Turk J Biol
28 (2004) 1-7

Characterization of Ammonia Oxidizing Bacteria in an Activated Sludge Plant using a Fluorescent in situ Hybridization Method

Gülnur COŞKUNER
Department of Environmental Engineering, Faculty of Engineering, Cumhuriyet University, Sivas - TURKEY

Received: 03.12.2003

Abstract: The spatial arrangement of functionally important ammonia oxidizing bacteria (AOB) in activated sludge flocs is largely unknown and activated sludge flocs are regarded as heterogeneous agglomerations of bacterial communities. This study aimed to determine both the location of AOB microcolonies within flocs and the size variation of these microcolonies based on their location.

Fluorescent in situ hybridization (FISH) was used in combination with a confocal scanning laser microscope (CSLM) to achieve these goals. Grab samples taken from a full-scale plug flow wastewater treatment plant were examined with probes targeted to AOB β-Proteobacteria (Nso1225) and a genus specific probe targeted to the genus *Nitrosospira* (Nsv443). Frequencies of AOB microcolonies were observed to increase towards the center of activated sludge flocs (ASFs). In addition, the size of AOB microcolonies was found to rise from the edges to the centers of flocs.

This study indicates that ASFs might have a non-random agglomeration of AOB microcolonies. However, FISH is a relatively preliminary method for the aims targeted in this study and the results are not sufficient to justify definite conclusions.

Key Words: Activated Sludge Flocs, Ammonia Oxidizing Bacteria, Confocal Scanning Laser Microscopy, Fluorescent in Situ Hybridization

Bir Aktif Çamur Sisteminde Amonyağı Okside Eden Bakterilerin Yerinde Floresanlı Hibritleşme Yöntemi ile Tanımlanması

Özet: Amonyağı okside eden bakterilerin (AOB) aktif çamur flokları içerisindeki düzeni çoğunlukla bilinmemektedir ve aktif çamur flokları bakteri gruplarının heterojen bir topluluğu olarak kabul edilmektedir. Bu çalışmada, amonyağı okside eden bakteri mikrokolonilerinin floklar içerisindeki konumu ve bu mikrokolonilerin konumlarına göre büyüklüklerindeki değişimin belirlenmesi amaçlanmıştır.

Bu amaçları başarmak için, yerinde floresanlı hibritleşme (FISH) yöntemi lazer mikroskobu ile birlikte kullanılmıştır. Piston akımlı bir atıksu arıtma tesisinden anlık numuneler alınmış ve numuneler AOB β-Proteobakteri`yi hedef alan Nso1225 probu ve *Nitrosospira* cinsini hedef alan Nsv443 probu ile incelenmiştir. AOB mikrokolonilerinin aktif çamur floklarının merkezine doğru sayılarının arttığı gözlemlenmiştir. Ayrıca; AOB mikrokolonilerinin büyüklüğünün de aktif çamur floklarının merkezine doğru arttığı tespit edilmiştir.

Bu çalışma, AOB mikrokolonilerinin aktif çamur floklarında gelişigüzel dağılmadığını gösterebilir. Fakat; FISH yöntemi bu çalışmada amaçlanan hedeflere ulaşmak için henüz başlangıç niteliğindedir ve çalışmada elde edilen veriler kesin sonuçları kanıtlayacak derecede yeterli değildir.

Anahtar Sözcükler: Aktif Çamur Flokları, Amonyağı Okside Eden Bakteriler, Lazer Mikroskobu, Yerinde Floresanlı Hibritleşme

Introduction

Recently, the removal of nitrogenous components in wastewater has gained great significance in terms of environmental protection. Nitrification is the first step necessary in the complete removal of nitrogen by nitrification-denitrification processes since wastewater almost always contains reduced forms of nitrogen, mainly as ammonia and organic nitrogen. Nitrification is managed by 2-step biological oxidation. In the first step ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB),

which are often represented by the genus *Nitrosomonas*. In the second step, nitrite oxidation is carried out by nitrite oxidizing bacteria (NOB) to produce nitrate. NOB are often represented by the genus Nitrobacter. Nitrifying bacteria have slow growth rates and they are also very sensitive to toxic shocks, and pH and temperature changes. Therefore many plants have difficulty in maintaining stable nitrification. AOB were thought to be a rate limiting step in the biological nitrification process. Therefore, it is a very important functional group of treatment plants.

