AMMONIA REMOVAL BEHAVIOR IN THE DOWNFLOW HANGING SPONGE BIOREACTOR

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Abstract

Ammonia removal behavior in the DHS has been studied extensively including apply a DO microelectrodes and a gold method of microbial identification, i.e. Fluorescence *In Situ* Hybridization (FISH) approach by means of 16S rRNA-targeted oligonucleotide probes. With the HRT of 2 h, the ammonia removal rate in the bioreactor was achieved 0,24 $kgN.m^{-3}.d^{-1}$, and nitrogen-losing rate was approximate 0,08 $kgN.m^{-3}.d^{-1}$. Moreover, the most important of this system is no requirement external aeration input. FISH of the DHS sludge revealed that nitrifiers could be detected with Nso190, NSR1156, or NIT3 probe, form dense clusters, and they mainly settled in the surface region of the heterotrophic aggregates. Ammonia oxidizers and nitrite oxidizers grown in DHS sludge in separated solitary occurrence. *Nitrospira* spp. were more likely responsible to oxidize of nitrite rather than *Nitrobacter* spp.

Key words: fluorescent *in situ* hybridization, nitrification, nitrifying bacteria, sewage

1. INTRODUCTION

The use of an upflow anaerobic sludge blanket (UASB) technology for sewage treatment has been explored as a feasible option in many developing nations like Colombia, Indonesia, Brazil, China, and India (Alaert *et al*., 1993). Application of the UASB process has been reviewed, with examples from Europe, Asia, and the Americas, appears today as a robust technology for sewage treatment (Seghezzo *et al*., 1998). Unfortunately, the anaerobic digestion does not enable nitrification process. These compounds contribute significantly to the eutrophication of aquatic environments and acts as a strong fish poison (Jansen *et al*., 1997). Therefore, to meet a future effluent requirement of many developing countries, UASB effluents need to be improved.

Recently, several conventional types of process have been proposed as a post-treatment unit for polish-up of UASB effluent treating sewage, by either a rotating biological contactor (Castillo *et al*., 1997), a submerged aerated biofilters (Goncalves *et al*., 1999), or dissolved air flotation (Penetra *et al*., 1999). Downflow Hanging Sponge (DHS) bioreactor has been proposed for a post-treatment unit of a UASB treating municipal sewage (Machdar *et al*., 1997). The DHS bioreactor provided simultaneously carbon and nitrogen removal, and nearly perfect SS removal as well as total BOD removal.

The DHS bioreactor was constructed by hanging a certain dimension of polyurethane foam (sponge) in open atmosphere. The sponge is provided for attached-growth or immobilized biomass. Influent wastewater is supplied at the top of the bioreactor and naturally permeating by means of gravity into the sponge inside. The most important feature of the DHS bioreactor is no requirement of external intended aeration and maintaining a very long SRT that is favorable for nitrification process.

The study present here is to analyze the behavior of the DHS bioreactor regarding ammonia removal process. Fluorescence *in situ* hybridization (FISH) approach by means of 16S rRNA-targeted oligonucleotide probes and DO micro-electrodes were applied to the accumulated biomass in the bioreactor to identify and enumerate nitrifier populations and to examine the mechanisms of nitrification.

2. MATERIALS AND METHODS

Experimental set-up and bioreactor operation

The UASB reactor has a working volume of 155 liter consisting of a 120-liter column portion and a 35-liter gas/solid separator. The right triangular prism polyurethane foams (side: 3 cm and length: 75 cm) were used to construct the DHS bioreactor. The foams were tilling onto both surfaces of a vertical plastic-made rectangular-sheet (75 cm in width by 200 cm in length). A distribution system was installed at the top of the bioreactor to provide

a uniform distribution of the wastewater over the sponge surface with 2 h of HRT. The DHS and UASB reactor were maintained at 25°C in order to simulate annual average ambient temperature in most of developing countries. The schematic diagram of UASB and DHS is shown in Figure 1.

