USING OLIGONUCLEOTIDE PROBES TO CHARACTERIZE NITRIFICATION IN A TWO-STAGE PILOT SCALE BIOLOGICAL AERATED FILTER SYSTEM

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ABSTRACT

A pilot-scale, two-stage (carbon oxidation stage one, ammonia oxidation stage two) fixed film biological aerated filter (BAF) process was operated on-site at a domestic wastewater treatment plant. Over the study period, hydraulic loadings to the system were varied, generating a range of organic and ammonia loading conditions. Nitrification was monitored along the length of the filters by measuring chemical nitrogen species and activity levels of ammonia oxidizing bacteria (AOB). During the first phase of the study, nitrification performance was characterized during the wintertime and compared with oligonucleotide probing results using an ammonia-oxidizer specific probe. Overall nitrification efficiency for wintertime conditions (average temperature 12.4 ± 0.1 ^{o}C) was greater than 90 percent when ammonia-N loadings to the second stage were 0.6 kg/m^{3}-day or less. Nitrification efficiency started to deteriorate at loadings beyond this point. Biofilm and liquid samples were collected along the distance of the two columns at high and low ammonia loadings. The degree of activity observed by ammonia oxidizing bacteria in the biofilm corresponded with the disappearance of ammonia and the generation of nitrate as water passed through the columns. During the second phase of the study, the probing methods were investigated and results of two approaches of analysis were compared to chemical nitrogen profiles. It was found that probe signals normalized to mass of total bacterial nucleic acid corresponded better with chemical profiles than

using a novel method of standardizing against known nucleic acid mass standards. During both phases of the study, zones of ammonia oxidizing activity progressed along the length of the columns as organic and ammonia loadings to the system increased. The oligonucleotide probe data suggest that this shift in the location of the nitrifier population is due to higher BOD loads to the second stage, which supported higher levels of heterotrophic growth in the second stage of the system. It was concluded that the strongest influence on nitrification performance in this type of BAF system is likely to be competition between heterotrophs and autotrophs.

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IV. EXECUTIVE SUMMARY

Biological processes are an integral part of municipal wastewater treatment. Conventionally, the most prominent application of these processes has been the use of activated sludge for degrading soluble organic pollutants and nutrients such as nitrogen and phosphorus. Extensive research has been done on activated sludge mechanisms, kinetics, and performance, and the process is relatively well understood and successfully operated at large scale facilities. In recent decades, alternatives to activated sludge have been developed and investigated. Most prominently, research efforts have focused on biofilm processes, returning full circle to the fundamental component of trickling filters, which were some of the first successful biological treatment operations. However, the new applications of biofilms in wastewater treatment have not been as thoroughly investigated as activated sludge, primarily because there are not nearly as many full-scale biofilm systems as there are activated sludge systems. With the growing prominence of biofilm processes in municipal and industrial applications, more research efforts have been devoted to developing a more complete understanding of the capabilities, limitations, and operation of these systems.

Current biofilm processes have taken the form of circulating bed reactors (CBRs) and biological aerated filters (BAFs). The former utilizes a suspended carrier media to support biofilm growth for soluble pollutant removal, while the latter adds the benefit of removing suspended solid material, as well. When designed and operated well, these systems can be as efficient as, and competitive with, a conventional activated sludge process. While these types of processes have not been widely implemented in the USA, there are numerous full-scale facilities in Europe. Primary advantages of biofilm systems

include low hydraulic retention time (HRT) due to high biomass retention, and, in the case of BAFs, the elimination of secondary clarification for solids removal since solid material is filtered out. A consequence of these advantages is a reduced footprint area, resulting in lower capital construction costs and a significant advantage when available land is limited. However, the requirement of pumping water upwards through the filter is an added cost. Thus, selection of biofilm processes over an activated sludge system is a site-specific choice that must be evaluated on an individual basis.

Information and understanding are needed to sucessfully operate BAFs on a cost-competitive basis compared to conventional processes. Some areas of necessary research include:

- Maximum loading conditions that can be treated to acceptable levels
- Fundamental understanding of biofilm structure, bacterial populations, and mass transfer
- Effects of operating conditions on system performance

The goal of this research is to better understand these characteristics by operating a pilot-scale BAF system using a real municipal wastewater.

As wastewater treatment technology has progressed over several decades, primary goals of removing solid material and easily biodegradable organic matter (and oxygen demand) in wastewaters are now easily achieved through clarification, grit removal, and aerobic oxidation. The next step in preservation of our natural waters is the removal of nutrients such as nitrogen and phosphorus. Phosphorus removal can be achieved through biological or chemical means, and nitrogen removal has been established as a common practice through the biological processes of nitrification for ammonia removal and denitrification for nitrate removal. Activated sludge systems are, for the most part, fully

capable of nitrification, but more operational attention is required for nitrification than for carbonaceous oxidation alone. The biological process is more sensitive to environmental conditions and characteristics of the wastewater. For this reason, efforts are continuously underway to improve nitrification performance and efficiency.

In BAFs, nitrification is also achievable, and as in activated sludge systems, investigations into ammonia removal performance are widespread. Issues known or suspected to influence nitrification in BAFs include oxygen transfer, boundary layer mass transfer, substrate loadings, and bacterial population dynamics (Grady et al., 1999) The last of these has proven the most difficult to examine. The bacteria responsible for nitrification are slow-growing autotrophs, and traditional culturing techniques have been shown to be insufficient and inaccurate for measuring nitrifier growth (Belser, 1979). However, more recent techniques are now being applied to identify and quantify these bacteria. Methods developed in the field of molecular biology allow for identification of organisms based on their genetic codes. From an isolated organism of a certain group, DNA can be extracted and sequenced. From this sequence, complementary sequences can be generated and used to probe for that type of organism. From activated sludge and biofilm samples, RNA can be extracted and subjected to this technique, which has shown effectiveness in identifying different groups of bacteria such as nitrifiers. By determining location and abundance of nitrifiers in biofilm systems, a better understanding can be gained of the fundamental nature of the bacterial population and resulting ammonia removal. Further, by altering operational characteristics of the systems on a global scale and measuring the changes in these organisms, nitrification performance can be optimized.

This study focuses on nitrification performance in a two stage pilot-scale BAF system treating a municipal wastewater. Molecular techniques have been applied to characterize the bacterial population of nitrifiers and the changes in this population under different operating conditions.

V. LITERATURE REVIEW

Biological Aerated Filtration

Recent advances in biological wastewater treatment have been accompanied by a renewed interest in fixed-film systems, returning to the principals utilized by some of the earliest treatment technologies, trickling filters. However, new approaches have expanded on this simplified approach by incorporating submerged beds and novel carrier and support materials. Upflow biofiltration systems, such as biological aerated filters (BAFs), are one such technology that shows promise in both municipal and industrial applications, and they have a particular advantage in instances where available land is limiting. These systems utilize an inert media to support bacterial growth over which wastewater and nutrients are passed. Much of the early research of recent biofilm systems was directed towards quantification of empirical parameters and mass transfer in these systems (Reiber and Stensel, 1985, Lee and Stensel, 1986). Performance studies followed in applications of aerobic carbonaceous oxidation (Lee and Stensel, 1986), aerobic nitrification (Peladan et al, 1997; Pujol et al., 1998), anoxic denitrification (Æsøy et al, 1998), sulfate reduction (Amann et al., 1992), treatment of specialty chemicals (Hekmat et al, 1997), and combinations of processes (Ohashi et al., 1995, Lazarova et al., 1998, Gilmore et al., 1999). With the versatility of these systems, a potential exists for broad application to wastewater treatment.

A developed biofilm may be similar to a suspended growth system in its microbial diversity and ability to treat different types of wastewater. However, the spatial distribution of bacteria leads to unique relationships between populations of organisms. Biofilms appear to offer competitive advantages over suspended growth systems for

multiple reasons. First, symbiotic or obligate relationships often develop when one type of organism produces a compound that is a favorable substrate or cometabolite for another type of organisms. Because of the structure of the biofilm, the second organism can grow proximate to the first and have readily available nutrients. Second, a robust ability to suffer variations in environmental quality is an observation from natural environments that engineered systems attempt to utilize. In the context of nitrification, it has been shown that ammonia oxidizers recover more quickly from periods of starvation than free-cell suspensions, possibly due to cell density regulation and production of signal molecules (Batchelor et al., 1997). This 'quorum sensing' is a current topic of investigation of various types of biofilm systems.

Nitrification

For the past decade, removal of ammonia from wastewater has become a reasonable goal for most municipal and many industrial wastewater treatment facilities. It is likely that legislation will ultimately include nitrification as an integral part of wastewater treatment regulations in many areas of the United States.

The removal of ammonia from wastewater is accomplished by biological nitrification, a two step process that converts ammonia to nitrate. The process proceeds as follows and is mediated by two distinct groups of bacteria:

$$NH_4^+ \xrightarrow[\text{Ammonia oxidizers}]{} NO_2^- \xrightarrow[\text{Nitrite oxidizers}]{} NO_3^-$$

The process is a precursor step to biological denitrification, resulting in complete nitrogen removal. Ammonia removal is important because, if discharged to receiving waters, it exerts an oxygen demand that will eventually contribute to lower dissolved oxygen

concentrations in lakes, streams, and rivers. In addition, ammonia is toxic to fish in concentrations that could be discharged from wastewater treatment plants. Along with nitrate and phosphorus, ammonia contributes to eutrophication in natural systems (Cole, 1994). Along with phosphorus, nitrogen species are the most important limiting nutrients in natural systems, and discharge of these nutrients can cause significant changes in receiving water quality. For this reason, ammonia removal is critical in achieving the goal of zero discharge.

Although the benefits of the process are clearly enumerated, the practice of achieving nitrification in engineered systems has proven more difficult. The process can be upset by such environmental factors as temperature extremes (Neufeld et al., 1983), solids residence time (Hall and Murphy, 1985), high organic loads (WEF and ASCE, 1992), low dissolved oxygen concentrations (Siegrist and Gujer, 1987), and inhibitory compounds (Hockenbury and Grady, 1977). This sensitivity can lead to operational difficulties in treatment plants achieving nitrification, and the goal of most current research on the subject is to overcome these difficulties. Furthermore, because two groups of organisms are involved, the populations of both ammonia- and nitrite-oxidizers are of interest when studying the process.