Little experimental research has been carried out on the spatial distribution of microbial communities. This is mainly due to the lack of suitable experimental techniques to study this subject. The sampling and resolution of the measurements are extremely difficult (1). However, FISH in combination with CSLM is a very powerful tool to study the spatial distribution of microbial populations. Conventional epifluorescence microscopy is adequate for thin samples where all information within the field of view is found in a single focus plane. CSLM offers the third dimension by a technique known as optical sectioning of thick samples without the need for mechanical sample sectioning (2). Stacks of these optical sections taken at successive focal planes can be reconstructed to produce a 3-dimensional view of the specimen with CSLM. In addition, the application of CSLM significantly minimizes the effects of background and out of focus fluorescence (3). However, CSLM has its limitations; for example even the best CSLM is limited in depth penetration to about 100 µm. Therefore, anything thicker than this requires mechanical sectioning prior to analysis with CSLM (2).

Recently, CSLM has been introduced in the field of microbial ecology in wastewater treatment, and initial studies regarding nitrification process have been performed with FISH (4-8). These studies have mainly investigated the diversity of nitrifying bacteria. In addition, the relationship between the structure and function of nitrifying bacteria was studied by using both FISH and microelectrodes to measure oxygen, nitrite and nitrate profiles in the biofilm structure. However, there is little information on the location of nitrifying bacteria and their abundance at various places in activated sludge flocs (ASFs). In this study, variations in size and frequencies of AOB at different parts of ASFs were investigated with FISH in combination with CSLM.

Materials and Methods

Sampling

Grab samples of mixed liquor were taken from a plug-flow reactor receiving domestic wastewater (Wanlip, Leicester, England). The activated sludge unit contained an anoxic zone with mixer, an oxic zone with twin surface aerators and tapered aeration with fine bubble diffused air. Samples were preserved immediately with absolute ethanol (1:1, v/v), carried to the laboratory at 4 ºC and stored at -20 ºC until they were used. Samples were fixed with 4% paraformaldehyde as described by Amann et al. (9).

Operational Parameters

Average daily flow was about 125,000 m^3 in the Wanlip sewage treatment plant. The total capacity of this unit was 23,200 m^3. In addition, mixed liquor suspended solids MLSS = 2500 mg l^{-1} and the F:M ratio was 0.25 kg BOD/kg MLSS.d. The influent BOD (settled wastewater) and effluent BOD concentrations were 150 mg l^{-1} and 20 mg l^{-1} respectively. The influent and effluent ammonia concentrations were 32 mg l^{-1} and 4 mg l^{-1} respectively. Operational parameters were provided by Severn Trent Water Ltd. (Leicester, England).

Oligonucleotide probes

The following oligonucleotide probes were used for the in situ detection of AOB: (i) S-*-Ntros-1225-a-A-20 (Nso1225) with a sequence 5'-CGCGATTGTATTACGTGTGA-3' targeted to AOB β -*Proteobacteria*, (ii) S-*-Ntros-0444-a-A-19 (Nsv443) with a probe sequence 5'-CCGTGACCGTTTCGTTCCG-3' specific for the genus *Nitrosospira*. In addition, S-D-Bact-0338-a-S-18 with a sequence 5'-ACTCCTACGGGAGGCAGC-3' with no target organisms (an antisense Bact338 probe; Non338) was used in negative controls. Notations were used as described by Alm et al. (10).

In situ Hybridization

Fixed activated sludge samples (200 µl) were placed in Eppendorf tubes. Then the samples were dehydrated in 60%, 80% and 98% ethanol successively. After the final dehydration step, supernatant was discarded and the pellet was resuspended in 38 µl of hybridization buffer (20% NaCl, 10% Tris HCl, 1% SDS). Then 100 ng of examined probe was added to the samples for positive controls. Negative controls were also prepared. The