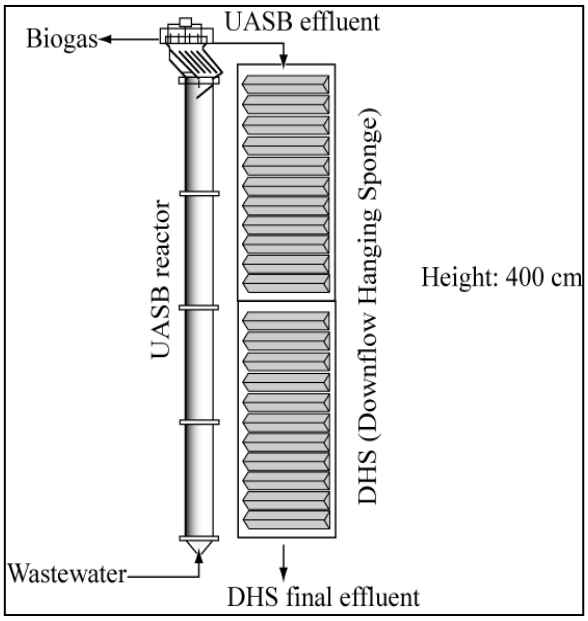

Figure 1. Schematic Diagram the Downflow Hanging Sponge Bioreactor

Batch experiment

The experiments were conducted by using sludge harvested from the DHS sponges located at three different heights: 10 cm, 200 cm, and 400 cm from the inlet. The collected sludge was, after washing by centrifugation to remove background concentration, resuspended in a 10 mM phosphate buffer solution (pH 7.0).

For determination of oxygen consumption rate, the resuspended sludge, after saturated with oxygen, was divided into three parts. A first part was used for measurement of endogenous respiration rate (no substrate added). A second part of the sludge was added with 25 mg NH_4-N.L^{-1} and 125 mg glucose.L^{-1}, and a last part was added substrate similar to the second one with follow added 5 mg.L^{-1} allylthiourea to inhibit nitrification. The rate of oxygen consumption determined in Winkler's bottle and the changed dissolved oxygen concentration was measured with a digital dissolved oxygen meter (YSI-5100).

Dissolved oxygen profile measurement

Clark-type dissolved oxygen (DO) microelectrodes were used to measure the profile of DO in the downward flowing liquid along the DHS bioreactor vertical distance (from inlet to exit point). The microelectrodes were prepared, which have a tip end with 50-100 μm in diameter, and a 90% response time of less than 30 s.

Sludge sampling and cell fixation

Sponges were collected from three different locations of the DHS bioreactor, i.e. 10 cm, 200 cm and 400 cm from the top of the bioreactor. For each location, two pieces of sponges (each had 2 to 3 cm width) were removed from the bioreactor, and were immediately kept in an icebox and transferred to laboratory. The outer part (refer to "surface" below) and the inner part (called "inside") indicates a deeper location from the outer part. Sludge samples were extracted from the sponges by squeezing. The extracted sludge was immediately used for quantifying the ratio of the live cells to the total cells in the sludge. The remaining samples were then washed twice with phosphate-buffered saline (PBS : 130 mM NaCl, 30 mM Na_2HPO_4, pH 7,2), and were fixed with a freshly prepared paraformaldehyde solution (4% paraformaldehyde in PBS) for 6 h at 4°C. The fixed samples were again washed with PBS, and were subjected to in situ hybridization and total cell count.

Fluorescence In Situ Hybridization (FISH approach)

Whole cell in situ hybridization was performed according to Amann (1995) with minor modifications. The fixed samples were first immobilized on gelatin-coated glass slides, and were then dehydrated through 50, 80, and 96% ethanol series. For hybridizations, it was used the following rhodamine- or Cy5-labeled oligo-nucleotide probes complementary to specific regions of 16S rRNA (Table 1). Hybridization was performed at 46°C for 3 h with hybridization buffer containing 0,9 M NaCl, 20 mM Tris-HCl (pH 7,2), 0,01% sodium dodecyl sulfate, and 5 ng.μL^{-1} of each labeled oligonucleotide probe. The washing step was done at 48°C for 20 min with washing buffer. Double staining of cells with two different types of probes was performed as described previously (Wagner *et al.*, 1995). The hybridized samples were mounted with SlowFade light antifade kit (Molecular Probes, Inc., Eugene). The slides hybridized with the probes were observed using a confocal laser-scanning microscope (OLYMPUS FLUOVIEW BX50).

