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Detection of Ammonia-oxidizing Bacteria (AOB) in the Biofilm and Suspended Growth Biomass of Fully- and Partially-packed Biological Aerated Filters

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1. Introduction

Nitrification is a two step process namely ammoniacal oxidation and nitrite oxidation. Oxidation of ammonium to nitrite is carried out by autotrophic bacterium mainly *Nitrosomonas* (e.g. *N. europaea*, *N.oligocarbogenes*) and Nitrosospira while conversion of nitrite to nitrate is performed by *Nitrobacter* (e.g. *N. agilis*, *N. winogradski*) and Nitrospira. However, ammoniacal oxidation is considered as the limiting or critical process in nitrification since the ammonia-oxidizing bacteria (AOB) has very low growth rate (Metcalf and Eddy 1991).
Various approaches, both culture dependent and independent have been applied to analyze and compare the microbial structure of biomass. However, culture dependent methods are biased by the selection of species which obviously do not represent the real dominant structure (Wagner et al 1995; Lipponen et al 2002). Recently, the development of culture independent molecular techniques, like fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR) or denaturing gradient gel electrophoresis (DGGE) improved the analysis of environmental samples.
Whole cell fluorescene *in situ* hybridization (FISH) is a technique that uses fluorescently labelled phylogenetic oligonucleotide probes to detect specific whole cells/organisms in biological samples. It can be a valuable tool for the study of microbial dynamics in natural environments (Li et al 1999; Liu et al 2002, Eschenhagen et al 2003). These probes could be designed using the wealth of 16S and 23S rDNA sequence data available to target species, genera subdivisions or divisions in-situ and could be labelled with fluorescent groups, radioactive groups or antigens for immunological detection (Amann 1995).
A combination of the FISH approach with the application of scanning confocal laser microscopy (SCLM) allows non-destructive studies of the three dimensional arrangements of bacterial population identified and out-of-focus fluorescence (Wagner et al 1995).
Biological Aerated Filters (BAFs) also have a long history of successfully removing nitrogen in wastewater treatment plants (Chen et al 2000; Quyang et al 2000; Chui et al 2001). Biofilm in the reactors bears great potential for simultaneous and efficient removal of nitrogen (Fdz-Polanco et al 2000). Therefore, an assessment of nitrogen removal efficiency has been made to detect any deterioration to the performance. A possible adverse effect of reduced mass of biofilm in the partial-bed reactor was foreseen for the reason that the slow-growing nitrifiers

will be more easily washed out at lower mean solids retention times (SRT) (Gieseke et al 2002). The denitrification process may also be disrupted because the biofilm provides potential anaerobic conditions in which denitrification flourishes.

Fdz-Polanco et al (2000) pointed out the importance of understanding the spatial distribution of the microbial population, and its activity, for the optimisation of nitrogen removal performance in reactors treating wastewater. The performance of the full and partial-bed reactors for nitrogen removal has been examined (Fatihah 2004). It was verified that the full- and partial-bed reactors have the capacity to remove 79.3 ±7.7 % and 79.4 ±3.6 % nitrogen at carbon organic loadings of 5.71 ±0.16 kg COD/m^3.d, corresponding to nitrogen loadings of 0.24 ± 0.02 kg N/m^3.d. At this condition, the organic carbon removal efficiency was 5.34 kg COD/m^3.d for the full-bed and 5.22 kg COD/m^3.d for the partial-bed. The successful removal of nitrogen indicates the existence of ammonia-oxidizing bacteria (AOB) in both reactors.

From the perspective of engineering design, it is important to be able to predict the functional groups of bacteria that are most favoured by various applied reactor conditions. In this respect, knowledge of their activities is more important than that of the detailed microbial population (Beer and Muyzer 1995). The nitrogen removal process in such systems is typically initiated by chemoliautotrophic ammonia-oxidizing bacteria converting ammonia to nitrite and traces of oxidized nitrogen gases. Subsequently nitrite-oxidizing bacteria catalyse the oxidation of nitrite to nitrate, and the process is then completed by denitrification (Metcalf and Eddy 1991). Clearly the oxidation processes of nitrification are an essential prerequisite for the whole removal process. In addition, retaining a large amount of nitrifying bacteria within the reactor can be difficult to achieve, due to their relatively low rates of respiration, and their subsequent sensitivity to DO and temperature, thereby making nitrification the rate-determining microbial system in the entire nitrogen removal process (Tsuneda et al 2003).

Since the number and the physiological activity of the ammonia oxidizers are generally the rate-limiting parameters, the rapid and reliable identification of this autotrophy is an important task. The aerobic ammonia oxidizers belong to a very restricted group of autotrophs with Nitrosomonas and Nitrosospira being the best-known oxidizers (Sliekers et al 2002), dominated by β-Proteobacteria (Wagner et al 1995; Eschenhagen et al 2003). Rowan et al (2003) found that detection of ammonia-oxidizing bacteria using PCR amplified 16S rRNA gene in a laboratory-scale BAF reflects the dominant AOB within a full-scale plant.

If the partial-bed reactor exhibited comparable nitrogen removal performance, intriguing questions would arise: would the slow-growing nitrifying bacteria's preference for attachment on biofilm thereby enhancing sludge retention time (SRT), be challenged by bacterial growth in suspension: or would there be other factors related to reactor configuration that satisfied the need for nitrifying bacteria to grow in the partial-bed reactor. Since, for any high rate system, the AOBs need to reside within the biofilm that has a longer SRT than the suspended growth, it is interesting to locate the microorganisms along the height of both the full- and partial-bed reactors. The detailed aspects to be evaluated in this part include:

- to detect and enumerate the presence of AOBs in the biofilm and suspended growth biomass using fluorescence *in situ* hybridization (FISH) technique in combination with confocal laser scanning microscopy (CLSM)

- to correlate changes in the proportion of AOBs to all bacteria along the reactor heights in relation to the reactor configuration
- to associate factors that contribute to the changes in the AOB proportion

2. Experimental system

Two identical reactors were built; each reactor was 14 cm in diameter and 100 cm in height, providing an empty bed volume of 15 l. A small amount of freeboard or headspace (2.8 litres) was provided at the top of the reactor. The reactors were constructed from PVC, a non-transparent material that prevents the growth of phototrophic organisms. The columns were built with considerations for process air and influent supplies, backwashing air and water requirement and sampling outlets.