Despite the sensitivity of the process, nitrification is commonly achieved in municipal systems by supplying sufficient aeration and maintaining SRTs higher than the nitrifier's net specific growth rate (Eckenfelder and Musterman, 1995). These systems are almost always single sludge systems, with the same sludge accomplishing both carbonaceous oxidation and ammonia removal. It is nearly impossible to keep pure nitrification systems from growing heterotrophic bacteria due to the scavenging of

endogenous autotrophs. As a result, it is often stated that nitrification is enhanced by the presence of heterotrophs. However, the physiology of the two groups of organisms are quite different. Heterotrophs have a half-saturation coefficient (K_S), with typical values of at least 10 mg/L, whereas, in contrast, the value for autotrophs is ≤1.0 mg/L as N (Sharma and Ahlert, 1977; Drtil et al., 1993). As a result of physiological differences, heterotrophs grow more quickly in single sludge systems and compete with autotrophs for oxygen. In fixed film systems, competition for space and substrate availability also dictate the proportional growth of heterotrophs and autotrophs. Therefore, there is likely a limit to the extent to which carbon and ammonia removal can be accomplished in the same system. The relationship between these two groups in fixed film systems such as BAFs is not fully understood and is the topic of much current research on nitrification. Ohashi et al. (1995) found that increasing C:N ratios caused a shift in population, causing incomplete nitrification in a previously fully-nitrifying system. Lazarova et al. (1998) saw a higher proportion of nitrifying organisms in a tertiary nitrification system than that observed in a carbonaceous and ammonia removal system.

Probing Research

With the recognition that nitrification is an important aspect of wastewater treatment comes the need for effective methods for measuring nitrification parameters and biological growth. While analyses for chemical species like ammonia, nitrate, nitrite, and total nitrogen are well established, means for monitoring growth of nitrifying bacteria are less adequate. An extensive review study by Amman et al. (1995) detailed the inadequacies of traditional culturing techniques in identifying specific groups within complex systems. The major shortcomings of these traditional techniques are twofold:

methods such as most probable number and selective plating underestimate cell numbers derived by direct microscope counts, and selective enrichment occurs when samples are analyzed in a laboratory setting, thus misrepresenting the abundances *in situ* of certain species of organisms. A pertinent example of this bias is the *Nitrobacter* genus, which has long been believed to be the primary genus responsible for the oxidation of nitrite to nitrate in biological nitrification. More recent molecular studies have shown little or no presence of this group in systems that actively nitrify (Wagner et al, 1997). Hence, it is important to recognize the limitations of traditional methods and look for more accurate techniques for describing microbial diversity.

The new methods that have been developed for studying microbial populations are based on the diversity of the bacterial genome. Since the advent of genetic sequencing, myriad species have been characterized and placed in a phylogenetic hierarchy showing a sort of bacterial "family tree." One region that has been shown to house particular specificity is the region of DNA that codes for the 16S rRNA molecule. This molecule contains a variety of regions that range from universal to species and enzyme specific. This characteristic of the 16S rRNA molecule has made it the genomic region of choice for developing oligonucleotide probes for environmental analyses.

While universal and specific probes can be derived from either genomic DNA or cloned RNA by reverse transcription, application of the probes has been mainly to extracts of RNA. The abundance of RNA in cells is the primary reason for this trend. Bacteria may contain from 10^3 to 10^5 ribosomes and copies of rRNA (Amann, 1995b), while genomic DNA coding for this rRNA is far less abundant. Therefore, in order to be able to measure differences in species nucleic acids, it is far easier to obtain a measurable

quantity of RNA than DNA. However, inherent in this approach is another potential bias: a small number of highly active cells may contain the same amount of rRNA as a large number of cells with low activity (Amann, 1995b). For this reason, probing of nucleic acids extracts yields insight into relative activity of a certain group of organisms, rather than a direct correlation to cell numbers.

These rRNA-targeted molecular techniques were first applied to immobilized biofilms in 1992 by Amann et al. in a study of a sulfate reducing biofilm. Polymerase chain reaction (PCR) primers were used to isolate 16S rRNA fragments from sulfate reducing organisms, probes were designed based on these sequences, and the probes were applied to visualize target organisms *in situ*. Other studies have used probes designed against less highly conserved regions of the 16S rRNA. Manz et al. (1992) used probes specific for the alpha-, beta-, and gamma-subclasses of Proteobacteria. By working with relatively general probes in this manner and also very specific probes, the "top to bottom" approach discussed by Amann (1995a, 1995b) can be used to obtain a hierarchy of microbial populations in a community.

Stahl et al. (1988) used probes to quantitatively characterize a mixed population in a study of the ecology of a ruminal system. Nucleic acids were extracted from samples and immobilized on membranes before probing with universal and group- specific oligonucleotides to describe the population of a system that had previously been difficult to culture. This approach favors whole cell and *in situ* studies when quantification is important, for the reason that single cells will contain different amounts of rRNA, depending on growth rate. By extracting and quantifying nucleic acids, and 16S rRNA in particular, prior to hybridizing with universal and specific probes, a percentage can be

found for the specific target groups. While these values cannot be assumed to represent the proportion of cell numbers, the percentage does show the relative contribution of activity of the specific groups to the whole culture because of the correlation between rRNA content and cell activity (Amann et al., 1995a). Growth rate has been correlated to rRNA content in several studies dating back to 1958, when Schaechter et al. established the relationship in *Salmonella typhimurium* (Schaechter et al., 1958; DeLong et al., 1989; Kemp et al., 1993). With whole cell and *in situ* studies, cell numbers and spatial positions can be determined, and by using more than one probe at a time, targeting two distinct specific regions of one organism's rRNA, more specificity can be obtained (Amann, 1995b) because both probes must hybridize to detect a specific cell.

Disadvantages of using *in situ* hybridization are mainly due to the complexity of the process. Since the method of extraction and blotting is relatively simple compared to whole-cell techniques (Amann, 1995b), it has the potential to be used often in preliminary studies or where resources are limited. In *in situ* methods, sample preparation is more rigorous than the extraction of nucleic acids and cell permeability to probes becomes an issue Activity based on rRNA content must be measured by digital analysis of individual cell signal intensity (Poulsen et al., 1993) or using flow cytometry (Wallner et al., 1993). Furthermore, whole-cell techniques depend on the absence of aggregated colonies of bacteria (Wallner et al, 1993), such as are commonly found in activated sludge and biofilm samples. For the best characterization of environmental samples using rRNA probing techniques, a joint approach of quantitative extraction/blotting and whole cell *in situ* probing is most useful. Contribution of specific groups of bacteria to the total activity

of a sample, as well as location and numbers of different types of organisms in that sample, can be obtained.

Application of Probing to Nitrification

The physiology of nitrifying organisms makes them a prime candidate for rRNA probing analysis. Species of ammonia-oxidizing bacteria are known to be slow-growing (Koops and Muller, 1991) and they are difficult to culture in a laboratory setting for this reason. The slow growth rate of the ammonia oxidizers makes the conversion of ammonia to nitrite the rate limiting step in most nitrification systems (Wagner et al., 1995). Wagner et al. has shown that species of *Nitrosomonas* are easily detected using rRNA probes, and Amann et al. (1995b) postulated that the reason for this was the high levels of ammonia monooxygenase needed for the low yield metabolism, and a high ribosomal content is probably needed to sustain this enzyme.

In a comprehensive probing study, Mobarry et al. (1996) developed a group of oligonucleotide probes for ammonia oxidizers within the Beta subclass of the *Proteobacteria*. These probes were shown to work well in quantitative slot blot hybridizations of biofilm samples and in situ probing of reactor samples. Other studies have applied nitrifier probes to activated sludge systems with good success (Wagner et al., 1996, Mobarry et al., 1996, Wagner et al, 1997, Ballinger, et al., 1997). These studies have shown that traditional ideas limiting nitrifying organisms to *Nitrosomonas* and *Nitrobacter* species may underestimate the abundance of novel nitrifiers.

As nitrification in engineered systems has been transplanted from activated sludge to biofilms, the application of probing techniques has been applied to nitrifying biofilms. Early studies were performed using solely nitrogenous synthetic wastewater to determine

optimal conditions for the process (Peladan, 1997, Pujol et al., 1998). Ohashi et al. (1995) used probing techniques to characterize the microbial population of a bench-scale BAF system similar to the design studied here, fed with a synthetic wastewater containing both ammonia and organic material. Nitrifier probe signals were measured at up to 24-30% of universal probe signals. Lazarova et al. (1999) measured nitrifier fractions from 20-30% and as high as 50% in another type of fixed film system. The study presented here is one of the first to investigate a two-stage BAF system treating a real wastewater.

Advances in microscopic chemical probes have also been used in conjunction with probing techniques to further confirm the results obtained from probing. Schramm et al. (1996) used these probes to measure changes in dissolved oxygen and nitrate within nitrifying biofilms to characterize the regions within the films where nitrification takes place. These studies have helped to gain understanding about both the mechanisms of nitrification and the nature of chemical transformations in biofilm processes. On the other hand, molecular approaches to studying populations may not always have direct operational applications. Wagner et al. (1995) showed that nitrifiers were still detected by in situ hybridization even in samples where nitrification was inhibited, suggesting that ammonia oxidizing bacteria can sustain ribosomal content even during periods of inactivity or inhibition. Because of this observation, the study suggests that hybridization techniques may not be suitable for monitoring nitrifier health in treatment plants. However, the knowledge gained from using these techniques will likely lead to conclusions that enable us to more effectively accomplish nitrification and other processes of engineering significance.

The purpose of this study is to apply molecular probing techniques to characterize nitrification in a two stage biofilm system. Most of the studies to date involving nitrification have focused on activated sludge systems or tertiary nitrification-only systems. This research is unique in that it involves characterization of a dynamic system that accomplishes both secondary carbonaceous treatment as well as nitrification, and the boundary between these two processes is dynamic and dependent on wastewater composition and operating conditions. Because the search for effective probes for nitrite-oxidizing bacteria is ongoing, ammonia oxidizers are used as the primary indicator of nitrification activity. The effects of operating conditions on the populations of ammonia oxidizers are examined to provide insight into biofilm wastewater processes.