samples were left to hybridize at their certain hybridization temperatures. After hybridization, the samples were washed in a 0.5 ml washing buffer (20 mmol l^{-1} Tris HCl, 0.1% SDS, 5 mmol l^{-1} EDTA, 180 mmol l^{-1} NaCl) twice for 15 min. Then they were washed in 0.5 ml molecular grade water. Next, the samples were centrifuged and the pellet was resuspended in 20 µl of filtered distilled deionized water. Then 10 µl of sample was spotted onto a gelatin-coated slide and allowed to air dry. A drop of Citifluor (Citifluor, Kent, UK) was added to the sample and a coverslip placed over the preparation. The edges of the coverslip were sealed using nail varnish and prepared slides were stored in the dark at 4 ºC before viewing. Optimum hybridization and washing conditions for the probes used in this study are summarized in the Table. The NaCl concentration in the washing buffer (WB) for probe Nsv443 was 32 mM as it was optimized in a previous study (11).

Microscopy

Slides were examined with a BIORAD MRC 600 CSLM equipped with a Kr/Ar-ion laser. Images were captured using the COMOS program (Bio-Rad, Hemel Hempstead, United Kingdom). Optical sections of ASFs with 1 µm increments were obtained by CSLM. It was assumed that each section consists of single layer of cells, since the average size of AOB cells is smaller than 1 µm.

Results

Detection of AOB in Activated Sludge Samples by FISH

The AOB group specific probe Nso1225 and genus specific probe Nsv443 targeted to *Nitrosospira* were applied to confirm the presence of AOB in activated sludge samples. A probe targeted to the genus *Nitrosomonas* was not used in this study since the genus Nitrosospira was found to be predominant in the Wanlip sewage treatment plant samples in previous a study. Details about how *Nitrosospira* was found to be the dominant genus can be found in a previous publication (11). AOB were observed as dense ellipsoidal microcolonies within ASFs throughout the reactor except from raw sewage. Typical AOB microcolonies detected by FISH obtained by the Nso1225 probe are shown in Figure 1.

Frequencies of AOB Microcolonies at Different Depths of ASFs

CSLM allows optical sections at different depths of the specimen under examination. In this study, the depth of ASFs was divided into optical planes with same distance theoretically and each corresponding plane was recorded on the computer. The number of AOB microcolonies observed in each plane was used to create the charts in Figures 2 and 3. Figure 2 shows the distribution of AOB microcolonies hybridized with the Nsv443 probe throughout the depth of ASFs and Figure 3 represents the distribution of AOB microcolonies hybridized with the Nso1225 probe throughout the depth of ASFs.

Size Variation of AOB Microcolonies within ASFs

The optical plane corresponding in the middle depth of ASFs was assumed to be the center of the floc (50% of ASFs) in the z-direction. From center to edge, the optical planes are named 40%, 30%, 20%, 10% and 0% with 10% increments in depth. It was assumed that the depth between 0% and 30% represents the edge of ASFs while 30% to 50% indicates the center of ASFs.

Figure 4 shows the variation in the average size of AOB microcolonies at different depths of ASFs labeled with Nsv443 and Nso1225 separately. The size variation in AOB microcolonies between the edges and centers of ASFs was significantly different based on the Kruskall-Wallis test (12) for Nsv443 labeled AOB microcolonies. At the same time, it was observed that the AOB

Table. Optimum hybridization and washing conditions for the probes used in this study.

Probe	Hybridization Temp. T_d (ºC)	Formamide (%)	Washing Temp. (ºC)	NaCl in WB (mM)
Nso1225	51	35	51	180
Nsv443	52	30	48	32
Non338	37	30	37	180