The control reactor was filled with 10.9 l cascade rings (Glitsch UK) whilst the second reactor was only partially packed with 5.5 l cascade rings. The media were stationary and held in place by a rigid polypropylene mesh with 15 mm diameter holes placed at the top and bottom of the packing. Three ports were placed along the height of the reactors for sample collection.

A synthetic waste prepared in the laboratory was used to provide a consistent organic substrate for all loadings. The basic make-up of the influent organic strength material used in the study was whey powder, glucose and meat extract (Lab Lemco powder) which contributed approximately 38%, 33% and 29% of the total soluble COD content of the substrate respectively. In order to guarantee that organic carbon was the limiting nutrient, a COD:N: P ratio of 25:5:1 was adopted. Nitrogen component of the feed came from whey powder (24.7%), meat extract (63.7%), and ammonium-dihydrogenphosphate (11.6%). 1 l of the prepared mixture produces a concentrated feed around 40000 mg/l COD.

2.1 Suspended biomass and biofilm sampling

The collection of samples for this study was carried out at the end of the steady-state condition of 0.24 ± 0.02 kg $N/m^3.d$ nitrogen loadings. Samples of the biofilm and suspended growth biomass were taken at different depths of the reactors. The in-situ characterization followed a top-bottom approach. Fig. 1 illustrates the exact locations where the samples of suspended biomass and biofilm were obtained from the reactors.

Samples of suspended biomass were taken from port 1, port 2 and port 3 respectively. At each port, about 50 ml of reactor aliquot was wasted before sample collection to ensure that any debris or anaerobic bacteria residing in the pipeline was discarded. A 10 mL volume of aliquot was taken and immediately fixed with 1:1 absolute ethanol. Samples were then stored at -20^{o} C.

For sampling the biofilm, the liquid was first drained from port 1 in order to allow access into the upper bed layer. Tongs were used carefully to remove the media from the upper layer. A random piece of media from the specified level was chosen. The biofilm was gently scraped off the plastic material using a sterile surgical knife before washing the media with 10 ml phosphate-buffered saline (PBS) solution. This procedure was repeated four times until all the biofilm attached to the media was completely removed. To homogenize the biofilm, the sample was sonicated for 2 minutes using an ultrasonic homogenizer (Bandelin Electronics D-1000, Germany). 10 ml of the aliquot was put in a universal bottle and fixed with 1:1 absolute ethanol before storing at -20^{o} C. The sampling of biofilm at the second location was subsequently continued by draining the liquid from port 2. The same procedures were repeated until the media at the bottom were sampled. To detect the AOB in

the samples, the FISH technique (Coskunur 2000) was applied in order to produce the fluorescent sites in the cells, and these were detected through the use of confocal scanning laser microscopy (CSLM).

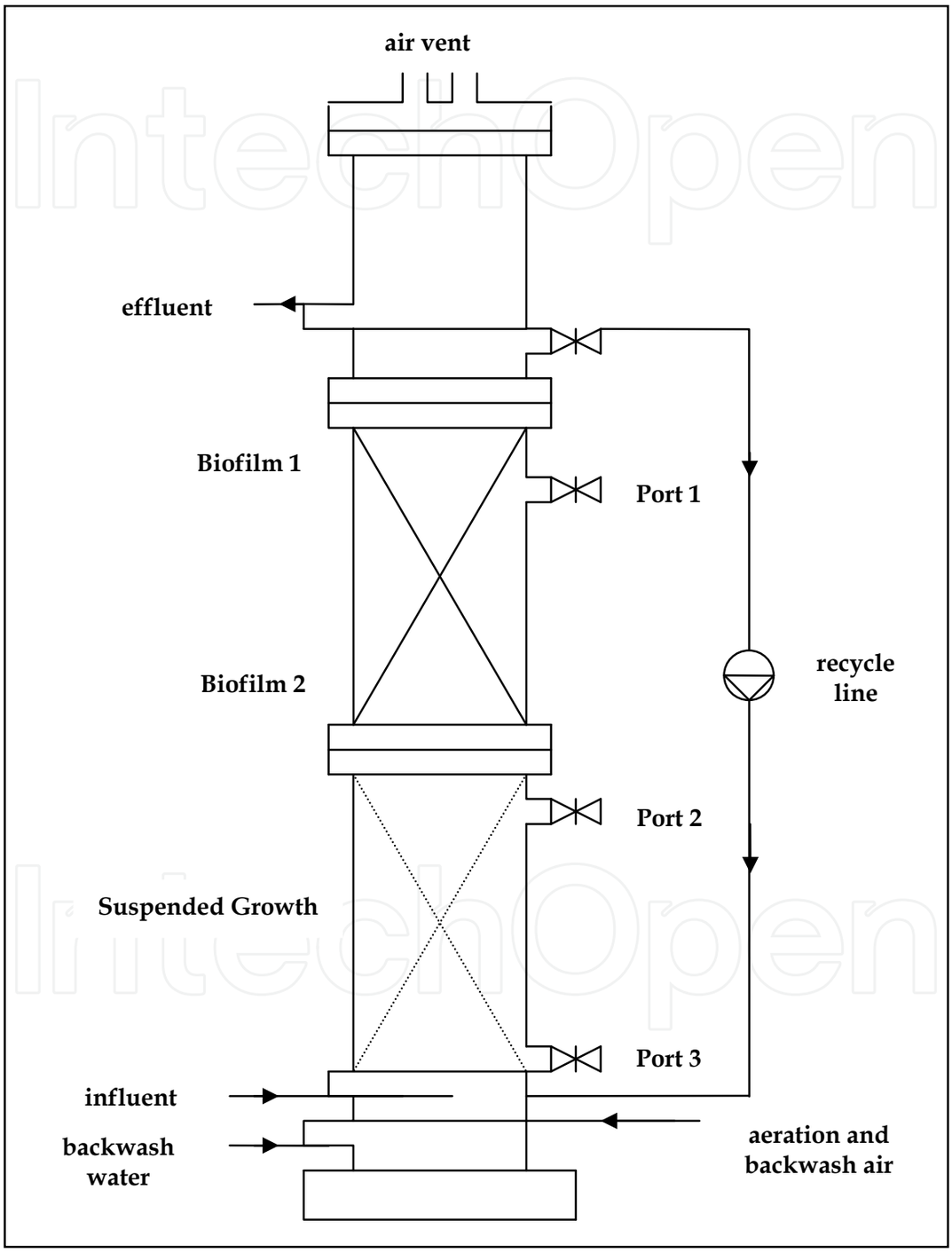

Fig. 1. Sampling locations for biofilm and suspended growth biomass along the reactor's height

2.2 Fluorescent *in situ h*ybridization (FISH) technique (coskunur 2000)

This method was applied to determine the presence of ammonia oxidizing bacteria (AOB) and to quantify them in the reactors. The steps involved fixation of the samples, permeabilization and hybridisation with probes, and finally detection with confocal laser scanning microscope (CLSM).

