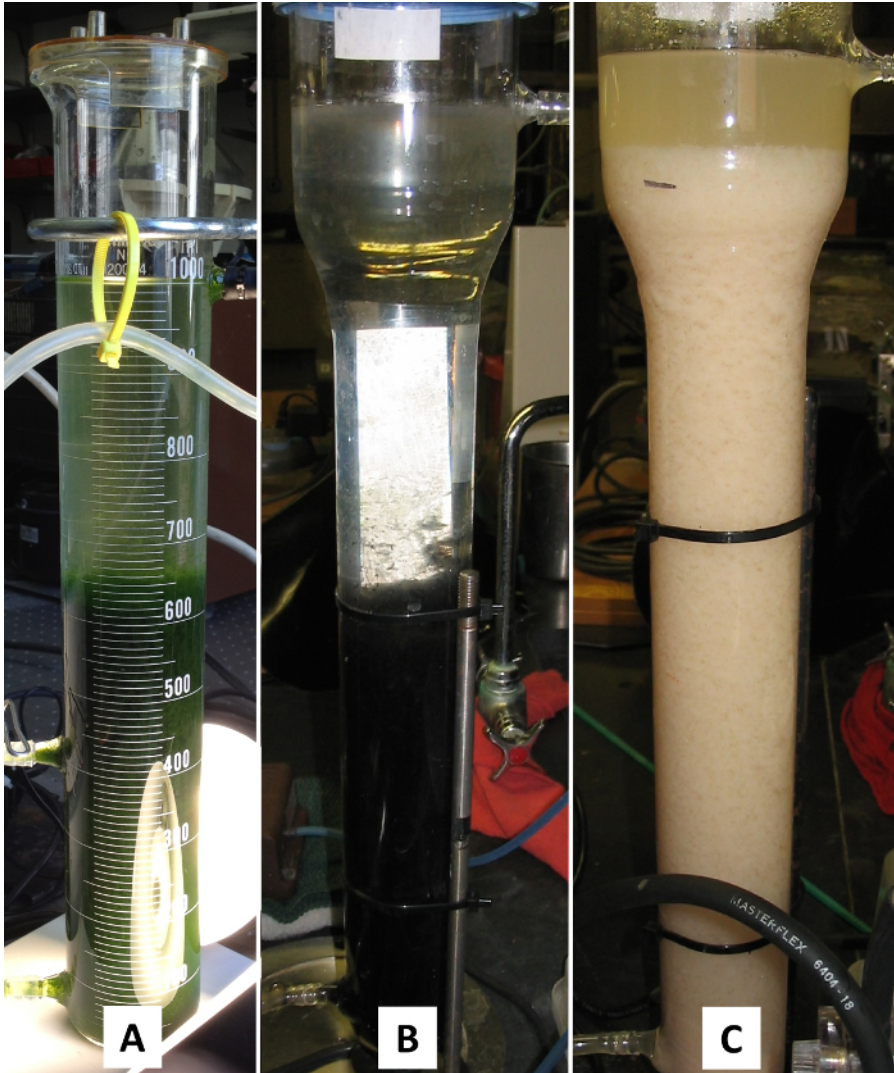
The removal of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  are plotted against the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  feed composition in **Figure 3**. A simple linear regression model was used to assess the significance of relationships between the removal of N species and the feed composition<sup>18-20</sup>. Removal of ammonia was observed at all ranges of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  composition (**Figure 3A** and **Figure 3B**, respectively). Neither  $\text{NH}_4^+$  nor  $\text{NO}_3^-$  feed composition affected the removal of  $\text{NH}_4^+$  over the conditions tested (n=44, p=0.993 and n=44, p=0.610 respectively). On the other hand, removal of  $\text{NO}_3^-$  was found to be negatively related with  $\text{NH}_4^+$  feed composition (n=44, p=0.000) (**Figure 3C**) and varied positively with  $\text{NO}_3^-$  feed composition (n=44, p=0.000) (**Figure 3D**).