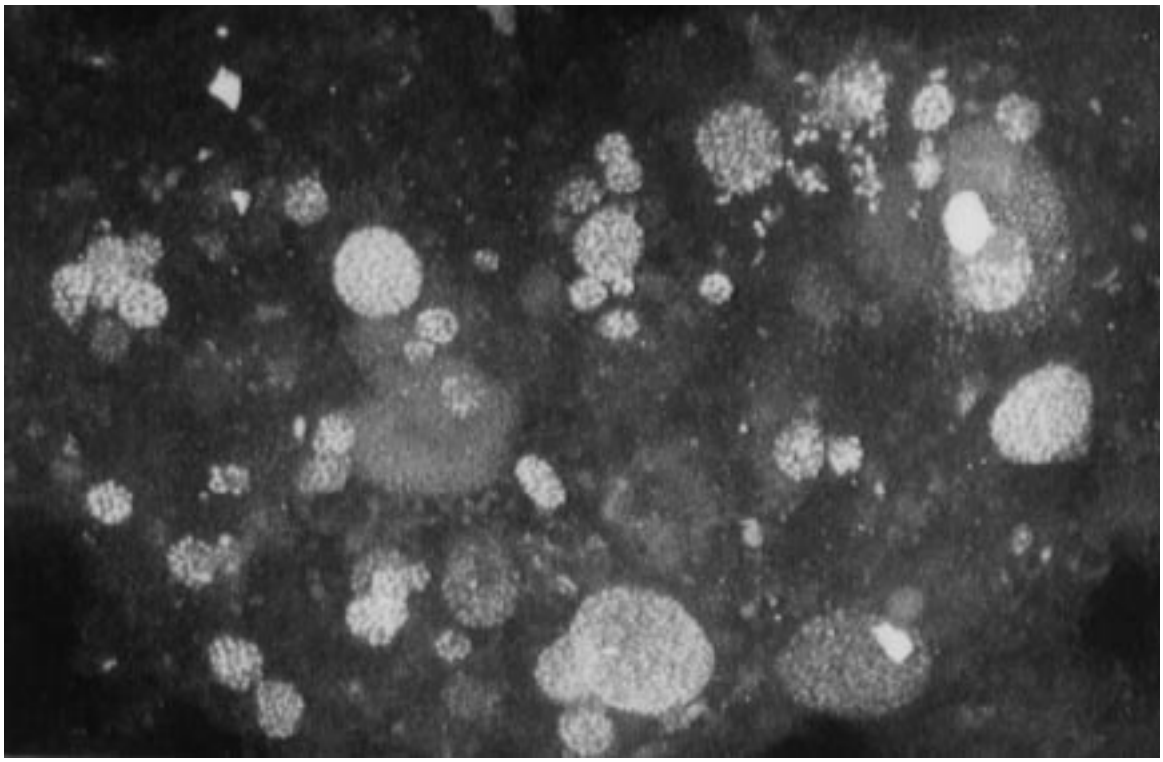

Figure 1. Hybridization of activated sludge sample with Nso1225 probe.