2.2.1Paraformaldehyde Fixation and Permeabilization

Generally, the samples used for this technique have undergone short term fixation where absolute ethanol was added in a volume ratio of 1 sample: 1 ethanol in sterile universal bottles and stored at -20^{o} C.

A 1 ml volume of the stored sample was transferred to a 1.5 ml eppendorf tube and centrifuged at 13000 x g for 3 minutes. The supernatant was removed and the sample was washed with phosphate buffered saline (PBS) by adding 1 ml of the solution, mixing using vortex and centrifuging at 13000 x g for 3 minutes before removing the supernatant again. The resulting pellet was resuspended in 0.25 ml PBS and 0.75 ml PFA fixative and vortexed. A 4 % paraformaldehyde fixative solution was prepared fresh for every time of use, the procedure of which tabulated in Appendix 4.1. The suspension was incubated for at least 3 hours, or overnight, at 4^{o}C.

After fixation, the cells were washed by centrifuging at 13000 x g for 3 minutes, removing the supernatant, adding 1 ml PBS and mixing. The samples were centrifuged again at 13000 x g for 3 minutes. The supernatant was removed and the sample was kept with PBS and absolute ethanol at 1:1 (v/v) and mixed. It was then stored at -20^{o}C.

2.2.2 Hybridization

A volume of 250 µl of fixed sample was centrifuged at 13000 x g for 3 minutes and the supernatant was removed. The sample was washed once by adding 1 ml PBS and centrifuged again. The sample was then divided into four tubes: a negative control containing no probe to observe autofluorescence, a negative control to observe non-specific binding events, a positive control where a universal eubacterial probe was added (Bact 338) and a sample to be hybridised by a specific AOB detection probe. The samples were serially dehydrated in successively increasing concentrations of molecular grade ethanol (60%, 80%, 100% v/v). After adding 1 ml of the ethanol solution, the sample was vortexed and left for 3 minutes. The sample was then centrifuged at 13000 x g for 3 minutes and the supernatant was removed.

The following step is to hybridize the samples. Hybridisation buffer (HB) was prepared according to Amann et al (1990). HB was added so that the final volume including the probe will be 40 µl. Thus, for the negative control for autofluorescence, 40 µl HB is added. For a hybridisation containing only one probe (2ul), 38ul HB is added. For a hybridisation containing two probes (2+2 µl) 36 µl HB is added. The samples were prehybridized for 15 minutes at the hybridisation temperature. After prehybridisation, 2 µl of probe (50 ng/µl) was added to the samples that were then incubated at the optimal hybridisation temperature for the given probe (Table 1) for at least 4 hours (or overnight).

Following hybridisation, the samples were centrifuged at 13000 x g for 3 minutes and the supernatant was removed. A volume of 0.5 ml of wash buffer was added and the sample was mixed using a pipette before being incubated for 15 minutes at the same temperature as the hybridisation step. The washing step was again repeated.

Probe	Sequence	rRNA target	Target	Formamide ; Temperature	Reference
nonEUB	ACTCCTACGG GAGGCAGC		None (negative control)	0% ; 37°C	Amann et al (1990)
EUB338	5′GCTGCCTCCC GTAGGAGT-3′	16S	Eubacteria	20% ; 37°C	Amann et al (1990)
Nso1225	5′-CGCGATTGTAT TACGTGTGA-3′	16S	Ammonia oxidizing β-Proteobacteria	35% ; 51°C	Mobarry et al (1990)

Table 1. Features and conditions of probes during hybridisation

The samples were centrifuged again at 13000 x g for 3 minutes, the supernatant was removed and 1 ml of MilliQ water was added. Finally, the samples were centrifuged, the supernatant removed and the samples resuspended in 100 ul MilliQ water.
A 10 ul aliquot of the sample was added to a gelatine-coated slide with Teflon-coated wells of a known diameter (Appendix 4.1) and allowed to dry in a hybridization oven at 30°C. The sample spot on the slide was mounted in a small drop of the antifadent-Citifluor (AFI, Canterbury, UK). A cover glass was sealed carefully on the top of the slide by applying clear nail varnish to the edges to prevent movement during microscopy. The slide was then stored at -20°C in the dark and was prepared for viewing.

2.2.3 Scanning on a confocal laser microscope

The distribution of hybridized cells was subsequently visualised by means of a Leica TCS SP2 UV confocal laser scanning microscope (CLSM) equipped with Leica DMRXA microscope. Images were captured and processed using LCS V2.5.1040-1 software. For observation x 60 Na 1.32 lenses were applied.
The CLSM was run in the following mode: single channel for Fluorescene and double channel for Carbocyanine-5. Fluorescene was detected using excitation at 488 nm and a long pass emission filter in the range of 500-530 nm. Cy5 was detected using excitation at 633 nm and a long pass emission filter of 650-680 nm. The artificial colours green and red were assigned to the monochrome images acquired in the fluorescene and Cy5 channels respectively. The LCS software actively mixed colours so that a cell emitting red and green (the AOB) would appear yellow. For each sample, only 5 fields of view were randomly recorded in view of the time and budget available for the process.

2.2.4 Enumeration technique

An Excel spreadsheet constructed by Coskunur (2000) was used to carry out the calculation based on Equation 1 below:

$$K = \frac{(Nx2xA1)}{(A2x0.01x10xODF)} \quad (1)$$

where

K = average number of microcolonies in one ml of sample
A1 = area of sample spot (the area can be calculated from the diameter of the sample spot , $[\pi(D/2)^2]$)

A2 = area of one field view
N = average number of ammonia oxidizer microcolonies/field of view
V = volume of sample applied
Vo = original volume of sample
ODF = other dilution factors not considered above may be required (e.g. volume of sample spun down). Where no ODF, default value = 1

The spreadsheet was designed for the quantification of AOB population in wastewater treatment plants following FISH and quantification typically using CLSM produced images. It requires that the user inputs data concerning the number of AOB microcolonies, the shortest and longest diameter of the microcolonies, area measurements of the fields of view and sample spots and dilution factors used in FISH. The spreadsheet returns the average number of microcolonies and geometric mean diameter. This data sheet can also be used to calculate the concentration of AOB in mg/l, the % AOB in terms of total bacterial population (measured by volatile suspended solids, VSS), following an empirically determined conversion factor, in terms of total cell numbers.