VI. MANUSCRIPT 1

INFLUENCE OF ORGANIC AND AMMONIA LOADING ON NITRIFIER ACTIVITY AND NITRIFICATION PERFORMANCE FOR A TWO-STAGE BIOLOGICAL AERATED FILTER SYSTEM

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ABSTRACT

A pilot-scale, two-stage (carbon oxidation stage one, ammonia oxidation stage two) fixed film biological aerated filter (BAF) process was operated during the wintertime on-site at a domestic wastewater treatment plant. Over the study period, hydraulic loadings to the system were varied and generated a range of organic and ammonia loading conditions. Nitrification performance was monitored based on water quality along the length of the filters, effluent water quality, and activity levels of ammonia-oxidizing bacteria within the biofilm using an oligonucleotide probe. Overall nitrification efficiency for wintertime conditions (average temperature 12.4 ± 0.1 ^{o}C) was greater than 90 percent when ammonia-N loadings to the second stage were 0.6 kg/m^3-day or less. Nitrification efficiency started to deteriorate at loadings beyond this point. Biofilm and liquid samples were collected along the distance of the two columns at high and low ammonia loadings. The degree of activity observed by ammonia oxidizing bacteria in the biofilm corresponded with the disappearance of ammonia and the generation of nitrate as water passed through the columns. The zones of ammonia oxidizing activity progressed along

the length of the columns as organic and ammonia loadings to the system increased. The oligonucleotide probe data suggest that this shift in the location of the nitrifier population is due to higher BOD loads to the second stage, which supported higher levels of heterotrophic growth in the column.

KEYWORDS

Biofilm, biological aerated filter, population dynamics, nitrification, competition

INTRODUCTION

Biological aerated filtration (BAF) is an alternative to the traditional activated sludge process commonly used in biological wastewater treatment. The primary advantages of the process are a reduced footprint resulting from the elimination of secondary clarifiers and their associated operational difficulties, and a low hydraulic retention time due to high biomass retention in the system. BAFs incorporate an inert media, which acts to support biomass and filter out suspended solids. Wastewater flows either upward or downward through the media bed, which remains submerged during operation. Air is introduced at the bottom of the column through a diffuser system to facilitate oxidation of organic and/or reduced nitrogenous substances. A dense granular or floating media may be used, but in the latter case a retaining barrier must be used to keep the media in the bed. Granular media systems are backwashed in a co-current mode while floating media are backwashed in a countercurrent mode; both are backwashed using water and air scour. While BAF systems are in more widespread use in Europe, facilities in the United States are demonstrating greater interest in this technology, especially for application to areas where available land is limited for expansion of existing facilities or for construction of new facilities.

Ammonia oxidation is an important goal for many wastewater treatment systems. While removal of organic material is not difficult to achieve in most domestic wastewaters, removal of ammonia requires more operational attention and a better understanding of the bacterial processes involved. Nitrifying organisms are adversely impacted by temperature extremes (Neufeld et al., 1983), solids residence time (Hall and Murphy, 1985), high organic loads (WEF and ASCE, 1992), low dissolved oxygen concentrations (Siegrist and Gujer, 1987), and inhibitory compounds (Hockenbury and Grady, 1977). Limitations to traditional biological methods that involve pure culture isolations and cell quantification have been shown to be inadequate in defining the composition of activated sludge cultures (Wagner et al., 1993). More recently, techniques have been applied to identify nitrifiers using molecular oligonucleotide probes (Head et al., 1993; Wagner et al., 1996;). The 16S rDNA gene sequences for a range of bacteria that are known or suspected to nitrify have been sequenced, and portions of the gene have been used to design a range of group-specific oligonucleotide probes. Nitrifiers can be identified *in situ* within a suspended culture or biofilm (Wagner et al., 1995; Wagner et al., 1996), or their metabolic activity can be quantified by detecting levels of 16S rRNA within a nucleic acid extract from a sample (Mobarry et al., 1996). These techniques can be used in biofilm systems to determine the presence of nitrifiers, their location and activity, and for correlation with soluble water chemistry measurements that imply nitrification.

The substrate loading limits for BAF systems have not been well defined, particularly with regard to field-verified nitrification in two-stage systems where carbon oxidation and

nitrification occur in series. The objective of this study was to determine limitations to nitrification for a granular media, upflow, two-stage BAF system (Biofor®, Infilco Degremont, Inc., Richmond, VA) during wintertime operation. These limitations were determined based on effluent quality and metabolic activity patterns for ammonia oxidizing bacteria.

METHODS

System Setup and Operation. Two pilot-scale BAFs were provided by Infilco Degremont, Inc. (Richmond, VA) for this study. The units were located at the Peppers Ferry Regional Wastewater Treatment Facility (PFRWWTF) in Radford, Virginia, and received a mixture of domestic and light industrial wastewater. Effluent from a primary clarifier was used as influent to the BAF system. A schematic of the BAF pilot-plant system is shown in Figure 1. Two columns that enable carbon oxidation (C column) and ammonia oxidation (N column), respectively, comprised the pilot-plant system. The two units, operated in series, were both 5 m (16.4 ft) in height and 0.61 m (2.0 ft) in diameter. The columns contained a clay-based media approximately 2 to 3 mm in diameter with an average specific surface area of 1600 to 2300 m^2/m^3. The C and N column contained 3.9 m (12.8 ft) and 3.7 m (12.1 ft) of media, respectively. Air was provided to the columns through patented diffusers (Oxazur, IDI) in a distribution manifold located at the floor of the columns, beneath a stone underdrain layer. Each column was backwashed in a co-current manner at regular intervals (12 hours for the C column, 48 hours for the N column). Cycle times and flow rates were controlled automatically. The system was operated in three distinct phases based on hydraulic loading to the C and N columns, and include: Phase I: 4.7 and 3.9 m/hr, Phase II: 7.9 and 6.8 m/hr, and Phase III: 11.4 and 10.2

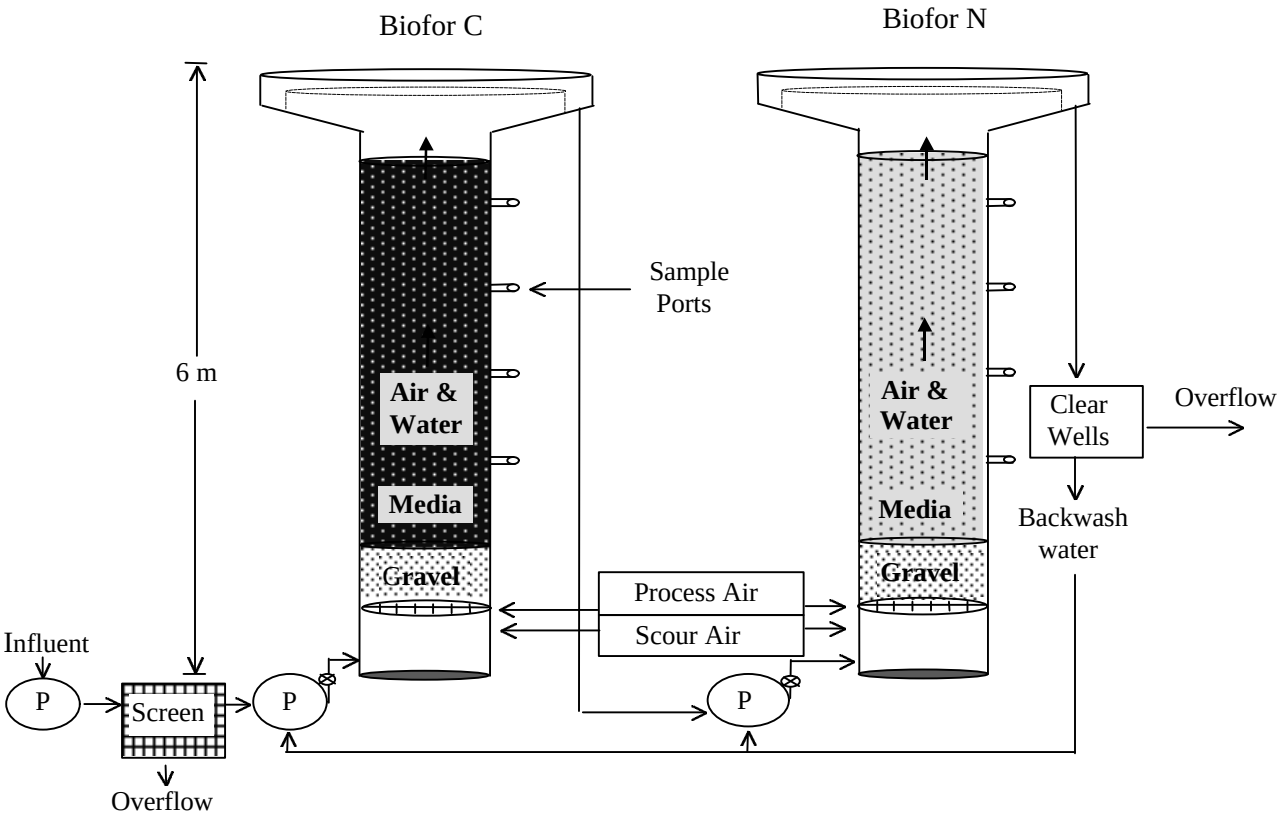

Figure 1. Schematic of Pilot-Scale Biofor® BAF System. (P=pump).

m/hr. The data presented here were collected over 3 winter months when the wastewater temperature ranged from 8.3 to 15.8 and averaged 12.4 ± 0.1 ^{o}C. Periodically, operational problems at the treatment plant led to the shutdown of the primary clarifier. Whenever this occurred, hydraulic loadings to the pilot-plant system were decreased, and operation and data collection continued.

The columns were seeded with bacteria by passing primary treated effluent through them at a hydraulic loading rate of 2.5 m/hr or less for 6 weeks prior to the beginning of the study period.