$\text{NO}_3^-$  was observed to accumulate (negative removal) within the reactor for most of the influent compositions (34 out of 44 samples).  $\text{NO}_3^-$  removal was only observed when  $\text{NH}_4^+$  feed concentrations were below 10 mg  $-\text{N L}^{-1}$  and  $\text{NO}_3^-$  feed concentrations were above 15 mg  $-\text{N L}^{-1}$ . Oxygen, which is being added to the reactor via aeration in the external recycle tank and by the algae, can serve as an electron acceptor for ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively). If aerobic conditions dominate within the reactor via high recycle flow rates, bacteria that may carry out dissimilatory (heterotrophic) denitrification will prefer to utilize oxygen as an electron acceptor<sup>4</sup>. If the rate of  $\text{NO}_3^-$  production and input from the feed exceeds the assimilatory conversion of  $\text{NO}_3^-$  to organic nitrogen or dissimilatory denitrification,  $\text{NO}_3^-$  can accumulate in the reactor. The removal of  $\text{NH}_4^+$  and accumulation of  $\text{NO}_3^-$  suggests that AOB and NOB are present and active within the community as algae are not known to catalyze the conversion of  $\text{NH}_4^+$  to  $\text{NO}_3^-$ . These results demonstrate the ability to use this reactor system to study nitrogen flux dynamics and kinetics in a mixed algal-bacterial community.

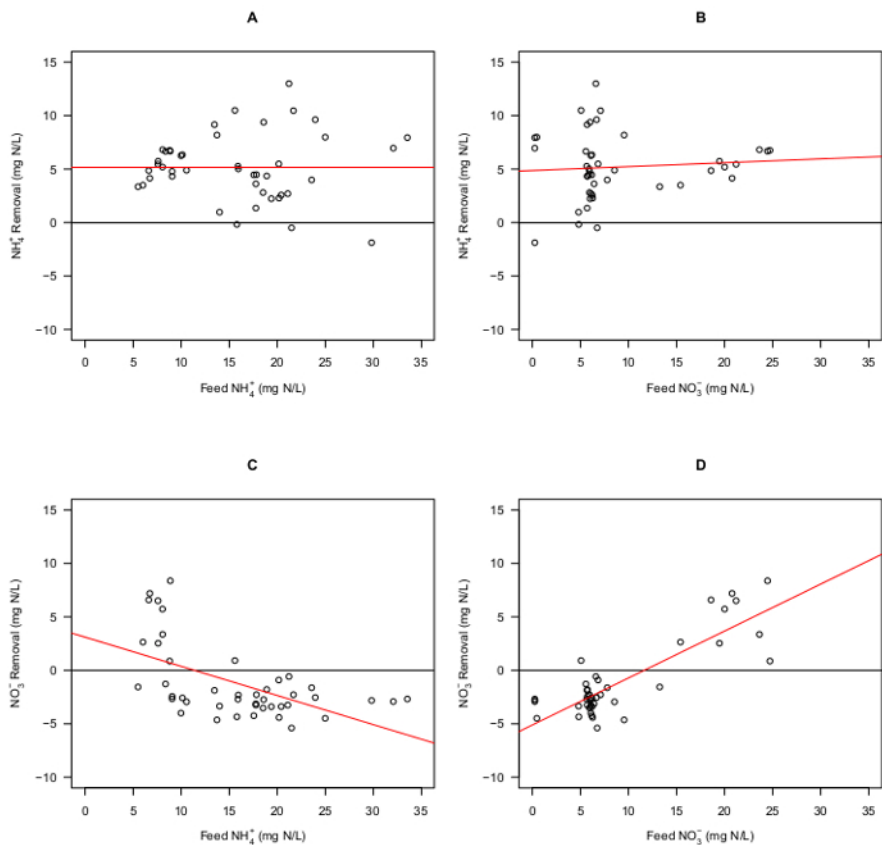
The authors have successfully maintained healthy algal communities in these HDBRs for over a year. Two reactor crashes, however, have occurred since the inception of this project, both as the result of severe changes to influent composition. The first was the result of a change of nitrogen species ratios with the total nitrogen flux being kept constant;  $\text{NH}_4^+$  was eliminated from the feed and  $\text{NO}_3^-$  concentrations were increased to compensate. The second crash occurred as a result of cutting the total nitrogen flux by 75 percent, from 40 mg  $-\text{N L}^{-1}$  to 10 mg  $-\text{N L}^{-1}$  (**Figure 4**). In both cases the distinct biomass zone boundary was observed to deteriorate over the course of two to three days coinciding with a sharp increase in suspended solids in the effluent (**Figure 4**). Effluent suspended solids increased to a maximum 6 days after the feed change as the reactor lost biomass (**Figure 4**). After the crash suspended solids in the effluent remained high (about 0.22 g SS  $\text{L}^{-1}$ ) and no new biomass was observed to accumulate within the reactor, preventing the continuation of the experiment. The current design lacks a safety mechanism to retain cultures if they do not remain well-flocculated.