3. Comparison of AOB Cells in the biofilm and suspended growth samples

3.1 Cluster size

The relative frequencies of AOB cluster diameters for all the samples investigated are presented in Fig. 2.

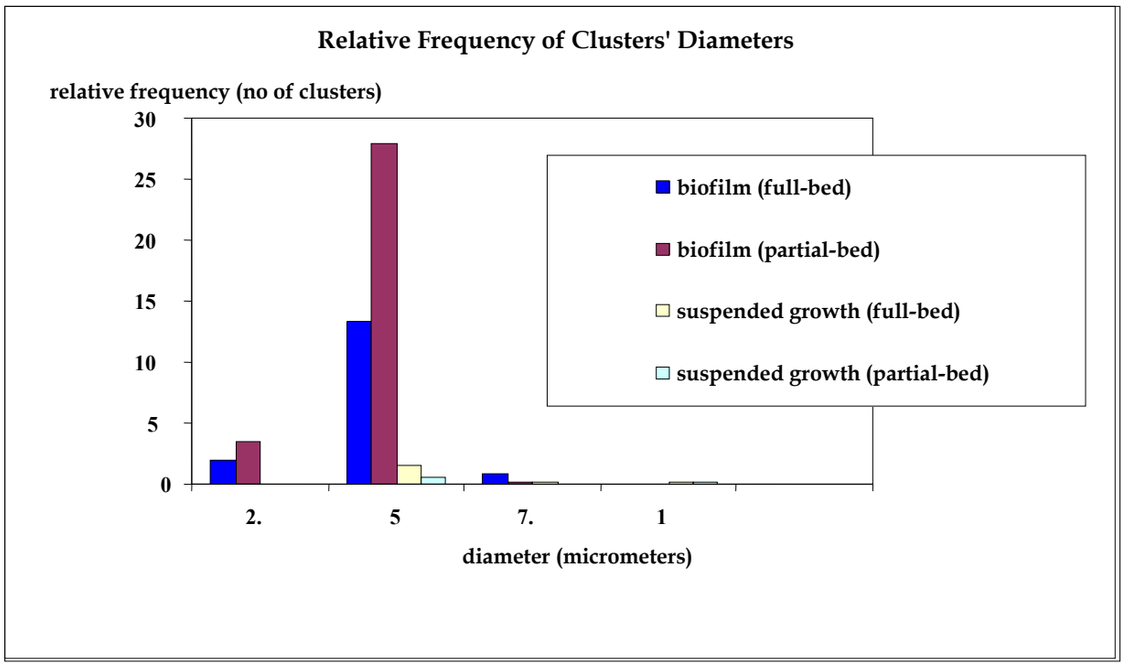

Fig. 2. Size distribution of cell clusters in the full- and partial-bed reactors

The results show that the majority of the clusters had diameters of 5 µm with the largest being 10 µm. These findings are quite consistent with the results obtained by Kloep et al (2000). Using probe Nsm 156, the majority of the hybridized clusters was found to be smaller than 10 µm and only a few were larger than 15 µm. Wagner et al (1995) also detected

clusters hybridized with probe Neu 23 having diameters between 3 µm and 20 µm from samples of municipal sewage treatment plants. Nitrifier agglomerates are therefore small, for example well below those particle sizes (>100 µm) effectively removed by conventional primary sedimentation (Kiely 1998). Their retention in the system must therefore be mainly due to interactions with the biofilm attached to the media elements in the bed.

By visual observation, yellow clusters emerge on all biofilm samples as shown on Plates 1- 4. The AOB appear yellow due to double bindings of the fluorescene-labelled probe EUB 338 (emitted as green) and Cy5-labelled probe Nso 1225 (emitted as red). The formation of cluster growths is a feature of ammonia-oxidizing bacteria, in particular Nitrosomonas sp (Wagner et al 1995; Mobarry et al 1996). The clusters were spherical to oval shaped and appeared over diameters ranging from approximately 2.5 to 12.5 µm.