Table 1. 16S rRNA-Targeted Oligonucleotide Probes Used in This Study

Probe	Specificity	Sequence (5'-3')	Formamide concentration (%)*
EUB338	Domain *Bacteria*	GCTGCCTCCCGTAGGAGT	20
Nso190	Ammonia-oxidizing members of the subclass of the *Proteobacteria*	CGATCCCCTGCTTTTCTCC	55
NIT3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	40
Nsr1156	*Nitrospira* spp.	CCCGTTCTCCTGGGCAGT	30

* Formamide concentration in the hybridization buffer

Total, probe-positive, and live cell counts

For total and probe-positive cell counts, the fixed sludge samples were homogenized with a sonicator for 2 to 3 min prior to the immobilization. For enumeration of the total cells in the sludge, the homogenized samples were immobilized on polycarbonate filters (pore size 0.2 μm), and were then stained with 5 ng μL-1 of 4,6-diaminido-2-phenylindole (DAPI) solution for 15 min. For probe-positive cell counts, the immobilized samples on glass slides were hybridized with oligonucleotide probes as described above. In addition, live and dead cell counts were performed using LIVE/DEAD BacLightTM Viability Kit (Molecular Probes, Inc., Eugene) essentially according to the manufacturer's instructions. Live and total cell counts were obtained by examining at least 10 randomly selected microscopic fields on the slide (or over 1,000 of cells were counted to determine the live and total cell counts).

Analytical methods

Ammonia, nitrate, and nitrite-nitrogen were determined by an ion-chromatography system using conductivity detection (Shimadzu CDD-6A). COD was measured using COD reagent kit supplied by Hach and digested in a COD reactor (Hach). The Kjeldahl-nitrogen concentration was colorimetrically determined with Nessler reagent after digestion in a digestion apparatus (Hach).

3. RESULTS AND DISCUSSION

Nitrification performance of the DHS bioreactor

Nitrification performance is shown in Figure 2. The result is given in the course of nitrification and denitrification process, i.e., total nitrogen and ammonia-nitrogen concentrations of influents and effluents (Figure 2a), and oxidized-nitrogen production (Figure 2b). During ten days after start-up, a negligible amount of intermediate product of nitrite-nitrogen concentration (2-8 $mgNO_2\text{-}N.L^{-1}$) was observed. With increasing the operation time, nitrite-nitrogen in the effluent fell below the detection limit. This was accompanied by an increased in the nitrate-nitrogen concentration in the effluent streams.

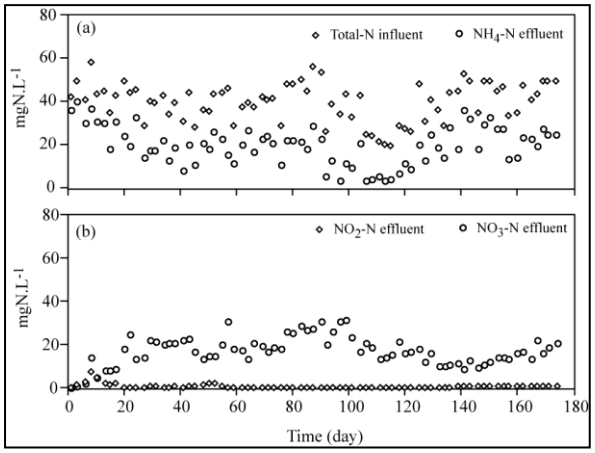

Figure 2. Time Course of Nitrogen in The DHS Bioreactor

Nitrogen mass balance in the DHS bioreactor

In order to verify the study, nitrogen mass balances on the DHS bioreactor were evaluated (presented in Table 2). It is a need to justify that the nitrogen mass balance was not occupied by ammonia stripping since the pH was always less than 8.