**Figure 1. Schematic of a High Density Bioreactor (HDBR) (not to scale).** The Reactor (R) is composed of a 1,000 ml graduated cylinder with ports (hose barbs, outside diameter 3/8") installed at the 100 ml and 1,000 ml levels. Reactor fluid is cycled through the reactor using peristaltic Pump A (PA), entering at the bottom of the reactor and flowing upwards through the biomass zone (BZ) towards the top port. Fluid exits the reactor at the top port and is directed to the recycle tank (RT) under gravity. The RT is composed of a 600 ml glass beaker; it has two ports installed, one located at the bottom of the beaker and the other at the 500 ml mark. Reactor fluid is returned to the reactor via the bottom port (and PA). Effluent leaves the reactor via the top port of the RT and is collected in a Waste container (W). Diffusive aeration is provided in the RT with the use of an aerator (A). The aeration process also drives mixing within the MV. Peristaltic Pump B (PB) delivers influent from a tank containing feed/influent (FT) into the RT. [Please click here to view a larger version of this figure.](#)

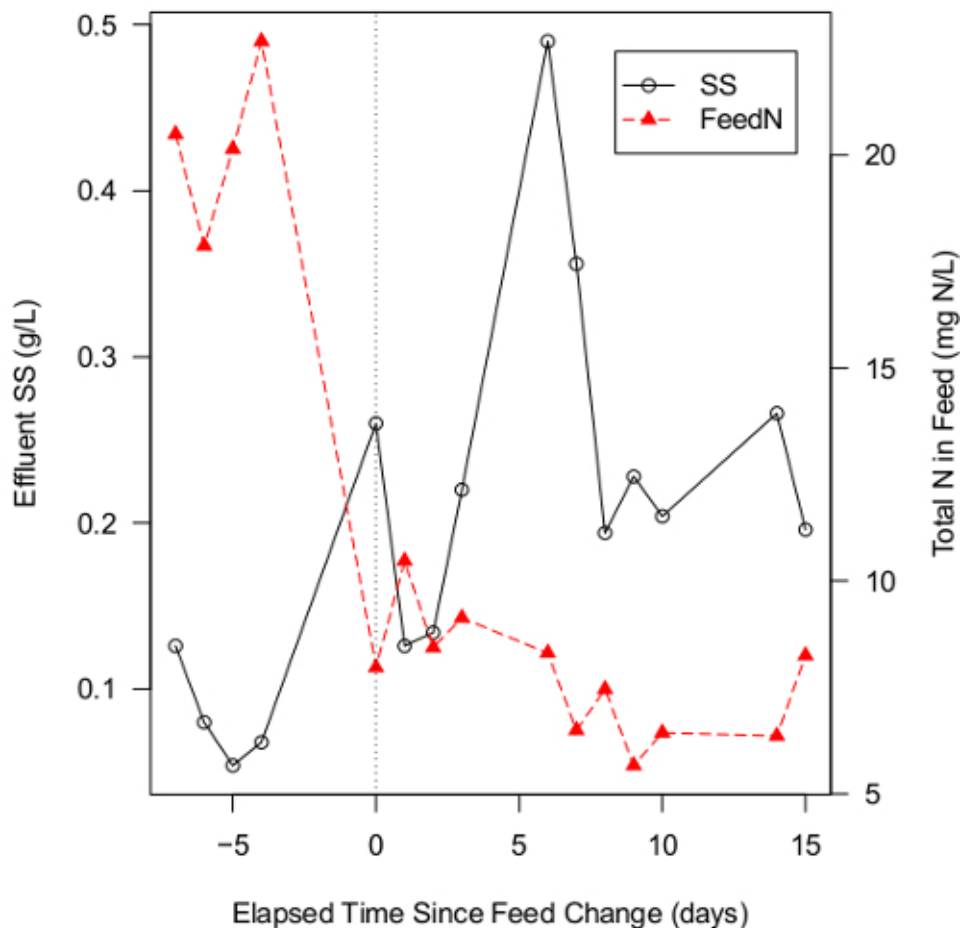


**Figure 2. Examples of biomass/reactor fluid separation within a high density bioreactor (HDBR).** Panel A shows a high density algal community ( $2.83 \text{ g SS L}^{-1}$ ) being cultured within an HDBR. A distinct boundary occurs when the settling velocity of the photosynthetic microbial community exceeds that of the reactor fluid. Panel B displays the microbial matrix formed by activated sludge in the recycle conditions discussed in Sales and Shieh (2006)<sup>1</sup>. Panel C shows yeast being cultured on an influent composed of glucose for the production of ethanol via fermentation (results not published). In all three of these reactor configurations the novel reactor design has eliminated the need for a separate clarifying or settling process in the reactor system. [Please click here to view a larger version of this figure.](#)

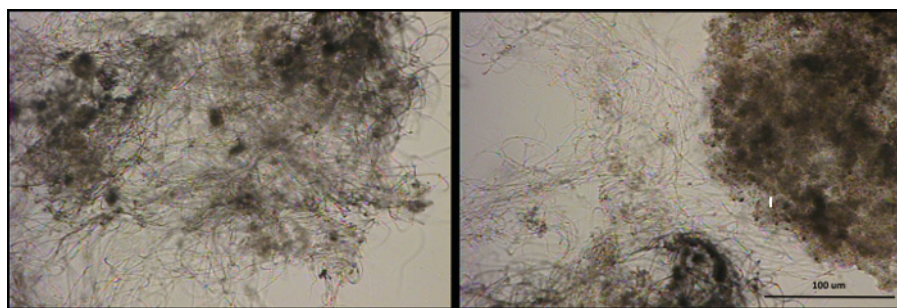