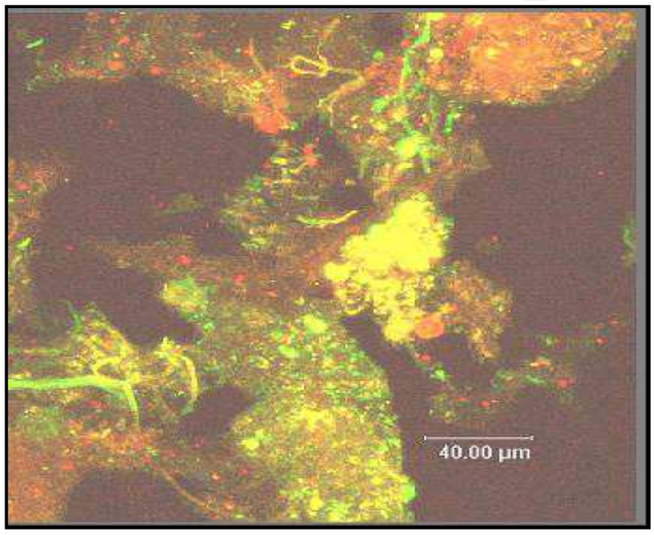

Plate 1. CLSM image of a biofilm sample from the top of the full-bed reactor

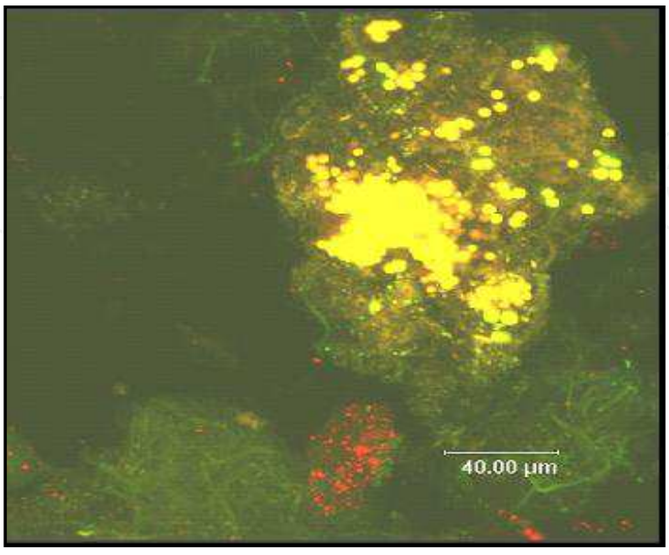

Plate 2. CLSM image of a biofilm sample from the middle of the full-bed reactor

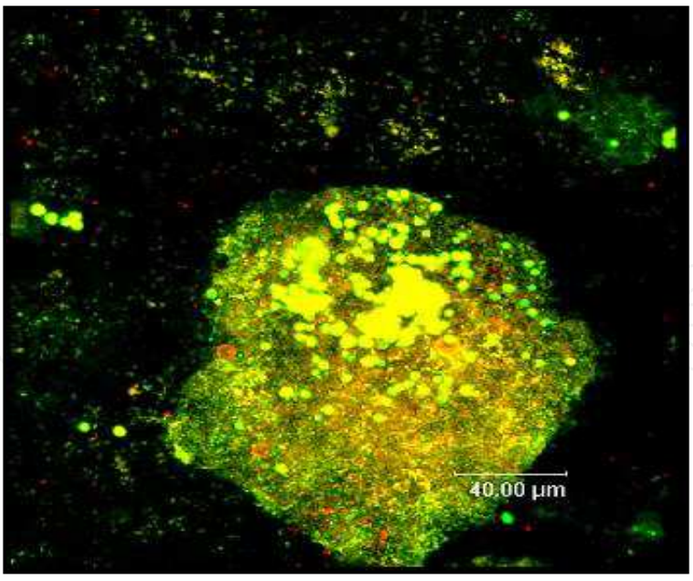

Plate 3. CLSM image of a biofilm from the top of the partial-bed reactor

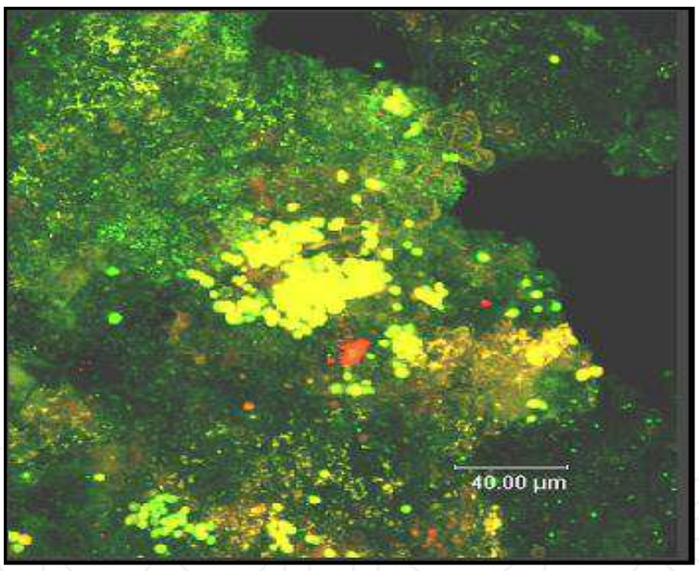

Plate 4. CLSM image of a biofilm from the middle of the partial-bed reactor

Plates 5 - 7 of suspended growth samples from the full-bed reactor show fewer AOB clusters than Plates 1 - 4. Layers of filamentous bacteria can be seen dominating, especially the suspended biomass samples from the top and middle parts of the reactors.

For the CLSM images of the suspended growth biomass samples from the partial-bed reactor, intense diffuse, green coloured fluorescence was often observed. This could have been due to debris, inorganic particles or the bacterial cells. A large number of coccoid structures was detected using the EUB 338 probe. They usually occurred in characteristic clumps and appeared ring shaped. MacDonald and Brozel (2000) observed the same phenomena in their study of bacterial biofilms in a simulated recirculating cooling-water

reactor and suggested that this could result from dense chromosomal material at the cell center, leading to a concentration of ribosomes at the periphery of the cells.

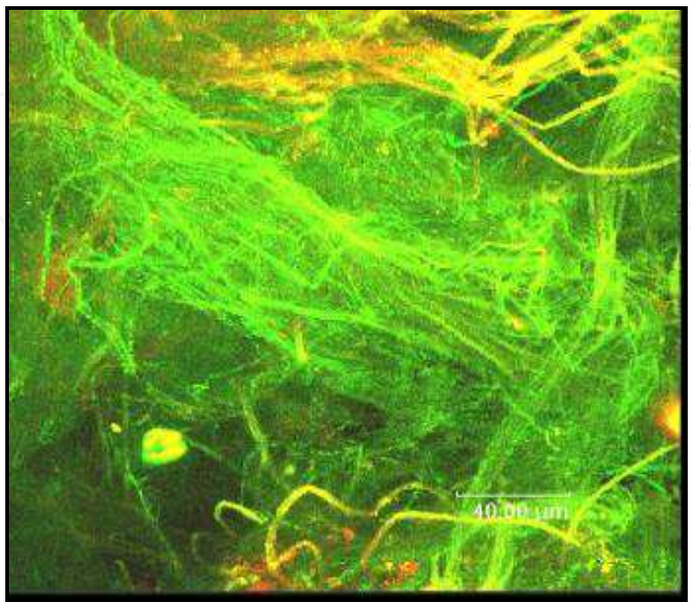

Plate 5. CLSM image of suspended growth biomass from the top of the full-bed reactor

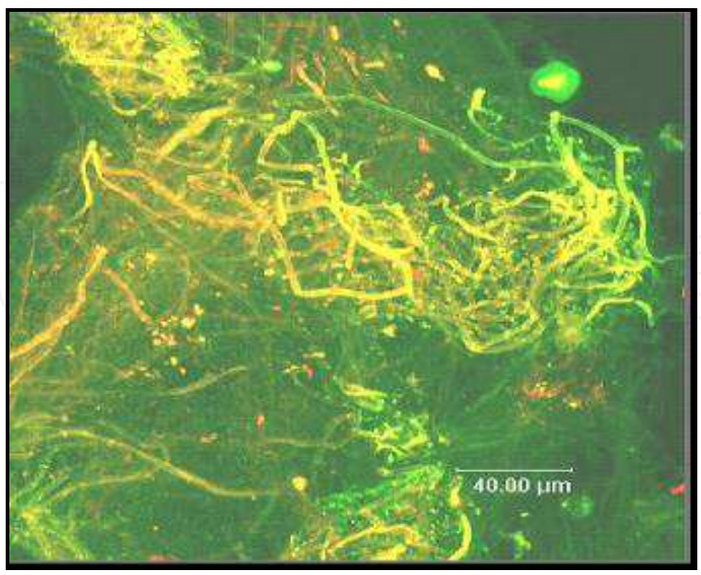

Plate 6. CLSM image of suspended growth biomass from the middle of the full-bed reactor