Of the influent nitrogen to the DHS, nearly 16,4% of total nitrogen was removed, the remaining constitutes in effluent sludge (3,5%), in oxidized-nitrogen forms (37,9%), and in ammonia-nitrogen form (41,6%). Ammonia-nitrogen removal rate and nitrogen losing rate given in Table 2 are the calculated average rate over the whole mass balance period made. The results give that the ammonia elimination rate and the nitrogen losing rate are 0,24 $kgNH_4\text{-}N.m^{-3}.d^{-1}$ (1,8 $gNH_4\text{-}N.m^{-2}$ media sponge.d^{-1}) and 0,08 $kgNO_3\text{-}N.m^{-3}.d^{-1}$ (0.6 $gNO_3\text{-}N.m^{-2}$ media sponge.d^{-1}), respectively. The obtained nitrification rates from the DHS bioreactor are comparable to those of in various nitrifying reactors which known used forced aeration. They are, for example, a submerged filter has the rate of 1,82 $gNH_4\text{-}N.m^{-2}.d^{-1}$ and an aerated floating filter has the rate 1,35 $gNH_4\text{-}N.m^{-2}.d^{-1}$ (Toettrup *et al*., 1993).

Table 2. Nitrogen Mass Balance

	Input (%)	Output (%)
Particulate organic nitrogen	7.9	3.5
Soluble organic nitrogen	8.8	0.6
NH_4-N	83.3	41.6
(ammonia removal rate)	0.24 kgN/m^3 media sponge d^{-1}	
NO_2-N		1.6
NO_3-N		36.3
Nitrogen losses		16.4
(nitrogen lossing rate)	0.08 kgN/m^3 media sponge d^{-1}	
Total	100.0	100.0

() rate elimination or losses

Profile analysis

Figure 3 gives representative profiles of nitrogen concentrations, and pH. The ammonia oxidation rate was found in the upper portion only around 0,070 kgNH_4-N.m^{-3}.d^{-1} (0,53 gNH_4-N.m^{-2} sponge material.d^{-1}). Although nitrification process with a higher sludge retention time can be achieved at DO concentration in the range of 0,5-1,0 mgDO.L^{-1} (Stenstrom and Poduska, 1980), the DO within a DHS biomass may be considerably less than the bulk liquid concentration. Boller and Gujer (1986) illustrated that nitrification efficiency dropped in the upper part of a trickling filter due to nitrifying biofilm was covered by heterotrophic organisms.

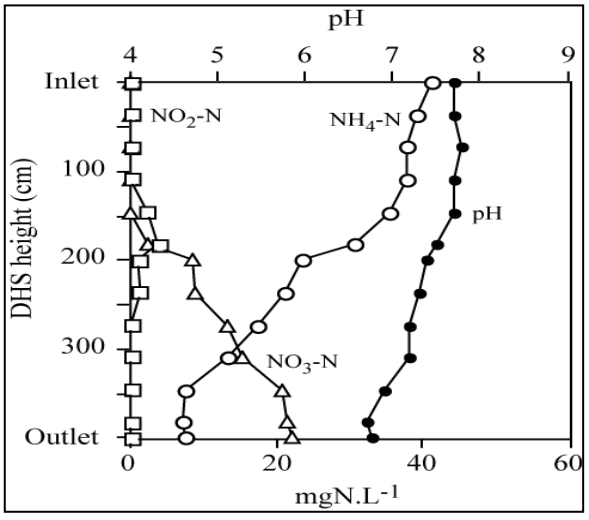

Figure 3. Representative Profile of The DHS Bioreactor

Oxygen profile and oxygen consumption rate

The oxygen consumption rates of accumulated biomass along the bioreactor are given overlap with oxygen profile in Figure 4. Despite there was no forced aeration input to the DHS unit, DO concentration of downward-flowing wastewater increased from zero at the inlet up to almost saturated at the exit.

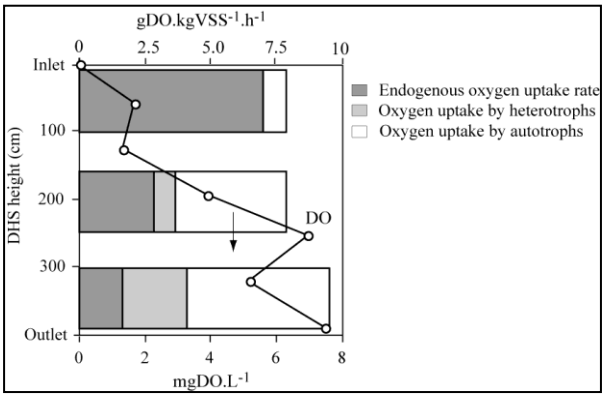

Figure 4. Profile of Oxygen Consumption Rate and Dissolved Oxygen along the DHS Bireactor

The results show the endogenous respiration rate has about 7,1 gDO.kgVSS^{-1}.h^{-1} in the upper part and decreased to 1,7 gDO.kgVSS^{-1}.h^{-1} of in the lower part the bioreactor. This range value indicates no easily degradable organic matter available in the sludge.