Water Sampling and Analysis. BAF influent (primary treated wastewater) and effluent from both columns were collected as refrigerated, 24-hour composite samples and routinely analyzed for the following parameters: TSS, VSS, BOD_5 (total and soluble), COD, ammonia, nitrate, nitrite, pH, alkalinity, and temperature. TKN was monitored

periodically. All analyses were performed in accordance with Standard Methods (APHA, 1995).

The columns were equipped to allow liquid sampling at four points along the height of the columns. Liquid samples collected from these ports were transported by cooler to the Environmental Engineering Laboratories and processed immediately, or preserved and stored for later analysis. These samples were analyzed for ammonia, nitrate, nitrite, soluble and total TKN. Although liquid samples were collected from ports at a high velocity in an attempt to prevent short-circuiting of water along the column walls, it was discovered during the study that liquid concentrations varied across the radius of each column. Higher concentrations occurred near the walls. Therefore, samples taken from the liquid sampling ports are comparable on a relative scale within a given profile, but do not represent a radial average at each port. Due to this wall effect, actual average concentrations at a given depth in the columns were lower than detected via the liquid sampling ports. Consequently, performance with depth at full-scale would be expected to be better than represented by the profiles presented here.

Media Sampling and Oligonucleotide Probe Analysis. The columns were also equipped to allow for media sampling at four points along the height of the columns via ports which were located next to the liquid sampling ports. Media samples were collected during system operation using a core sampler inserted horizontally across the full width of the column. Some of the biofilm biomass was loosely connected to the media and the coring unit sheared this biomass off the media in the process of removing the sample. Both support media samples and the associated liquid phase containing the sloughed

biomass were collected, transported on ice back to the Environmental Biotechnology Laboratory, and processed immediately or frozen as whole cells.

Bottles containing media, liquid and sheared biomass were vigorously shaken by hand for one minute prior to removing well mixed biomass slurry samples. Total nucleic acids were extracted from field samples, pure cultures of *Nitrosomonas europaea* (ATCC 19718) harvested at late-log phase, and nitrifying activated sludge samples, according to the method of Stahl et al. (1988). The two latter samples were used as positive controls for the probing method. Cells were lysed using bead beating (Biospec Products, Bartlesville, OK). Each 2 mL screw-cap vial contained 1.5 g glass beads (0.1mm diameter), 0.7 mL sample, 50 µl 10% SDS, and sufficient phenol equilibrated to pH 5.0 with 0.1 M Tris and preheated to 58 to 65^{o}C, for a final volume of 2 mL. Cells were beaten for two-90 second intervals with 30 seconds rest. Samples were centrifuged briefly at 175 x g to consolidate glass beads, and the entire liquid phase was transferred and centrifuged for 5 minutes at 11,500 x g. The aqueous phase was collected and nucleic acids were isolated by two extractions with phenol (pH 4.3):chloroform:isoamyl alcohol (100:24:1; preheated to 58 to 65 ^{o}C) and one extraction with chloroform:isoamyl alcohol (24:1). Nucleic acids were ethanol precipitated and incubating at –20^{o}C overnight. Extract quality was confirmed by agarose gel electrophoresis and observation of 16S RNA bands and A_{260}/A_{280} values for extracts were between 1.7 and 2.0. Nucleic acid mass was quantified spectrophotometrically (1 A_{260}=40 µg/ml, Stahl et al., 1988). After denaturing with glutaraldehyde, 5 µL dot blots were placed on nylon membranes (Boehringer Mannheim, Indianapolis, IN), and baked at 120^{o}C for 20 minutes. Membranes were air dried and stored for later hybridization.

The oligonucleotide probe Nso190 (S-Nso190Sb-19, 5'-CGATCCCCTGCTTTTCTCC-3') was used for this study to identify activity levels of ammonia oxidizing bacteria (AOB) in the biomass (Mobarry et al., 1996). Probes were labeled with digoxygenin (DIG) at the 3' end using the Genius™ 5 Oligonucleotide 3'-End Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Labeled probe concentrations were measured spectrophotometrically. The probe was hybridized to sample nucleic acids at the specified optimum temperature (Mobarry et al., 1996). Prehybridization and hybridization were also performed according to the Genius™ 5 kit. Membranes were air dried and stored prior to detection with anti-DIG alkaline phosphatase and the chemiluminscent substrate CSPD (Boehringer Mannheim, Indianapolis, IN). Detection was performed according to the Genius™ 7 Luminescent Detection Kit (Boehringer Mannheim, Indianapolis, IN), with the following modifications: membranes were incubated at 37°C for 15 minutes and at room temperature for an additional 30 minutes, then exposed to chemiluminescent detection film (Boehringer Mannheim, Indianapolis, IN) for 80 to 90 minutes. Signal intensity was quantified directly from films using transmissive densitometry (CS9000, Shimadzu).

Degree of variability in reported data are presented as the standard error of the mean.

RESULTS AND DISCUSSION

N Column Wintertime Operational Performance. Ammonia removal by the N column was particularly dependent upon ammonia mass loading, as shown in Figure 2. It can be seen that ammonia removal was consistently at or near 100% at loadings up to 0.6 kg NH_4^+-N/m^3-day. On some days, the concentration of ammonia applied to the N column

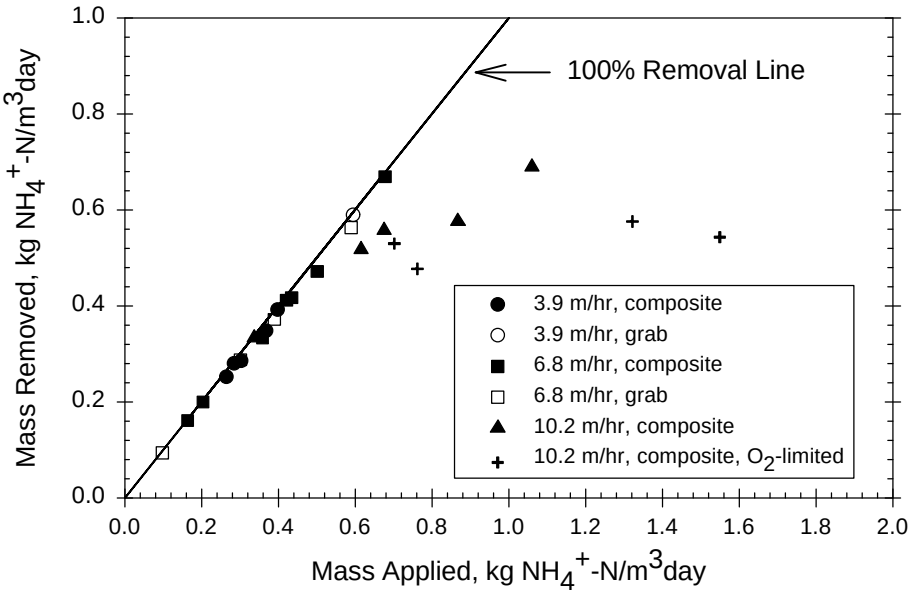

Figure 2. Ammonia removal performance for the second stage N column during wintertime operation.

increased suddenly and high ammonia loadings were experienced. This occurred whenever the treatment facility's belt filter press was in operation to dewater anaerobically digested biosolids and filtrate was recycled back to the primary clarifiers. Whenever this occurred, influent ammonia concentrations to the pilot plant system increased up to 2.5 times the average composite ammonia concentration (10.3 ± 0.6 mg/L NH_4^+-N) when filtrate was not being recycled. For this reason, ammonia concentrations were difficult to predict ahead of time and on some of these days, the N column became oxygen-limited because insufficient air was supplied.

Hydraulic loading did not influence ammonia removal rates at applied ammonia loadings up to around 0.6 kg NH_4^+-N/m^3-day, after which nitrification efficiency started to decrease. Previous studies conducted with single-stage Biofor® systems suggest that nitrification efficiency improves with this treatment design as hydraulic loading increases (Peladan et al., 1997, Pujol et al., 1998). In one of these studies, an N column fed a synthetic wastewater containing 25 mg/L NH_4^+-N and no BOD demonstrated a 90 percent

nitrification efficiency at an ammonia loading of 4 kg NH_4^+-N/m^3-day and a hydraulic loading of 30 m/hr at 17.5 ^oC (Peladan et al., 1997). The second study, conducted over two years with a full-scale Biofor® located downstream of an activated sludge system, demonstrated nitrification efficiencies greater than 80 percent at loading rates up to 1.6 kg NH_4^+-N/m^3-day and BOD_5 loads typically at or below 2 kg/m^3-day (Pujol et al., 1998). The results presented in Figure 2 initially suggest that nitrification performance deteriorated at higher hydraulic loadings, contrary to the results presented in the two studies referenced above. However, another factor that could have caused this shift in performance at higher ammonia loadings is competition between heterotrophic and nitrifying bacteria for oxygen and space in the N column. Additional information was collected to elucidate the nature of the biomass located in the N column under two extreme ammonia loading conditions to further evaluate this observation.

N Column Profiles and AOB Activity. Figures 3 (Phase II) and 4 (Phase III) show soluble nitrogen species measured along the flow path of the C and N columns, when the influent ammonia-N loadings to the N column were 0.1 (grab sample) and 0.66 (24-hour composite sample) kg/m^3-day, respectively. These ammonia loadings represent two extremes of the loading conditions to the N column during wintertime operation, as shown in Figure 2. The corresponding intensity of Nso190-based AOB activity relative to the total mass of nucleic acid extracted from biofilm samples is also shown with distance along the columns. It can be seen that the levels of AOB activity along the columns correspond with levels of nitrification intensity, which is predominantly represented by changes in ammonia and nitrate concentrations. Nitrite concentrations remained low,

indicating that nitrite oxidizing bacteria (NOB) were present in sufficient quantity to prevent nitrite buildup at either ammonia loading.

Peak AOB activity levels during Phase III were significantly lower (2 times) than they were during Phase II. The rRNA activity level per AOB would reach a maximum at those points in the N column where the ammonia concentration was well above the Monod half saturation constant (K_{NH}) for ammonia oxidation, which is typically reported to be less than or equal to 1 mg/L NH_4^+-N (Sharma and Ahlert, 1977; Drtil et al., 1993). Ammonia concentrations were above 1 mg/L as N during both Phases II and III in the portion of the column where nitrification was most rapid and, therefore, rRNA levels per AOB would not be expected to differ ignificantly during these two phases. However, during Phase II, the 24-hour composite BOD_5 mass loading to the N column averaged 1.15 ± 0.13 kg/m^3-day.