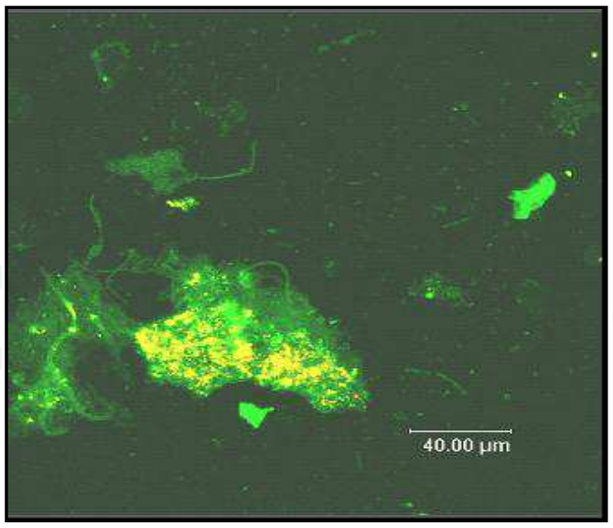

Plate 7. CLSM image of suspended growth biomass from the bottom of the full-bed reactor

3.2 Enumeration of ammonia-oxidizing bacteria

The number of AOB cells per ml of biomass was calculated from the counts based on cluster diameters using an Excel spreadsheet developed by Coskunur (2000). The numbers of AOB cells obtained are given in Table 2 below:

	Full-bed		Partial-bed	
	Biofilm	Suspended growth	Biofilm	Suspended growth
Top	1.720×10^5	2.149×10^4	5.589×10^5	1.075×10^4
Middle	2.204×10^5	1.344×10^4	2.929×10^5	ND
Bottom	6.451×10^4	1.345×10^4		8.075×10^3

Table 2. Number of AOB cells per ml of biomass in the biofilm and suspended growth samples

The higher number of AOB cells present in the biofilm samples than in the suspended growth samples could be due to the fact that AOB are slow-growing bacteria that need long mean solids' retention times to become established. Nitrifying bacteria, when compared with the heterotrophic organisms, are very much slower growing. Watson et al (1989) observed that the doubling times of these bacteria range from 8 hours to several days and that they have a tendency to attach to surfaces and to grow in cell aggregates referred to as zoogloeae or cysts (Lipponen et al 2002). In order to maintain an effective population of nitrifying bacteria within a biological reactor, a long retention time is required (Barber and Stuckey 2000). This is in accordance with the results obtained by Hidaka et al (2003), who discovered that in a biofiltration process for the advanced treatment of sewage, attached biomass contributed to most nitrification activity. Gerceker (2002) reported the loss of nitrification between SRTs of 0.9 and 2.4 days in a closely controlled jet-looped membrane bioreactor. Noguiera et al (2002) found that competition in biofilm results in a stratified biofilm structure, the fast-growing heterotrophic bacteria being drawn to the outer layers where both substrate concentration and detachment rate are high, whilst the slow-growing nitrifying bacteria stay deeper inside the biofilm. The heterotrophic layer has a positive

effect on the nitrifiers by protecting them from detachment as long as the bulk oxygen concentration is high enough to preclude its depletion in the biofilm.
It is a fact that biofilm is significant in controlling long SRTs in a system. The full-bed reactor, which has a higher mass of biofilm than the partial-bed, as a result of the greater volume and surface area of the fully packed reactor, has SRTs of 21.2, 27.5 and 11.1 days at the three backwashing rates used in the study. The partial-bed reactor, on the other hand, had much shorter SRTs of 3.3, 3.9 and 2.7 days. Meanwhile, the biofilm in the partial-bed reactor was kept thin and stable, and therefore was not easily washed out during the backwash operation. Therefore, the retention time of biofilm in the partial-bed reactor is actually longer than the overall SRT of the system. Chuang et al (1997) pointed out that satisfactory nitrogen removal is achieved at SRT > 10 days.
The suspended growth biomass in the reactors, and especially that of the partial-bed reactor, was always subject to being washed out by the backwashing operation and lost in the effluent.

3.3 Significance of AOB Cells in the biofilm and suspended growth cultures

Tests carried out to compare the significance of AOB cells in both types of cultures were based on nonparametric methods of one-way ANOVA. Table 3 lists the results obtained.

	Full-bed		Partial-bed	
	Biofilm	Suspended growth	Biofilm	Suspended growth
Mean	$1.523 \times 10^5 \pm 7.979 \times 10^4$	$1.613 \times 10^4 \pm 4.645 \times 10^3$	$4.259 \times 10^5 \pm 1.881 \times 10^5$	$6.275 \times 10^3 \pm 5.596 \times 10^3$
Pooled s.d.	5.651×10^4		1.0867×10^5	
p-value	0.042		0.024	

Table 3. Results of variance analysis of AOB cells (no. AOB cells/ml sample) in the biofilm and suspended growth samples

Table 3 indicates that in both reactors there is a significant difference in the number of AOB cells in the biofilm and suspended growth samples. At 95% confidence levels, the p-value for the full-bed reactor is 0.042 whilst that of the partial-bed reactor is 0.024. Since the p-values obtained are smaller than 0.05, this means that in both reactors, specific cell concentrations of AOB were found to be significantly higher in the biofilm samples as compared to the suspended growth samples.
It was found that the AOB cells are more numerous in the biofilm samples than in the suspended growth samples of both the full- (p=0.042) and the partial-bed (p=0.024) reactors. It is therefore interesting to compare the significance of the overall AOB cells in the full- and partial-bed configurations, knowing that the mass of biofilm is lower in the partial-bed reactor due to the reduced media volume compared to the full-bed reactor.
Table 3 also indicates that there is no significant difference between the concentrations of AOB cells in the biofilm samples of the full- and partial-bed reactors (p=0.099), and also in the suspended growth samples (p=0.079). To put the overall abundance of AOB cells in the full and partial-bed reactors side-by-side, the AOB cells in the biofilm and suspended growth samples for each reactor were combined, giving total concentrations of AOB cells for that particular configuration. The p-value of specific AOB concentrations comparing the

full- and partial-bed configuration is p=0.427. The value indicates an almost comparable AOB relative abundance in both the full- and partial-bed reactors. Higher mean AOB cells of the biofilm in the partial-bed reactor equate with the higher mean value of suspended growth samples in the full-bed reactor, resulting in almost equivalent mean AOB cells in both reactors.