**Figure 3. Illustration of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> removal rates versus influent composition.** Total influent N concentration was maintained at 40 mg -N L<sup>-1</sup> over the duration of the study. **(A)** NH<sub>4</sub><sup>+</sup> feed concentration is plotted against the removal of NH<sub>4</sub><sup>+</sup>; there was no significant effect (n=44, p=0.993). **(B)** NO<sub>3</sub><sup>-</sup> feed concentration is plotted against the removal of NH<sub>4</sub><sup>+</sup>; there was no significant effect (n=44, p=0.610). **(C)** NO<sub>3</sub><sup>-</sup> removal was found to be significantly and negatively related to NH<sub>4</sub><sup>+</sup> feed concentrations (n=44, p=0.000). **(D)** NO<sub>3</sub><sup>-</sup> removal was significantly and positively related to NO<sub>3</sub><sup>-</sup> feed concentrations (n=44, p=0.000). [Please click here to view a larger version of this figure.](#) [Please click here to view a larger version of this figure.](#)





**Figure 4. Increasing effluent suspended solids in response to significantly decreasing nitrogen flux through the reactor.** Influent nitrogen content was decreased from 40 mg  $-N L^{-1}$  to 10 mg  $-N L^{-1}$  (at time 0 in this figure, also denoted by the vertical line). Deterioration of the distinct biomass zone was observed after 2 days; after 3 days biomass loss was readily observable. A significant increase in effluent SS was observed after days after the change was enacted and maximum effluent SS occurred after 6 days. [Please click here to view a larger version of this figure.](#)