The BOD_5 loading was more than twice as high (2.70 ± 0.21 kg/m^3-day) during Phase III. More importantly, 0.60 ± 0.14 kg/m^3-day and 1.40 ± 0.26 kg/m^3-day of BOD_5 was removed by the N column during Phases II and III, respectively. This increase in BOD_5 loading to and removal by the N column during Phase III reflects an increase in the mass of heterotrophic bacteria that accumulated over time in the column relative to Phase II. Consequently, a greater fraction of the RNA recovered from the biomass was attributed to heterotrophic activity during Phase III. Therefore, it is reasonable that the normalized activity level of AOB decreased during Phase III. At higher substrate loadings, the region of the N column where maximum nitrification activity occurred was “pushed” further up the column. The results presented here suggest that this was due to the increased BOD_5

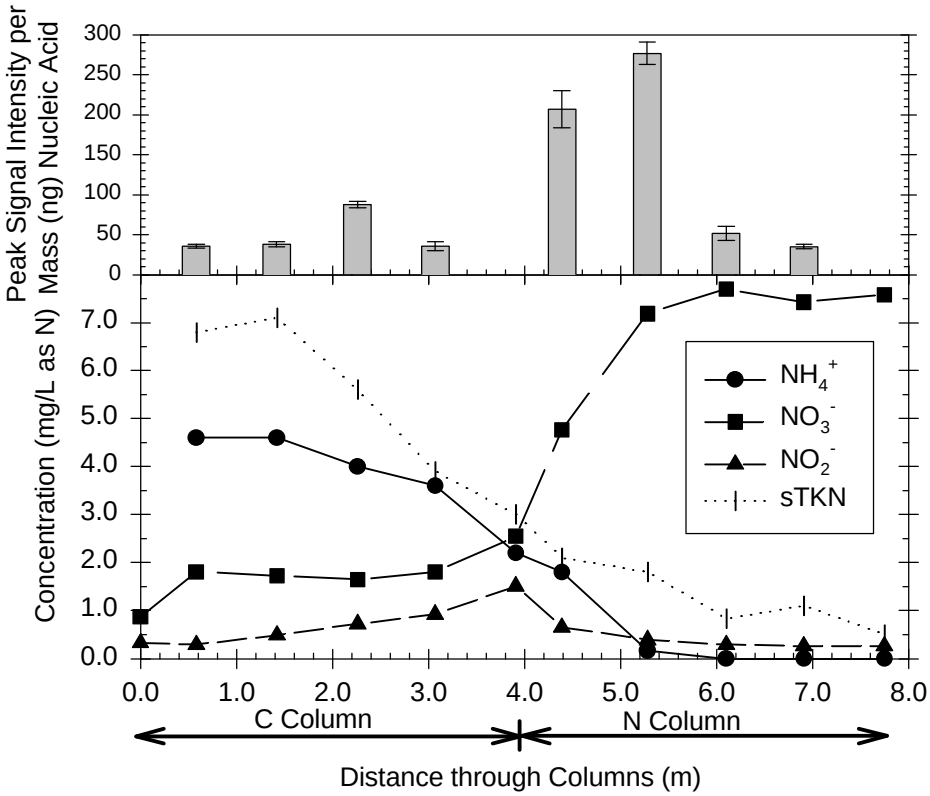

Figure 3. Profile of nitrogen species and Nso190 signal intensity normalized to mass of nucleic acids with distance through the two-stage BAF system. Data were collected during Phase II when the hydraulic loading was 6.8 m/hr. The ammonia load to the N column was 0.1 kg NH_4^+-N/m^3-day (grab sample).

load applied to the N column which interfered with nitrification. Summertime dissolved oxygen profiles collected along the N column when the hydraulic, ammonia and organic loadings were equivalent or greater than during Phase III suggest that nitrification is not limited by available dissolved oxygen at these high loadings, as long as sufficient air is applied (data not shown). Therefore, we do not believe that dissolved oxygen limitation played a role in pushing nitrification activity further up the N column.

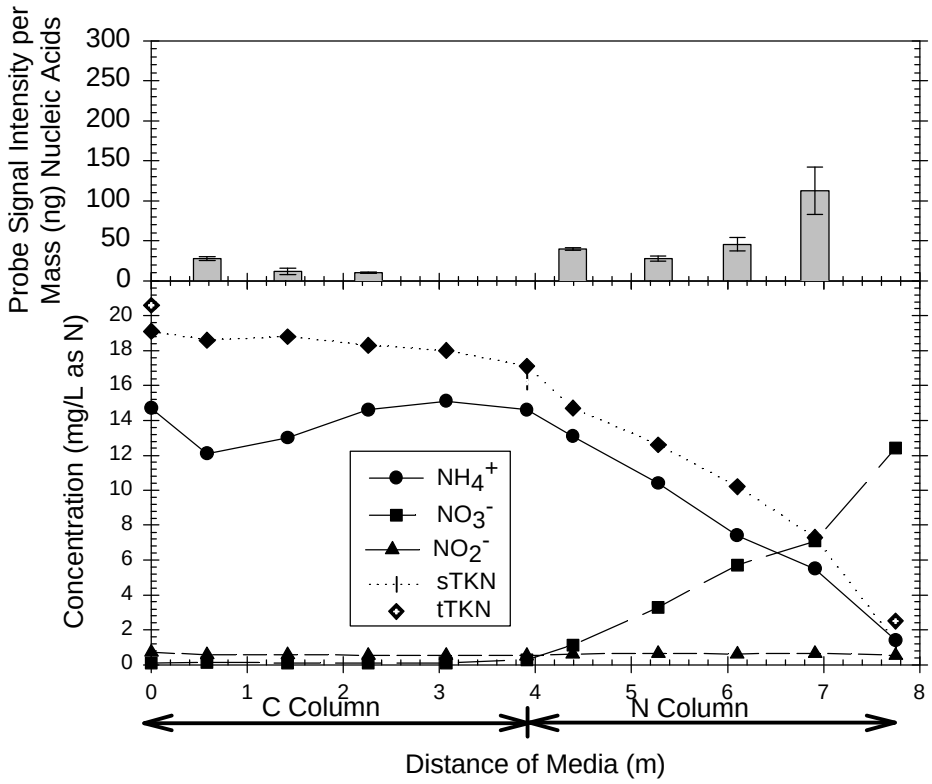

Figure 4. Profile of nitrogen species and Nso190 signal intensity normalized to mass of nucleic acids with distance through the two-stage BAF system. Data were collected during Phase III when the hydraulic loading was 10.2 m/hr. The ammonia load to the N column was 0.66 kg NH4+-N/m3-day $_4^+$-N/m^3-day (composite sample).

The relative activity of AOB measured during the two phases demonstrates the benefit of operating two physically distinct units within a BAF system, as the separation allows for independent optimization of both carbon oxidation as well as nitrification. The bacterial population structure in a nitrifying biofilm system is significantly influenced by the applied BOD load. As more organics bleed from the C column to the N column, the relative populations of heterotrophic to autotrophic bacteria will increase and nitrifying bacteria will be forced to compete with heterotrophic bacteria for dissolved oxygen and space. Therefore, minimizing effluent BOD from the first stage promotes preferential growth of nitrifiers in the second stage.

CONCLUSIONS

The second stage of a two-stage pilot plant BAF system was able to effectively oxidize ammonia during wintertime conditions at ammonia-N loadings up to 0.6 kg/m^3-day, independent of hydraulic loading. Nitrification performance deteriorated as loadings increased beyond this point, especially at higher average organic loadings. Within the two-stage BAF pilot plant system, chemical measurements of nitrogen species corresponded well with AOB activity levels based on rRNA hybridization with a group specific oligonucleotide probe, thus confirming the spatial location of AOB activity (and presumably NOB activity, based on the lack of nitrite build-up) along the length of the media. Additionally, the relative levels of AOB activity appeared to decrease as higher BOD loads were applied to the second stage N column, presumably due to an increase in the fraction of bacteria comprised of heterotrophs. Separation of nitrification from upstream carbon oxidation processes develops opportunities to optimize the levels and rates of treatment for each process individually. The system performed best and showed highest AOB activity levels when low average BOD loadings were introduced into the N column. This study is ongoing, and the investigation will be expanded to evaluate the effect of hydraulic loading at constant BOD and ammonia mass loadings on ammonia removal performance in the N column.

ACKNOWLEDGEMENT

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VII. MANUSCRIPT 2

MEASURING NITRIFYING POPULATIONS IN A BIOLOGICAL AERATED FILTER SYSTEM USING QUANTITATIVE DOT BLOTTING

K. R. Gilmore, K. J. Husovitz, A Delahaye, N. G. Love

ABSTRACT

A two-stage pilot scale biological aerated filter (BAF) system was operated during the summer and the bacterial population of ammonia oxidizers was examined. At four times during the study, profiles of chemical nitrogen species and samples of the biofilm throughout the system were analyzed to characterize the nitrification process in a BAF system. Changes in the population of ammonia oxidizing bacteria (AOB), measured with an oligonucleotide probe, were observed as operating conditions were changed. Nitrifier probe signals, normalized to total nucleic acid mass, corresponded well with chemical nitrogen profiles. It was concluded that the strongest influence on nitrification performance in this type of BAF system is likely to be the competition between heterotrophs and autotrophs. A second method of expressing the fraction of ammonia-oxidizing activity, was also investigated for accuracy; ammonia oxidizing activity was expressed as nitrifier nucleic acid mass as a percentage of total bacterial nucleic acid mass measured using a universal probe. Mass was determine based on standard curves of probe signal intensity versus nucleic acid mass developed from pure cultures of *Escherichia coli* (for universal probe standard) and *Nitrosomonas europaea* (for nitrifier standard). Differences between the two methods of expression were observed, and the method correlated more closely with chemical nitrogen measurements in the system. However, using the second method, percentages of activity attributable to ammonia oxidizers were

still in a reasonable range of 2% to 12% in the nitrifying stage of the system. It was determined that care must be taken in expressing proportions of activity when normalizing specific probe data to universal probe results.