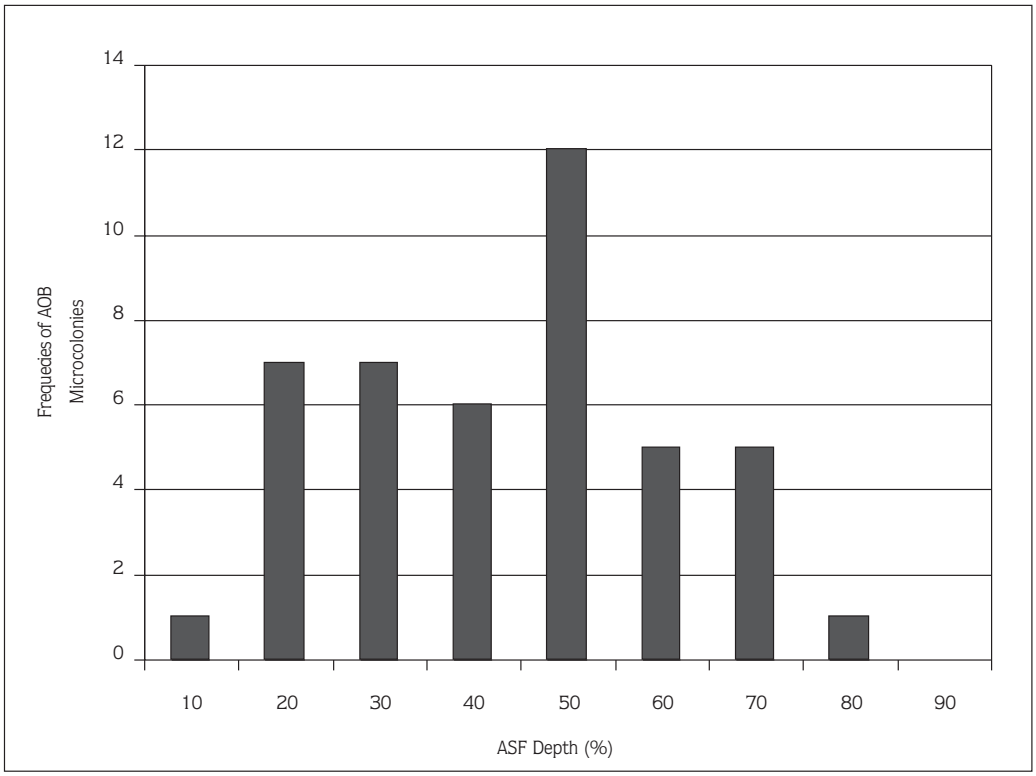

Figure 2. Distribution of AOB microcolonies hybridized with Nsv443 throughout the depth of ASFs.

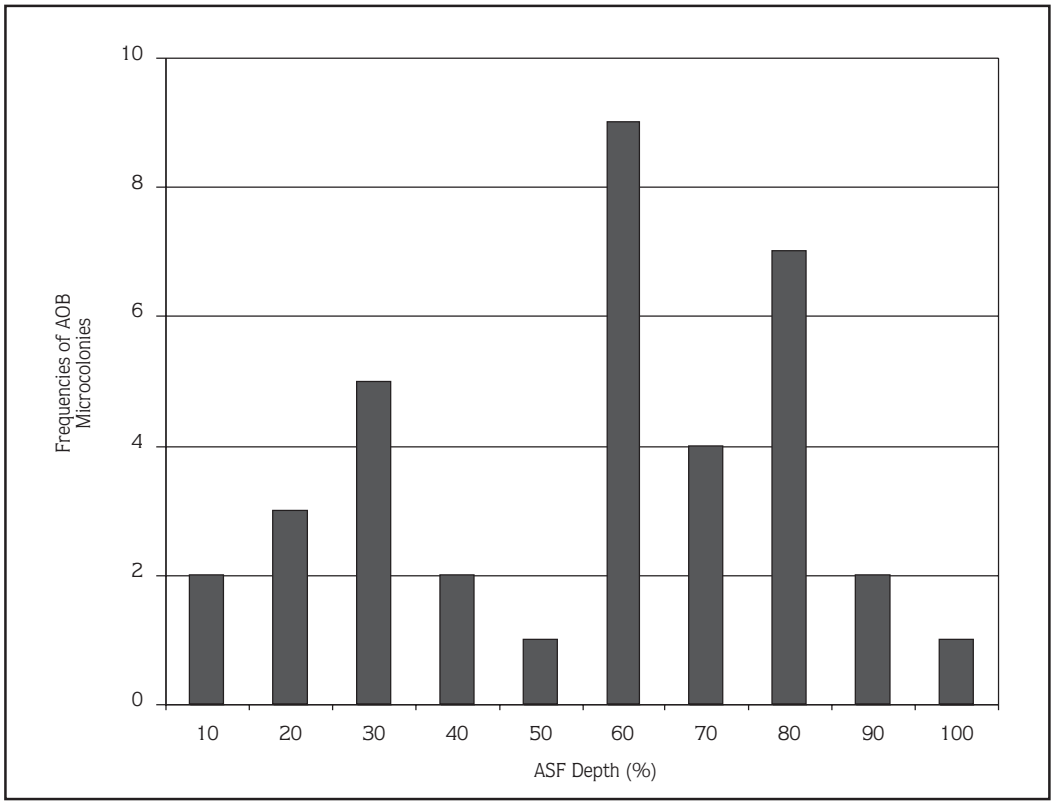

Figure 3. Distribution of AOB microcolonies hybridized with Nso1225 throughout the depth of ASFs.