Characterization of the DHS sludge

Since the DHS exhibited the high ammonia oxidation efficiencies, the sponges in the bioreactor were thought to be suitable for accumulating active nitrifier biomass. The amount of total bacteria along the bioreactor height determined by DAPI was slightly different, but similar in order with exception of total bacteria found at the surface of middle part. The total bacteria lay between 1.4 x 10^7 (SD± 0,3 x 10^7) cells.mgVSS^{-1} and 5,6 x 10^7 (SD± 1,4 x 10^7) cells.mgVSS^{-1}. However, according to the analysis of live/dead microbes, the sludge at the inlet point had a higher percentage of dead microbes (more than 50% of total cells) than those of the sludge at the middle and the lower portions.

These observations suggested that the upper portion of the DHS had effectively trapped the washout sludge in the UASB effluent. In contrast to this, the sludge at the lower portion of the DHS bioreactor contained the highest percentage of live microbes, probably indicating that an active microbial community had occurred at that position. At the lower portion, the dead cells were obtained only between 33,3% (SD± 12,0%) and 31,2% (SD± 11,6%). Comparison of sludge quality, the percentage of dead cells elevated from surface to inside of sponge in all location investigation which most likely associate with the available dissolved oxygen (Table 3).

Table 3. Total and Live/Dead Bacterial Profile

	Location from the inlet					
	10 cm		200 cm		400 cm	
	Surface (0-1 cm)	Inside (> 1 cm)	Surface (0-1 cm)	Inside (> 1 cm)	Surface (0-1 cm)	Inside (> 1 cm)
Total bacteria (cell $mgVSS^{-1}$)$x10^7$	1,9 (0,6)	1,4 (0,3)	14,0 (2,5)	1,7 (0,6)	5,6 (1,4)	6,4 (1,9)
Live bacteria (%)	49,8 (17,3)	41,3 (15,3)	65,0 (25,4)	52,5 (18,5)	66,7 (24,1)	68,8 (25,3)
Dead bacteria (%)	50,2 (17,5)	58,7 (21,3)	35,0 (14,0)	47,5 (16,9)	33,3 (12,0)	31,2 (11,6)

() standard deviation

In situ detection and enumeration of nitrifiers

Figure 5 presents representative picture of nitrifers found in the DHS bioreactor sludge performed by using fluorescence *in situ* hybridization approach and confocal laser scanning microscopy. *In situ* hybridization with Nso190 (*Nitrosomonas* sp) probe showed a number of coccoid cells in the DHS sludge. Enumeration of Nso190 probe-positive cells out of the total cells was attempted to elucidate the abundance and distribution of ammonia oxidizers in the sludge (Table 4). The sludge at the lower portion, particularly the surface of the sponges, contained the highest number of Nso190-positive clusters (4,07 x 10^3 [SD ± 3,06 x 10^3] clusters $mgVSS^{-1}$). The result was in good agreement with the previous data on the ammonia-oxidation rate.