INTRODUCTION

For the past decade, removal of ammonia from wastewater has become a reasonable goal for most municipal and many industrial wastewater treatment facilities. It is likely that legislation will ultimately include nitrification as an integral part of wastewater treatment regulations in many areas of the United States. However, the process remains sensitive to environmental factors such as temperature, (Neufeld et al., 1983), low dissolved oxygen concentrations (Siegrist and Gujer, 1987), and inhibitory compounds (Hockenbury and Grady, 1977; Love et al., 1999), and others. This sensitivity can lead to operation difficulties in treatment plants achieving nitrification, and the goal of most current research on the subject is to overcome these difficulties.

The application of molecular probing techniques to engineered systems has given engineers and scientists a powerful new tool for investigating bacterial populations. In wastewater treatment systems, nitrification has become a common target for these techniques because the process is an important one and the bacteria involved are suitable for probing (Amann, 1995a). Nitrifying bacteria have been studied in both activated sludge and fixed film systems (Wagner et al., 1995; Wagner et al., 1996; Mobarry et al., 1996; Lazarova et al., 1998), and the probes used for these organisms are continuing to be developed and refined. With continued research, there is hope that soon nitrification will be much more thoroughly understood than it was a decade ago.

With the growing implementation of fixed film systems in modern wastewater treatment, molecular probing techniques have been applied to characterize the structure and bacterial makeup of biofilms. The ability of a biofilm to treat wastewater will rely largely on the different groups of organisms populating the biofilm as well as their spatial distribution and accessibility to substrates. Upflow biofiltration systems, such as biological aerated filters (BAFs), are one such technology that shows promise in both municipal and industrial applications, and they have a particular advantage in instances where available land is limiting. Nitrification capabilities of BAF systems have been studied with solely nitrogenous synthetic wastewater to determine optimal conditions for the process (Peladan, 1997, Pujol et al., 1998). Ohashi et al. (1995) used probing techniques to characterize the microbial population of a bench-scale BAF system similar to the design studied here, fed with a synthetic wastewater containing both ammonia and organic material. Nitrifier probe signals were measured at up to 24-30% of universal probe signals. Lazarova et al. (1999) measured nitrifier fractions from 20-30% and as high as 50% in another type of fixed film system. The study presented here is one of the first to investigate a two-stage BAF system treating a real wastewater.

Recent probing studies of biological wastewater treatment systems have relied heavily on the use of universal and specific probes (Ohashi et al., 1995; Lazarova et al., 1998). In the case of nitrification in wastewater treatment systems, much of the current literature reports fraction of ammonia oxidizer activity as a percentage relative to signals obtained using universal probes. Without the use of fluorescent *in situ* hybridization (FISH), direct cell counts cannot be obtained. Therefore, the best that can be determined from “semi-quantitative” dot blot hybridization is an idea of the activity contribution of a

specific group of organisms to the total culture activity. During the course of this research, it was found that many factors contribute to variability when using this type of quantitative method where nucleic acids are extracted from cells. When a target sub-population such as ammonia oxidizers constitute a small fraction of the total bacteria, as may be the case in wastewater treatment systems, small sources of variability in probing methods can be very significant. The motivation behind the investigation of methods was to increase the confidence with which proportions of sub-populations are measured when analyzing complex systems. Since this method is relatively simple compared to whole-cell techniques (Amann, 1995), it has the potential to be used often in preliminary studies or where resources are limited. Hence, it is important to ensure that the method is as accurate as possible.

In a previous study, it was shown that nitrifier signals normalized to mass of nucleic acid blotted corresponded well with chemical measurements of nitrogen species (Gilmore et al., 1999). However, the following factors were identified as sources of variability and error in this approach to analyzing blotting methods: probe labeling efficiency, extraction efficiency, and relative proportions of RNA, DNA, and contaminants in the extracts. These factors contribute underlying variability that is compounded by statistical variability seen in measuring signal intensity, etc. For these reasons, a modified method for quantifying probe signals and bacterial populations was investigated, based on the generation of nucleic acid mass standards and comparison of sample hybridization signals to the signals obtained from the standards. This method was used to characterize the activity of ammonia oxidizing bacteria (AOB) throughout a BAF system and to observe changes in that population under different operating conditions.

METHODS

BAF System Setup and Operation. Two pilot-scale BIOFOR™ BAF units were provided by Infilco Degremont, Inc. (Richmond, VA) for this study. The units were set up on-site at a wastewater treatment plant receiving a largely domestic wastewater with a small amount of industrial flow. The column units were operated in series, the first stage (C column) and second stage (N column) designed to treat BOD and ammonia, respectively. The system was fed effluent from a primary clarifier. Specifications for the system have been previously presented (Gilmore et al., 1999). The system was operated at three distinct hydraulic loading rates, which are presented in Table 1. Performance was studied at flows up to approximately 0.95 m^3s^{-1} (0.02 MGD). For practical reasons, the loading rate to the second stage was always less than the loading to the first stage.

During each period, routine sample analysis was conducted to monitor performance of the system and to indicate a condition of steady-state. Influent to the system and effluent from each stage were collected as refrigerated, 24-hour composite samples and routinely analyzed for the following parameters: TSS, VSS, BOD_5 (total and carbonaceous), COD, ammonia, nitrate, nitrite, pH, alkalinity, and temperature. TKN was monitored periodically. All analyses were performed in accordance with Standard Methods (APHA, 1995).

At the end of four periods of operation, comprehensive sampling was done before and after each stage of the system, and at four points within each filter bed. Chemical species were measured and media samples were collected to characterize the population of ammonia oxidizing bacteria.

Media Sampling and Oligonucleotide Probing. Samples collected for bacterial analysis were taken using a coring device inserted across the width of the column. Ports were designated C1 to C4 in the C column, from bottom to top, and likewise, N1 to N4 for the N column. Media and biomass were recovered from the sampler and stored at –20°C until analysis. Nucleic acids were extracted from biofilm samples using the method of Stahl et al. (1988) with the following modifications: phenol was equilibrated with Tris at pH 4.3, and in some cases more than two extractions with phenol:chloroform:isoamyl alcohol (100:24:1) were performed to remove proteins from the sample. Sample extracts were then dot blotted, hybridized with digoxygenin (DIG)-labeled probes, detected using chemiluminescence, and analyzed using transmissive densitometry (Shimadzu, CS 9000). The full analysis is described previously (Gilmore et al., 1999).

Table 1. Operating Conditions for System Profiles

Phase of Operation	Hydraulic Loading rate, m/hr First and second stage, resp.
I	11.7 & 10.5
II	10.2 & 9.0
III	9.4 & 8.2
IV	9.4 & 8.2

Samples were probed with the universal probe EUB338 (5'-GCT GCC TCC CGT AGG AGT-3', wash temperature 58°C) and the probe Nso190 (S-Nso190Sb-19, 5'-CGA TCC CCT GCT TTT CTC C-3', wash temperature 53°C) specific for the ammonia oxidizers in the beta subclass of Proteobacteria (Mobarry et al., 1996). Hybridization signals obtained with the ammonia oxidizer specific probe were compared to profiles of nitrogen species throughout the system to determine if probing results correspond to chemical measurements.

Mass Standards. To investigate the accuracy of reporting fraction of nitrifying organisms by comparing nitrifier probe signals to universal probe signals, internal standards were created using nucleic acid extracts of pure cultures of *E. coli* and *Nitrosomonas europaea*. Cultures were grown in shake flask and nucleic acids extracted using the same method as for samples. Concentrations of total nucleic acids were measured by spectrophotometry, and a dilution series of known mass was blotted. Signals obtained from these standards were normalized to mass of nucleic acid blotted, and standard curves of signal vs. mass nucleic acid were generated. These membranes were hybridized along with samples during each analysis set in order to provide a basis for quantification to a known mass standard. One standard membrane was hybridized with EUB338 and one with Nso190. Signals obtained from sample blots were compared to the standard dilutions and the mass of nucleic acid binding with each probe was calculated. Mass of total bacterial nucleic acid was determined by standardizing the sample signal against *E. coli* hybridized with EUB338, and mass of nitrifier nucleic acid was determined by standardizing the sample signal against *N. europaea* hybridized with Nso190. The calculated mass of Nso190-hybridized nucleic acid was divided by the mass calculated for total nucleic acid and thus a percentage was obtained. These results were compared with another method for expressing nitrifier activity, normalizing the signal to total mass of nucleic acid measured by spectrophotometry. The chemical profiles of nitrogen species in the system were used to determine the accuracy of each method of expressing nitrifier activity.

RESULTS AND DISCUSSION

In preliminary studies and previous analyses, a reasonable percentage of nitrifiers could not be established simply by normalizing nitrifier probe signals to universal probe signals. It was decided that differences in probe structure, target sites, labeling efficiency and hybridization conditions were reasons for difficulties in using this approach. By creating mass standards with pure cultures, the goal was to reduce the effects of some of these factors and obtain more reliable results of nitrifier contribution to culture activity.