Lazarova et al (1994) made a point that the balance between biofilm losses and growth processes on the outside of the media was dominated by shear forces, exerted by the liquid as it flowed past the media surfaces in the reactor. In a study to evaluate the essential role of hydrodynamic shear force in the formation of biofilm, Liu and Tay (2002) pointed out that biofilm density quasi-linearly increases with the increase of shear stress. Chang et al (1991) discovered that the medium concentration and the turbulence indicated by Reynolds numbers, significantly affected biofilm density and thickness of a fluidized bed biofilm reactor. In this type of reactor, increasing medium concentration can be associated with increasing attrition due to particle-to-particle contacts and increasing turbulence correlates flow fluctuations that could create forces normal to the biofilm, i.e. the shear stress. Table 4 illustrates the results obtained in their study.

Glass beads concentrations (g/l)	Reynolds number	Shear stress (dyne/cm^2)	Biofilm density (mg VS/cm^3)	Biofilm thickness (μm)
664.0	0.55	8.30	56.0	10.6
457.0	0.61	6.77	18.5	32.0
463.0	0.61	6.82	21.0	31.3
684.4	0.55	8.42	41.50	8.8
604.1	0.56	7.90	30.5	15.4
609.4	0.56	7.90	28.5	15.3
502.9	0.79	8.26	52.0	11.0
542.0	0.78	8.58	62.0	7.1
269.7	1.16	7.44	14.5	21.4
258.6	1.17	7.31	14.0	23.2
265.2	1.16	7.38	9.9	22.1

Table 4. Measured and calculated values for experimental runs with the fluidised bed biofilm reactor (Chang et al 1991)

In this study, since the medium is fixed, there is no attrition effect. Therefore turbulence effect could be the major factor that increases the detachment pressures, and caused the biofilm to become denser and thinner.

3.4 Relative concentration of AOB at different filter heights of the full- and partial-bed reactors

Fig. 3 illustrates the percentage values of AOB concentrations with respect to VSS concentrations in biofilm samples from the full-bed reactor.

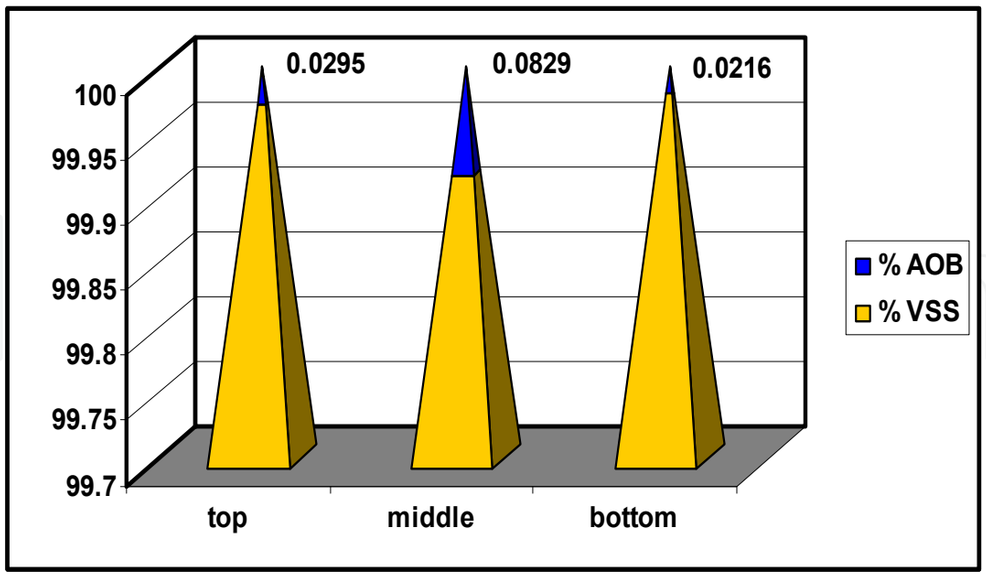

Fig. 3. Percentage values of AOB in the biofilm samples of the full-bed reactor

The highest percentage of AOB was found in a sample from the middle of the full-bed reactor (0.0829%), followed by the top part (0.0295%), whilst very little was found in the bottom part (0.0216%). A low percentage of AOB was obtained at the bottom despite the fact that the substrate and oxygen sources were supplied from here. This anomaly could best be explained by the fact that competition between heterotrophic and nitrifying bacteria for substrates (oxygen and ammonia) and space in the biofilms resulted in the fast-growing heterotrophic bacteria dominating the bottom part of the reactor. Plate 8 of biofilm sample from the bottom of the full-bed reactor show that AOB clusters are not dense as in Plates 1- 2 of the top and the middle positions.

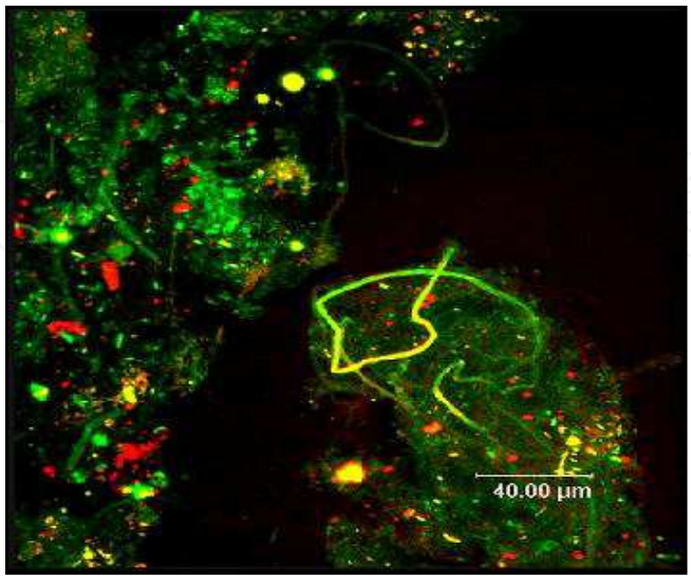

Plate 8. CSLM image of a biofilm sample from the bottom of the full-bed reactor

The trend of AOB growth in the biofilm samples of the full-bed reactor was followed through for the partial-bed reactor (Fig. 4):