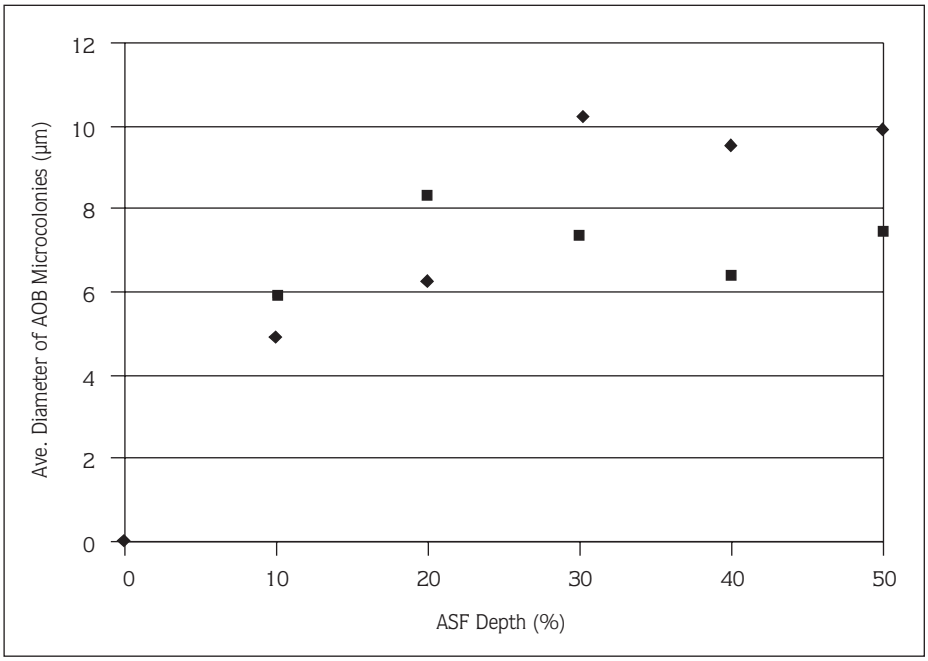

Figure 4. Variation of AOB microcolony sizes at different depths of ASFs.
(◆), Obtained with Nsv443 probing; (■), obtained with Nso1225 probing.

microcolonies detected by the Nso1225 probe were larger than the AOB microcolonies detected by Nsv443 at the edges (from 0% to 20%) and the AOB microcolonies detected by the Nsv443 probe were larger than the Nso1225 ones in the centers (from 30% to 50%).

Discussion and Conclusion

This study aimed to see how AOB microcolonies were distributed within ASFs and size variation of these AOB microcolonies. However, there are some drawbacks, which prevent us from drawing clear conclusions. FISH experiments were performed in Eppendorf tubes. Therefore, the pipetting steps may disintegrate large flocs and some artificial aggregates might form during the centrifugation steps in FISH experiments. Floc architecture should have been preserved to allow more determinative results. For preserving floc architecture, the flocs need to be embedded such as in a plastic resin prior to fixation, dehydration and FISH. Slide hybridization is preferable for this purpose. It is commonly accepted that ASFs are not stable (like biofilms) and their structure changes due to attachment or breakage caused by high sheer stress due to mechanical aeration in aeration basins. I think that although the structure of ASFs might be slightly distorted during FISH experiments performed in Eppendorf tubes, this distortion may be negligible particularly when compared with distortion that occurs in the aeration basin with mechanical aerators.

In this study, FISH detected very few AOB microcolonies at the edges of ASFs (Figures 2 and 3). In addition, there were no AOB microcolonies at the edges of some flocs. Most of the AOB microcolonies were observed in the centers of ASFs. It can be concluded that nitrifiers are not able to compete with heterotrophs at the edges of flocs when the carbon sources are high in the surface of the flocs, similar to biofilm systems. At the same time, it is known that heterotrophs can grow much faster than nitrifiers (13), and so slowly growing AOB might be forced to the inside parts of the ASFs because nitrifiers can compete only when the substrate for heterotrophs is depleted (14). This localization may also protect the AOB from the detachment effects of the flocs.

Figure 4 shows that larger AOB microcolonies were observed towards the centers of the flocs examined. There was a clear tendency in AOB microcolonies to increase in size from the edges to the centers of flocs with the Nsv443 probe. The average size of AOB microcolonies at the edges of flocs was significantly smaller than size of these in the central parts of flocs with the Nsv443 probe. Increased microcolony size at the center must reflect faster and/or more prolonged growth in these AOB. If the observation reflects faster growth, this would imply that more favorable conditions have existed for a sufficiently long period of time for the size differences to occur. This would only be possible if the floc had sustained a structure for that period of time. Similarly, the existence of an area of prolonged growth may imply the existence of a stable 3-dimensional structure. The frequent observation of AOB microcolonies in the central part of ASF and their larger sizes might be an indication that AOB microcolonies are not located randomly in ASFs.