Column Profiles

Nitrogen profiles conducted at different operating conditions showed expected results, with ammonia oxidation occurring primarily in the second stage of the system and shifting further through the system at higher loading conditions. This finding is consistent with results seen in other studies of nitrification in BAFs (Ohashi et al., 1995, Gilmore et al., 1999). The profiles of nitrogen species were compared to the probing results, using both methods of normalization: normalizing to universal probe through pure culture mass standards, and normalizing to mass measured by spectrophotometry. The spectrophotometric mass normalization method yielded activity profiles that corresponded well with chemical measurements. Figure 1 shows the first profile taken at 11.7 & 10.5 m/hr, where the region of nitrification activity measured by chemical species is located near the end of the second stage. For this profile, the first three ports of the system where ammonia removal was minimal were not sampled. At this operating condition, the system was not achieving complete nitrification and relatively high loadings of BOD were bleeding through to the N column (Figure 1c). It can be seen that the signal normalized to

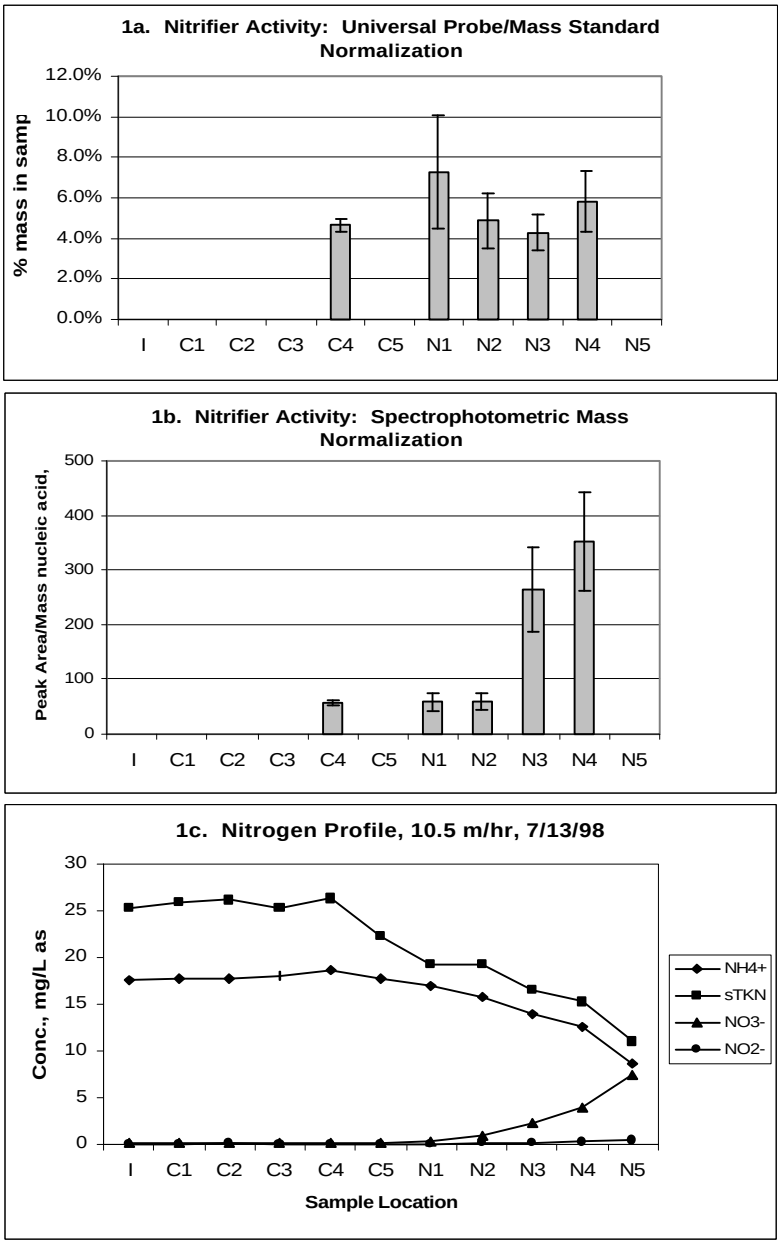

Figure 1. Column Profiles at 11.7 & 10.5 m/hr, 7/13/98. a. Nitrifier activity normalized to mass standards; b. Nitrifier activity normalize to mass of nucleic acids; c. Chemical nitrogen species.

nucleic acid as measured by spectrophotometry (Figure 1b) corresponds more closely with the chemical nitrogen profile than the nitrifier signal normalized using the pure culture mass standards (Figure 1a). The profile in Figure 2 was taken at 10.2 & 9.0 m/hr. Again the method normalizing nitrifier signal to spectrophotometic mass more accurately reflects the chemical nitrogen profile (Figure 2b), although at this time the activity measured in

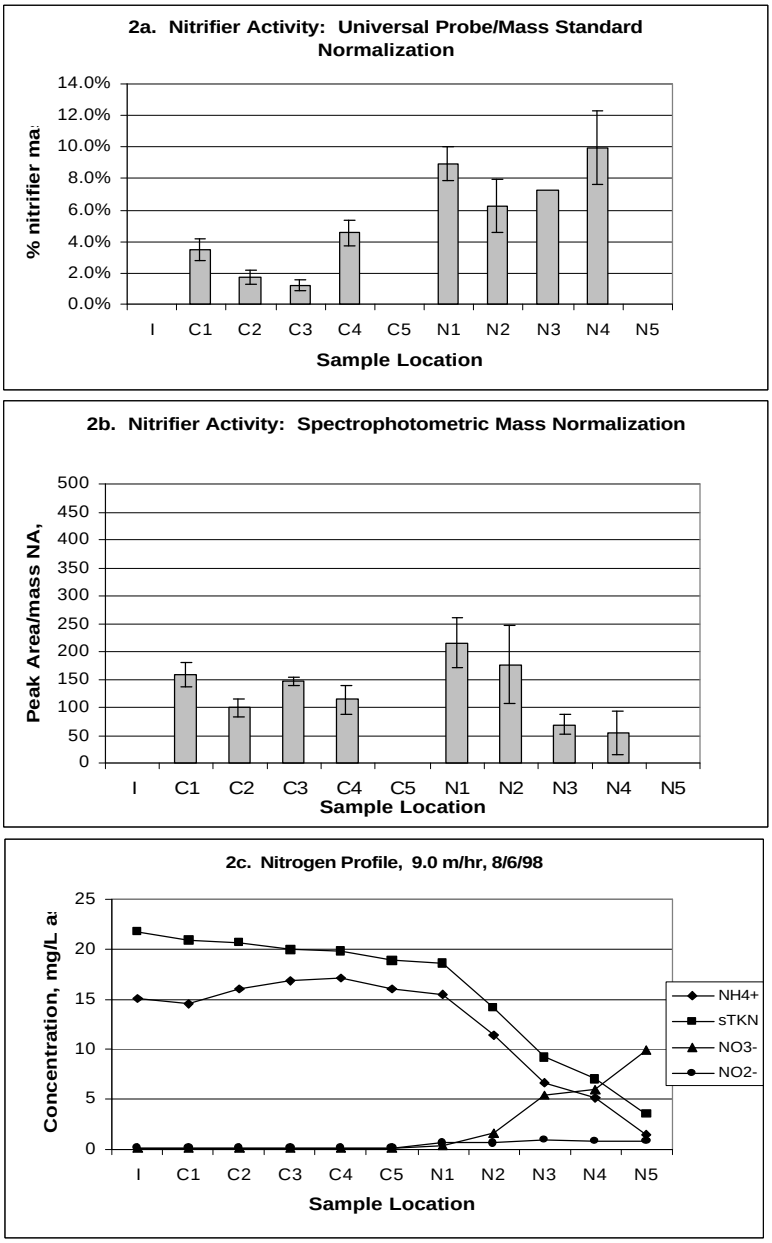

Figure 2. Column Profiles at 10.2 & 9.0 m/hr, 8/6/98. a. Nitrifier activity normalized to mass standards; b. Nitrifier activity normalized to nucleic acid mass; c. Chemical nitrogen species.

the first stage was significantly higher. This phenomenon was not explained by looking at the profiles of ammonia, NO_2^- and NO_3^-, and may be the result of a background population of nitrifiers growing on a substrate other than ammonia.. Nitrifier activity measured using pure culture mass standards does show higher activity in the N column, but activity does not correspond with chemical measurements of nitrogen species. During

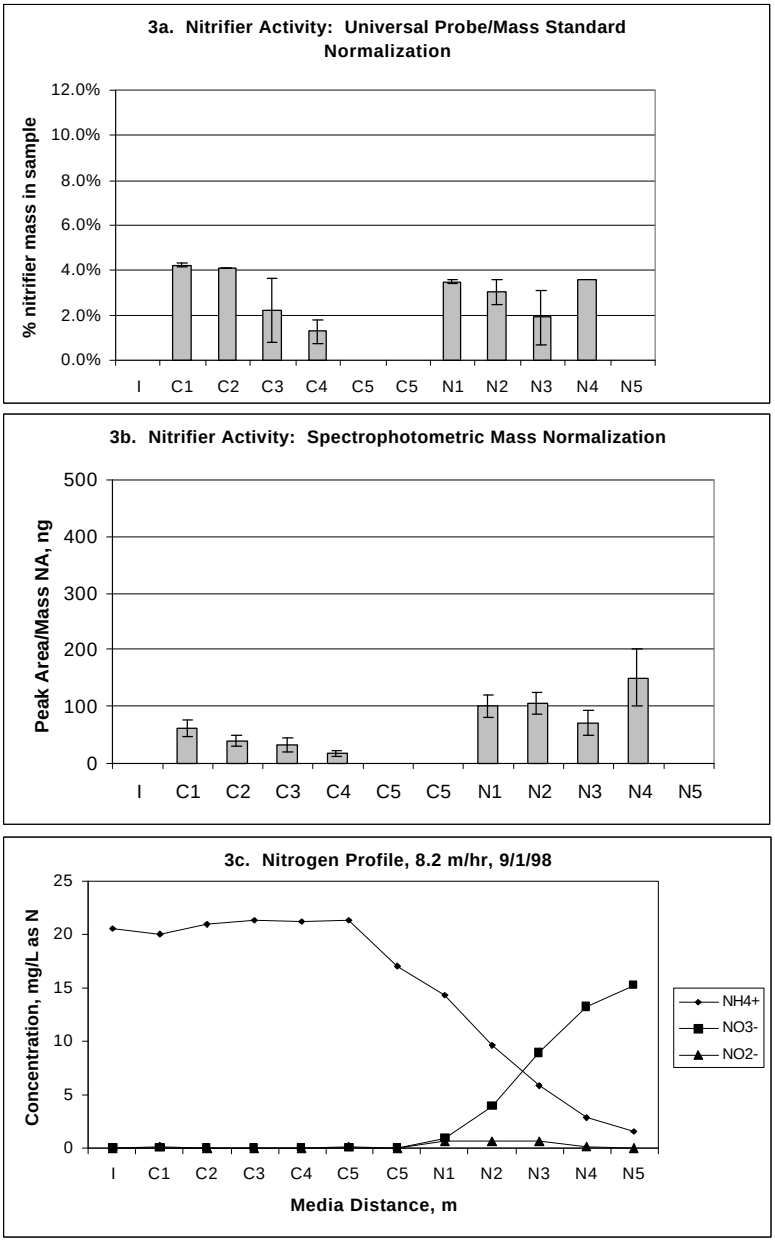

Figure 3. Column Profiles at 9.4 & 8.2 m/hr, 9/1/98. a. Nitrifier activity normalized to mass standards; b. Nitrifier activity normalized to nucleic acid mass; c. Chemical nitrogen species.