**Figure 5. Micrographs highlighting porous floc structure and interlocking filamentous bacteria.** Two micrographs exhibiting the porous structure formed by heterotrophic bacteria (activated sludge). Filamentous bacteria bridge the space between flocs, interlocking flocs in to the stabilized biomass zone. [Please click here to view a larger version of this figure.](#)

## Discussion

This section will start with a discussion of protocol variations needed to address possible operational issues as well as using different microbial communities. The strengths of this reactor design will be discussed, including the ability to govern control of oxygen flux and the formation of high density flocs within the reactor. Current challenges and possible avenues of investigation will also be mentioned.

### *Protocol nuances and variations*

The operation of HDBRs for cultivation of different types of cultures requires slight changes in operational protocol, depending the species under investigation. Sufficient mixing and expansion of the algal biomass zone is needed to increase exposure of all flocs to light and enable photosynthesis. Suspension of algal biomass within the reactor is driven by a combination of the reactor recycle rate and mixing bar speed. Care

should be taken in selecting the operating characteristics of both, such that there is adequate distance between the biologically active algae biomass zone and the port at the top of the reactor, as any biomass that leaves the reactor vessel may be lost in the effluent through the top port of the recycle tank. If biomass is observed leaving in the effluent, a filter can be fitted to the top port of the recycle tank. A plug of glass wool can be used as a filter. As the filter accumulates biomass it will need to be changed. If a filter is used, suspended solids samples must be taken from downstream of the filter in addition to the reactor fluid within the recycle tank in order to obtain the correct mass balance; accumulated biomass in the filter must also be accounted for. Under some operating conditions with algae and yeast the biomass zone does not always lead to clarified liquid, even when the community is healthy. In these cases there is a dilute suspension of cells noticeable above the biomass zone and within the recycle tank. We hypothesize that in the algal and yeast communities that have been cultivated in the HDBR thus far do not contain the filamentous bacteria to form the stable, interlocking biomass zone as seen in heterotrophic and the nitrification/denitrification bacterial cultures (Figure 5). Therefore, if the goal is to prevent cells from escaping in the effluent, such as is the case with algae and yeast, it may be necessary to use a membrane or filtration device at the effluent port.

An unexpected source of biomass disturbance, which could negatively impact the solid-liquid separation of the HDBR, is the accumulation of bubbles within the recycle pump lines. These bubbles are a product of aeration in the recycle tank. Care should be taken to regularly purge any buildup of gasses within the tubing. Squeezing the tubing in the direction of fluid flow will expedite this process and also serves to dislodge any biomass that has become fixed to the interior of the tubing. As the cells in these cultures tend to aggregate into flocs, they also have a tendency when released from the biomass zone to adhere and colonize the walls of the HDBR. Thus, if investigators notice the adhesion of biomass to the inside walls of the reactor and recycle tank, they should use a pipette or sanitized glassware brush to disturb and prevent excessive biofilm growth on the walls of the reactor.

The protocol described above must be modified when heterotrophic microorganisms or nitrifying/denitrifying bacteria are the community of interest. For instance, 4 g of heterotrophic microorganisms (measured as VSS) was used to seed the reactor, as described by Sales and Shieh<sup>1</sup>. When studying ammonia and nitrite oxidizing bacteria, 2 g of enriched AOB/NOB was used as described in Nootong and Shieh<sup>2</sup> and Ramanathan *et al.*<sup>4</sup>, and expanded upon in the appendix to that manuscript<sup>21</sup>. The exact amount of inoculum to start the reactor can vary and truly depends on the amount of source inoculum available and the actual volume of the reactor being used. To prevent biomass disturbance, the use of a stir bar is discouraged when using these mat-forming cultures.