Future studies may be performed to investigate the spatial distribution of different genera of AOB microcolonies considering the drawbacks mentioned in this study. It is important to know whether AOB microcolonies located in ASFs are random or non-random. If it is confirmed that they are not located in a non-random way with future studies, the examination of activated sludge systems can be performed on a microscale rather than a macroscale. At the same time, it may be possible to take some precautions in time during plant operation by observing the structure of functional groups of AOB in ASFs.

Acknowledgments

I would like to express my sincere thanks to the Civil Engineering Department of the University of Newcastle upon Tyne and Dr. Tom Curtis for allowing me to carry out this PhD study. I also thank Dr. Trevor Booth for guidance with the CSLM and Severn Trent Water Ltd for providing operational data relevant to the plant examined. In addition, I am very grateful to Cumhuriyet University for the financial support of this PhD study.

References

1. Okabe S, Hirata K, Ozawa Y et al. Spatial Microbial Distribution of Nitrifiers and Heterotrophs in Mixed-Population Biofilms. Biotechnol Bioeng 50: 24-35, 1996.
2. Wagner M, Noguera DR, Juretschcko S et al. Combining Flourescent In Situ Hybridization (FISH) with Cultivation and Mathematical Modeling to Study Population Structure and Function of Ammonia- Oxidizing Bacteria in Activated Sludge. Wat Sci Techn 37: 441- 449, 1998.
3. Wagner M, Aßmus B, Hartmann A et al. In situ Analysis of Microbial Consortia in Activated Sludge Using Fluorescently Labelled, rRNA Targeted Oligonucleotide Probes and Scanning Confocal Laser Microscopy J of Microscopy 176: 181-187, 1994.
4. Juretschko S, Timmermann G, Schmid M et al. Combined Molecular and Conventional Analyses of Nitrifying Bacterium in Activated Sludge: *Nitrosococcus mobilis and Nitrosospira*-Like Bacteria as Dominant Populations. Appl Environ Microbiol 64: 3042-3051, 1998.
5. Schramm A, Larsen LH, Revsbech NP et al. Structure and Function of a Nitrifying Biofilm as Determined by In situ Hybridization and the Use of Microelectrodes. Appl Environ Microbiol 62: 4641-4647, 1996.
6. Schramm A, Larsen LH, Revsbech NP et al. Structure and Function of Nitrifying Biofilm as Determined by Microelectrodes and Fluorescent Oligonucleotide Probes. Wat Sci Techn 36: 263-270, 1997.
7. Schramm A, Beer DD, Wagner M et al. Identification and Activity in situ of *Nitrosospira* and *Nitrospira* spp. as Dominant Populations in a Nitrifying Fluidized Bed Reactor. Appl Environ Microbiol 64: 3480-3485, 1998.
8. Schramm AD, Beer DD, Heuvel JCVD et al. Microscale Distribution of Populations and Activities of *Nitrosospira* and *Nitrospira* spp. Along a Macroscale Gradient in a Nitrifying Bioreactor: Quantification by In Situ Hybridization and the Use of Microsensors. Appl Environ Microbiol 65: 3690-3696, 1999.
9. Amann RI, Krumholz L, Stahl DA. Fluorescent-Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental Studies in Microbiology. J Bacteriol 172: 762-770, 1990.
10. Alm EW, Oerther DB, Larsen N et al. The Oligonucleotide Probe Database. Appl Environ Microbiol 62: 3557-3559, 1996.
11. Coskuner G, Curtis T. In Situ Characterization of Nitrifiers in an Activated Sludge Plant: Detection of *Nitrobacter Spp.* J Appl Microbiol 93: 431-437, 2002.
12. Sokal RR, Rohlf FJ. Biometry: The Principles and Practice of Statistics in Biological Research. W.H. Freeman and Company, New York, 1995.
13. Rittmann BE. Aerobic Biological Treatment. Environ Sci Techn 2: 128-136, 1987.
14. Rittmann BE and Manem JA. Development and Experimental Evaluation of a Steady-State, Multispecies Biofilm Model. Biotechnol Bioeng 39: 914-922, 1992.