the third profile, conducted at 9.4 & 8.2 m/hr, the spectrophotometric mass normalization data also corresponded better with chemical nitrogen measurements throughout the system (Figure 3). Both methods of activity normalization show low activities relative to other profiles, but the mass normalization method (Figure 3b) corresponds more closely with the chemical data (Figure 3c). The fourth profile, Figure 4, was taken at the same

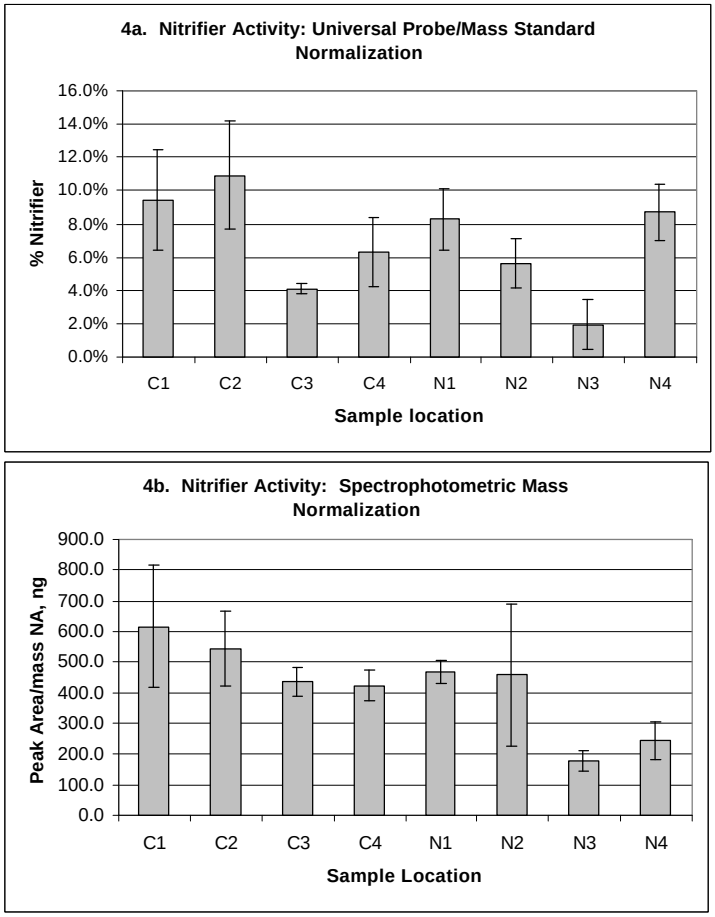

Figure 4. Column Profiles at 9.4 & 8.2 m/hr, 9/30/98. a. Nitrifier activity normalized to mass standards; b. Nitrifier activity normalized to nucleic acid mass.

operating condition as the third but one month later. The reason for this was to attempt to observe the effects of unique characteristics of the wastewater on the pilot system. These characteristics are discussed below. Since the purpose of this profile was to observe the ammonia oxidizing activity, no chemical measurements were taken at this time.

The first profile, at the highest hydraulic loading rate, was the earliest profile measured during the study. The activity levels measured using the spec mass normalization method were the highest seen until the very end of the study. The reason for this is that up to this point, the N column had been grown predominantly as a nitrification stage only. Prior to this condition, the system had not been stressed with organic material, and minimal BOD had been left undegraded in the C column to bleed

through into the N column. After this first operating condition was complete and the loading rate was lowered, the N column had already seen enough organic material to significantly boost the growth of a heterotrophic population, which remained for the duration of the study. It is probable that the normalized nitrifier activity levels are lower in the subsequent two profiles because of this higher heterotrophic population. The last profile was taken following a novel period in the operation of the system. The treatment plant at which the pilot system was operated was routinely operating a belt filter press and draining a stagnant holding tank. The liquid stream from both of these units were returned to the head of the plant, resulting in very high ammonia concentrations in the primary effluent fed to the BAF system. This would explain the higher activity levels measured throughout the system during this profile. In retrospect, a chemical profile of nitrogen species during this condition would have been useful for comparison to the probing data.

Methods Comparison

Theoretically, the method of normalizing to universal probe (EUB338) signal should be more representative of actual proportions of ammonia oxidizing activity, since the signals are normalized to total bacterial RNA as opposed to simply a spec measurement. The fact that the data using this method do not correspond well with the chemical measurements may be very significant. If the method of universal probe normalization is correct, than perhaps chemical nitrification activity cannot always be directly correlated with bacterial activity measurements. It has been shown that ammonia oxidizers can maintain high levels of ribosomes and also emit strong signals even when nitrification is limited or inhibited. Wagner et al (1995) showed that even after the addition of an inhibitor to a nitrifying culture, no changes in nitrifier signal intensity were

observed when compared to signal intensities from a non-inhibited culture. Hence, it is possible that nitrifying bacteria may show signs of activity and growth even when ammonia is not being metabolized. More research of this type is needed, wherein nitrification activity can be linked with spatial location and activity can be measured.

However, assuming that bacterial activity should coincide closely with the chemical conversion of ammonia to nitrate, this new method of utilizing mass standards does not yet overcome its inherent variability. The primary reason for this is likely the reliance on use of spectrophotometry in measuring nucleic acid concentration in extracts. Differences in extraction efficiency were considerable throughout the course of this study, and, as a result, composition of the nucleic acid extracts were quite variable, with values of A_{260}/A_{280} often ranging from 1.5 to 2.2. Furthermore, the lability of RNA leads to degradation throughout the processing of samples. Previous researchers have noted this problem and presented selected results due to RNA degradation in certain samples (Ohashi, 1995). Indeed, when RNA degrades in extracts, or when extraction efficiency is low due to aggregation in samples, measuring extremely low concentrations of nucleic acids greatly increases the variability in that measurement. Current methods work is being devoted to measuring only 16S rRNA bands by digital analysis of gel electrophoresis, in hopes of finding a more accurate basis for normalizing probe signals.

Differences were observed between pure culture extracts and system samples. With the same universal probe, pure cultures and environmental samples yielded different signal intensities per mass of nucleic acid blotted on membranes. The environmental extracts, when observed on agarose gels stained with ethidium bromide, showed more smearing and less distinct bands of RNA. The pure cultures contained less DNA and

other contaminants, likely due to more efficient cell lysis and less extracellular nucleic acids, proteins, etc. This observation shows that trying to normalize environmental samples to standards generated from pure cultures may introduce error, and the reliance on a spectrophotometric measurement is disadvantageous.

The efficiency with which different probes are labeled will certainly affect the final proportion of specific probes compared to universal probes. Probe labeling efficiency can be measured by direct detection of labeled probe with the antibody and substrate, and this efficiency can be incorporated into calculations. It was seen that probe labeling efficiency varied during the course of the study, with no particular preference for one probe over the other. The differences between labeling of probes EUB338 and Nso190 were not quantified because, by using the mass standards, labeling efficiency is inherent in the standard curves generated, as long as the same batch of labeled probe is used for sample hybridization. For each set of samples analyzed, a set of standard membranes must be hybridized to generate a new standard curve. The elimination of the need to check probe labeling efficiency is an advantage of this method.

Differences in probe sequence and target sites may also be a factor contributing to probing variability. Different probes, due to the composition of their respective target sequences, have different affinities for binding. This is accounted for by using specific wash temperatures in the hybridizations. However, it may also be that the accessibility of the target sites on the 16S rRNA molecule varies from one probe to the next. This may contribute to variability in comparing probes, but this phenomenon has not been thoroughly investigated and quantified.

In addition to all other sources of error, small considerations such as the blot volume were seen to have some effect on signal intensity although mass blotted was the same. This should also be considered when comparing sample signals.

It must also be stressed that only one probe was used in this study. There are other probes available for measuring ammonia oxidizers, and it is quite possible that, within the nitrifying population of this system, there is a shift in the predominant ammonia oxidizers at different locations in the columns. This could be due to small differences in optimal conditions for different species or competitive advantages for one nitrifier over another. For a better understanding, a comprehensive set of probes should be used to further characterize the nitrifying population. Furthermore, it is possible that complex systems such as wastewater treatment bacterial populations contain organisms that are closely-related nontarget organisms for the probe Nso190, but they have simply not yet been identified and sequenced. This factor would also interfere with accurately measuring ammonia oxidizers in sludge and biofilms.

Based on the chemical measurements throughout the system, it is likely that this type of system is not limited by oxygen concentration in the bulk liquid. Many attempts were made to measure oxygen concentrations throughout the column (data not shown), but it was difficult to obtain values with confidence. However, approximate concentrations were at or above 2 mg/L throughout the system and were similar at different operating conditions. Furthermore, the chemical profiles seem to show a dynamic region of nitrification that shifts position in the column, while oxygen profiles remained consistent from one operating condition to the next. Therefore, the reason for the change in nitrogen profile is most likely due to loadings of organic carbon to the

second stage. At higher carbon loadings, there is more competition for space and oxygen between heterotrophs and autotrophs, consistent with similar conclusions about these types of systems (Ohashi et al., 1995). Thus, at high loading rates, if carbonaceous BOD is not removed in the first stage of this type of BAF system, it may severely impact nitrification performance in the second stage. This should be taken into account in design of these types of systems.

CONCLUSIONS

The need to refine quantitative blotting methods in oligonucleotide probing techniques remains a challenge to be addressed. The mass standard method proposed here shows some promise, if concentrations of nucleic acids can be measured accurately. Further efforts to elucidate 16S RNA concentrations alone may prove more effective. In BAF systems, the region in which nitrification occurs is dynamic and mainly dependent upon competition with heterotrophs for space and oxygen. Operating conditions such as hydraulic and mass loadings will affect performance in such a two-stage system and proportions of organic material and ammonia will determine the resulting population that develops in the system. Care must be taken in design of BAFs to minimize the presence of BOD in stages where nitrification is a priority.

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