#### Manipulation of hydraulic characteristics

A primary advantage of the HDBR design is the ability to control the feed and recycle flow rates independently of one another. Investigators can target specific loading rates, recycle rates, or recycle ratios. For example, while studying reactor performance utilizing activated sludge to remove COD from synthetic wastewater, the recycle ratio varied from 3.5-21.5<sup>1</sup>. Initial studies of the reactor utilizing autotrophic nitrifying/denitrifying bacteria indicated that stable biomass zones could be maintained under recycle ratios of 2.5-24.3<sup>3</sup>. These estimates proved to be conservative as no problems were encountered when increasing recycle ratios up to 43 in a follow up study<sup>21</sup>. The ability to operate at high recycle ratios, and thus high recycle rates, is useful for studying the effects of fluid shear on the stability and characteristics of the biomass zone. In some cases, such as algae cultivation, the establishment and maintenance of a biomass matrix is not a requirement and high recycle rates and ratios are needed to assist in the suspension of the algal column. This reactor design is able to facilitate suspension assisted by high recycle rates (49.3 in this study), provided that investigators are able to maintain a distinct biomass/effluent interface within the reactor. In cases where the recycle ratio is high, the reactor hydraulic characteristics of the entire HDBR system behave more similarly to completely mixed reactors (CMFR) than a plug flow reactor (PFR), and thus allows the investigator to examine cultures over a spectrum of hydraulic mixing characteristics in a single system. The recycle flow rate also plays a role in the flux, mass transfer, and distribution of dissolved gaseous species throughout the reactor, as described below.

#### Control of O<sub>2</sub> flux from and degassing in the recycle tank

While studying combined nitrification/denitrification processes, dissolved oxygen concentrations were observed to be quickly depleted in the lowest portions of the active biomass zone as nitrification was carried out<sup>2,4</sup>, paralleling previous COD removal studies<sup>1</sup>; this may suggest that the flow regime within the biomass flow resembles that of a PFR<sup>1</sup>. With the upper biomass zone becoming anoxic, denitrification was carried out, resulting in the removal of dissolved nitrogen from the reactor effluent<sup>2,3</sup>. These observations demonstrated that the combination of external aeration in the recycle tank, combined with the ability to control the flux of oxygen to the upflow tank, via manipulation of the recycle rate, allows for gradients of oxygen to develop within flocs and along the length of the biomass zone, allowing for aerobic, anaerobic and anoxic reactions to occur in a single tank. As many reactions for biological treatment depend upon or are inhibited by oxygen, this reactor allows for an easier way to control oxygen mass rates into bioreactors; possibly enabling more efficient aeration practices. As aeration is one of the highest energy costs in wastewater treatment, this may serve to lower operating costs for municipalities<sup>22,23</sup>.

The control of oxygen flux through a reactor is not only a concern for heterotrophic and chemoautotrophic bacteria. Excess excitation energy (EEE) is the surplus light energy algal cells are exposed to, and results in oxygen (O<sub>2</sub>) being reduced to superoxide (O<sub>2</sub><sup>-</sup>) with excess electrons shunted from photosystem I or II (PSI and PSII)<sup>24</sup>. Superoxide anions can cause significant physiological damage in algal systems. A cellular framework exists to detect and neutralize O<sub>2</sub><sup>-</sup> before damage can occur to cellular components, but in highly stressed cells reactive oxygen species (ROS) can still form<sup>24,28</sup>. By controlling the recycle rate and the aeration in the recycle tank, investigators may be able to address issues arising from excess oxygen and the toxicity it can induce in algal cultures, and may further enhance the growth of algae in highly dense cultures, particularly in cases where supplemental light is being provided through the use of lamps.