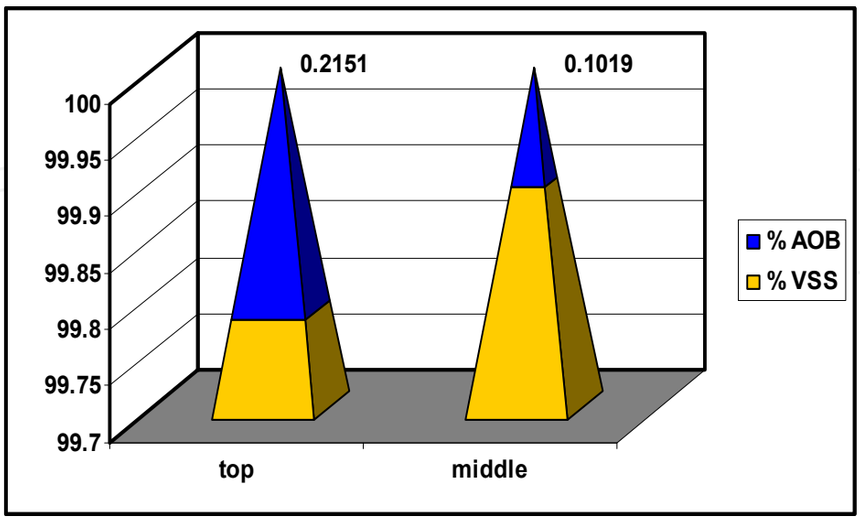

Fig. 4. Percentage values of AOB in the biofilm samples of the partial-bed reactor

The same argument of competition for substrates and space between heterotrophic bacteria and nitrifiers explained the lower percentage of AOB obtained in the middle (0.1019%) compared to the top part of the partial-bed reactor (0.2151%).
To validate the hypothesis made on AOB distribution in both the full and partial-bed reactors, a previous work by Wijeyekoon et al (2000) was used to investigate the effect of organic loading rates on nitrification activity. Table 5 summarizes the reactor conditions of their study.

Biofilters	A	B	C
Diameter (cm)	5	5	5
Height (cm)	50	50	50
Influent flow (l/h)	1.6	0.8	0.4
Influent conc. (mg/l TOC)	5	5	5
Influent nitrogen (mg/l NH_4^+-N)	5	5	5
OLR (kg COD/m^3.d)	0.19	0.098	0.097

Table 5. Unit dimensions and operating conditions of downflow biological filters (Wijeyekoon et al 2000)

The three reactors, packed with the same weights of anthracite, were equipped with sampling ports at depths of 6 cm (port 1), 18.5 cm (port 2) and 37.5 cm (port 3) from the top end of the filters. The specific rate of NH_4^+-N oxidation in the reactors was determined by the biomass extracted from those ports. It was discovered that the highest rates in filter A and B were obtained at the effluent ends of the reactors, but in filter C, the rates were comparably high from all ports. Also, among the three reactors, filter C produced the highest rates, with an average of 48.1 and 56.4 g N/(mg protein.hr) for ports 1 and 2 respectively. The conclusion derived from the study is that at high organic carbon loadings nitrifiers are non-uniformly distributed along the length of a filter, with excessive growth of heterotrophs near the feed end and nitrifiers at the effluent end under the influence of

comparatively higher organic loading. Meanwhile, at low organic loadings, the heterotrophs and autotrophs can coexist. Filter C had the lowest organic carbon loading and consequently had the lowest biomass density. Therefore, the nitrifiers in filter C may have experienced less competitive pressure from the faster-growing heterotrophic organisms for oxygen and space. The displacement of the nitrifying population by the heterotrophs is caused by the varying ratio of carbon and nitrogen entering the reactor.

The carbon loading used in this part of study, 5.71 ±0.16 kg COD/m^3.d, was much higher than the loadings used by Wijeyekoon (Table 9.4), and therefore nitrifiers were not only displaced further away from the feed source, but also buried deeper into the biofilm (Ohashi et al 1995). Fdz-Polanco et al (2000) also observed that as the amount of organic carbon entering the filter increases, the nitrification activity is displaced to the upper part of the filter in an upflow process. Quyang et al (2000) also argued that the differences in biological activity at different filter heights were due to their varying loadings.

Rowan et al (personal communication) also investigated the percent value of AOB in a full-scale BAF plant treating municipal wastewater and obtained a value of 0.65%. This value is almost three times higher than the highest percentage obtained in this study (0.2151% from Figure 9.4). The difference in values could be attributed to a number of factors including carbon loading, nitrogen loading, pH, DO, media type and size, direction of flow, backwashing regime and thus mean SRT and biofilm attachment characteristics.

4. Conclusion

The extent of comparable nitrogen removal in the two reactor configurations needs further microbiological evidence, specifically that of the existence of AOB. The formation of a dense biofilm as a result of higher turbulence would account for the higher number of AOB cells enumerated in the biofilm samples from the partial-bed reactor ($4.259 \times 10^5 \pm 1.881 \times 10^5$ no of AOB cells/ml sample) as compared to those from the full-bed reactor ($1.523 \times 10^5 \pm 7.979 \times 10^4$ no of AOB cells/ml sample). Although biomass was washed out in the treated effluent and during backwash operation, the SRT at the high organic loading of 5.71±0.16 kg COD/m^3.d was still maintained at 4.2 days for the partial-bed reactor and 7.6 days for the full-bed reactor. These SRTs were still longer than the limit noted by Sastry et al (1999), who claimed that a mean cell residence time > 3 days is desirable for nitrifiers to reach a stable population for effective nitrification, and Gerçeker (2002) who recorded a loss of nitrification below 2.5-2.7 days at an OLR of 5 kg COD/m^3.d and a temperature of 25^{o}C.

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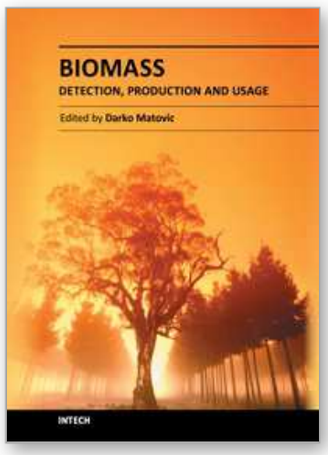

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Biomass has been an intimate companion of humans from the dawn of civilization to the present. Its use as food, energy source, body cover and as construction material established the key areas of biomass usage that extend to this day. Given the complexities of biomass as a source of multiple end products, this volume sheds new light to the whole spectrum of biomass related topics by highlighting the new and reviewing the existing methods of its detection, production and usage. We hope that the readers will find valuable information and exciting new material in its chapters.

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