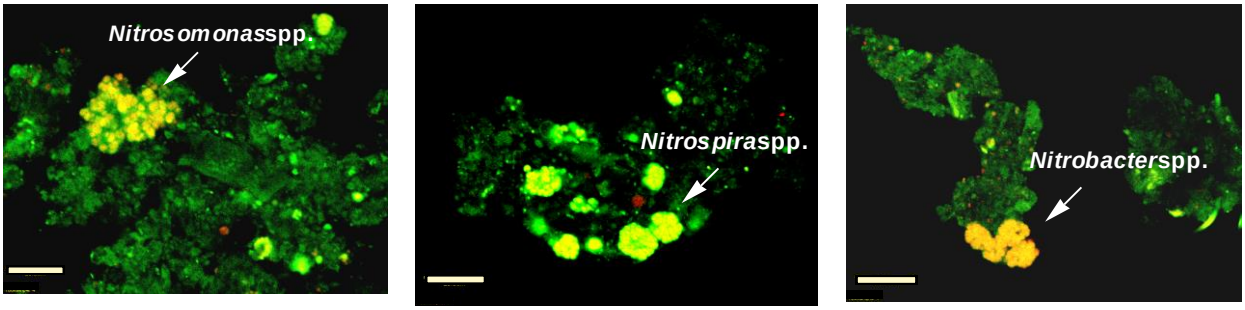

Figure 5. Representative Photographs of Nitrifiers in the DHS Bioreactor Sludge

NSR1156-positive cells (*Nitrospira* sp) were also enumerated according to the method as employed for Nso190-positive cells. According to this analysis, NSR1156-positive cells were counted nearly between 3,12 x 10^3 (SD ± 0,12 x 10^3) clusters.$mgVSS^{-1}$ and 6,18 x 10^3 (SD ± 0,87 x 10^3) clusters.$mgVSS^{-1}$, which were very similar to the values obtained for Nso190 positive cells at similar location (Table 4). Because the NSR1156-positive cells were frequently detected in the DHS sludge than that of the case for NIT3 probe (see below), NSR1156-positive cells, Nitrospira-like cells, might play an important role for nitrite oxidation in the DHS bioreactor.

Table 4. Enumeration of Nitrifier Clusters in the DHS Sludge. No Nitrifier Signal was Observed at the 10-cm Location from The Inlet

Parameter	200 cm location					
	Surface			Inside		
	Nso190	NSR1156	NIT3	Nso190	NSR1156	NIT3
No. of cluster $mgVSS^{-1}x10^3$	1,82 (0,37)	1,61 (0,37)	ns	2,87 (1,54)	2,08 (0,70)	ns
Ave. diameter cluster (μm)	4,5	9,9		5,0	8.8	
Maximum (μm)	12	15		10	14	
Minimum (μm)	2	6		2	5	
Bacterial surface density (cells μm^{-2})	1,80 (0,35)	0,56 (0,20)		1,79 (0,29)	0,52 (0,21)	
	400 cm location					
No. of cluster $mgVSS^{-1}x10^3$	4,07 (3,06)	3,12 (0,12)	1,64 (0,12)	6,2 (1,09)	6,18 (0,87)	ns
Ave. diameter cluster (μm)	4,8	9,7	3,5	6,4	9,6	
Maximum (μm)	15	18	6	12	13	
Minimum (μm)	2	4	2	3	6	
Bacterial surface density (cells μm^{-2})	1,65 (0,40)	0,56 (0,26)	1,27 (0,41)	1,66 (0,57)	0,49 (0,15)	

() standard deviation and ns: no signal

NIT3-positive cells (*Nitrobacter* sp) were detected only in the "surface" of the sponges at the lower

portion (Table 4). In addition, the frequency of the detection of NIT3 positive cells was relatively lower than that of the case of Nso190 and NSR1156 probes.

Simultaneous *in situ* hybridization with probe Nso190 plus NSR1156 probes or Nso190 plus NIT3 probes was performed to confirm the close associations of ammonia and nitrite-oxidizers in the DHS sludge samples. Through this analysis, it was elucidated that the ammonia-(Nso190-posotive cells) and nitrite-(NSR1156 and NIT3-positive cells) oxidizers did not always occur in juxtaposed locations.

4. CONCLUSIONS

The DHS bioreactor is suitably for nitrification of UASB effluents. The bioreactor performed ammonia removal rate of around 0.24 $kgN.m^{-3}.d^{-1}$, and nitrogen-losing around of 0.08 $kgN.m^{-3}.d^{-1}$. Regarding the nitrification rate, although no requirement of external aeration input, the DHS nitrification rate is comparable to other systems which is known using mechanical-device for supply oxygen. The combination of a DO microelectrode and specific oligonucleotide probes offered a reliable information of nitrification in the DHS sludge. The high population of nitrifiers was observed from the half to the end of the bioreactor, equivalent to HRT of 1-2 h. The dominant population of *Nitrospira* spp was observed as compared to *Nitrobacter* spp. in DHS sludge. Therefore, *Nitrospira* spp. was assumed to be responsible for nitrite oxidation.

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