#### Formation of flocs and/or biomass zone leads to diverse macro- and micro-environments

One of the most unique features of this reactor design is the elimination of a clarifying tank. We hypothesize that the good solid-liquid separation that is achieved in HDBRs can be attributed to either the formation of highly dense flocs (*i.e.*, the case with algae), or the formation of a stable, porous matrix of interlocking flocs and long-filamentous microorganisms (*i.e.*, with the heterotrophic and nitrifying/denitrifying cultures)<sup>1-4,7,16</sup> (Figure 5). The formation and stability of flocs is dependent on a number of physical, chemical and biological factors<sup>7,13,29-31</sup>. In fact, the formation of flocs is the primary goal of start-up and depends on sufficient mixing (shear force gradients) to increase collisions among the suspended inoculant<sup>13,30</sup> but also on the presence of well-flocculating microorganisms that produce compounds (flocculants) that allow cells to aggregate<sup>31,32</sup>. In these laboratory scale reactors, we have found that sufficient mixing for flocculation can be accomplished either by the upflow velocity profile or a mixing device, such as a stir bar, located at the bottom of the reactor. For cultures that require oxygen, the external recycle tank can be used as an external gas transfer tank (either for aeration or stripping of gases, for instance for removing oxygen produced by



photosynthesis reactions). The benefit of external aeration is it prevents excess mixing as well as air bubbles coming into contact with flocs and breaking them apart. In some instances, with the heterotrophic and nitrifying/denitrifying cultures that formed a stable, porous matrix, when gas bubbles were found to enter the reactor they could be found breaking apart parts of the matrix or become entrained within sections of the matrix causing them to float to the top of the reactor. Therefore, operation of the external gas transfer tank to prevent bubbles entering the reactor via the recycle line is key to maintaining good solid-liquid separation of the system.

#### Potential HDBR directions

Benchtop reactor studies, particularly those focused on PBRs, are often focused towards gathering kinetic data for a particular microbial species or community<sup>1,3,4,33,34</sup>. Historically many studies are done on axenic or antibacterial treated algal cultures despite mounting evidence of the importance of interspecies interactions between algae and bacterial communities<sup>35,36</sup>. Studies of mixed cultures promise to yield new and insightful conclusions about how these interspecies relationships work<sup>35-38</sup>. Recent studies of mixed cultures has expanded to include sample analysis with molecular biology tools such as quantitative polymerase chain reaction (qPCR) to quantify algae and bacterial growth rates<sup>33,34</sup>. Metagenomic and metatranscriptomic analysis has been used to elucidate further information about how algae and bacteria interact in both engineered and natural ecosystems<sup>39,40</sup>. In addition to molecular investigations of the microbial cultures in HDBRs, microscopy studies examining the size, structure, and organization of flocs and the porous biological matrix of the biomass zone would provide valuable information on the HDBRs ability to promote good solid-liquid separation.

Thus far, only a small range of reactor volumes and recycle ratios have been investigated using the HDBR design. As such, reactor performance in scaled up applications is currently unknown. Each of the tested reactor systems are less than 2 L in volume and composed of glass. As these reactors are not off the shelf components and must be constructed by a laboratory glassware specialist increasing the size of glass reactors may be difficult as the starting pieces must be carefully selected for appropriate wall thickness (Private correspondence: K. Carraro, 2014). Large glassware also runs a higher risk of being broken or damaged in comparison with a metal, plastic, or concrete reactor. Constructing larger reactors with metal or plastic for benchtop experiments may be an option but the viability of this option has yet to be investigated. Additionally the use of opaque or translucent materials may hinder visual observation of the reactors under investigation and would complicate the operation of these reactors in a PBR configuration.

This manuscript has outlined the assembly, startup, and operational procedures to operate a high density bioreactor (HDBR). Previous work has established HDBRs capacity to remove both COD and nitrogen species using heterotrophic and chemoautotrophic bacteria<sup>1-4</sup>. Here the authors demonstrate the ability of HDBRs for the culture of high density algal communities and the removal of nitrogen species from a synthetic waste streams. Following previous observations, a stable biomass zone was formed by the algae, and successful operation of the reactor without a clarifying process was achieved while removing 18.4% of total nitrogen species from the influent. Conversion between nitrogen species ( $\text{NH}_4^+$  to  $\text{NO}_3^-$ ) was observed, allowing the authors to suggest the presence and activity of AOB and NOB. The results presented in this manuscript from the current demonstration with algae and prior studies using the HDBR system support further use, as well as research and development, of this reactor design for high density cultivation of microorganisms for a variety of environmental and industrial applications.

## Disclosures

The authors have nothing to disclose and declare that they have no competing financial interests.

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