Int. J. Environ. Res., 7(2):479-484, Spring 2013
ISSN: 1735-6865

Monitoring Bacterial Diversity in a full-scale Municipal Wastewater Treatment plant in Dubai by Fluorescence *in situ* hybridization Technique

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Received 30 Jan. 2012; Revised 21 Dec. 2012; Accepted 7 Jan. 2013

ABSTRACT: In this study, the bacterial diversity in the activated sludge system of a full-scale municipal wastewater treatment plant in Dubai was monitored over a period of one year using ribosomal RNA (rRNA) targeted oligonucleotide probes for a defined phylogenetic group of bacteria by the Fluorescence *in situ* hybridization (FISH) technique. The largest fraction of the bacterial community in the sludge samples belonged to the gamma-subgroup of proteobacteria (25%) followed by gram positive bacteria of high G+C content(16%), gram positive bacteria with low G+C content (9 %), beta-proteobacteria (8%) and alpha-proteobacteria(5 %) with respect to the population percentages stained by DAPI (4,6-diamino-2-phenylindole).A specific nocardioform actinomycete, simultaneously targeted by both HGC69a and MNP1 probes, was predominantly found throughout the study period in all activated sludge mixed liquor samples. The nocardioform actinomycetes group members were detected in both branched and single cell morphotypes. Most of the previously published genus and species specific probes failed to hybridize to the sludge samples. In conclusion, the overall bacterial community populations detected by the sub-group specific 16S rRNA targeted oligonucleotide probes in FISH technique remained almost constant throughout the period of study irrespective of treatment plant conditions.

Key words: Fluorescence *in situ* hybridization, Bacterial community, Nocardioform actinomycetes, Activated sludge, Oligonucleotide probes

INTRODUCTION

Today activated sludge systems represent a widely used technology for domestic and municipal wastewater treatment in most countries (Eschenhagen *et al*, 2003; Jenkins *et al*., 2003). The engineering intensifies the treatment, but a basic understanding of the microorganisms and their activity under different conditions are key for its successful operation (Wagner *et al*., 1994). The health of anactivated sludge system thus depends upon its microbial diversity, which again is dependent on the influent wastewater, environmental parameters and prevalent operational conditions (Bitton, 2005; Wilderer *et al*., 2002; Martins *et al*., 2004). Monitoring of the microbial community in such plants can be instrumental in understanding and control of bulking and foaming which are caused chiefly by filamentous bacterial communities (Jenkins *et al*., 2003). The Eikelboom keys have been invaluable to wastewater professionals in identifying the bacterial filaments. However, it was found that one Eikelboom type might not consist of one single phenotype, and that morphological characters are not reliable indicators for distinguishing phylogenetic groups because the morphology of some filaments is known to change (Howarth *et al*., 1999). Over recent years, the growth of 16S and 23S ribosomal RNA sequence databases have enabled researchers to use rRNA-targeted hybridization for studying activated sludge biomass. Oligonucleotide probes targeting specific domains, genera, species, or even strains have been developed. Molecular probe based detection techniques like FISH have been successfully employed for this purpose (Amann *et al*., 2001; DeLong *et al*., 1989).

At present Dubai sewage treatment plant, one of the major wastewater treatment plants in Dubai city is frequently challenged by bulking and foaming

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episodes in its activated sludge system. In our earlier study (Faheem and Khan, 2009), various filamentous bacteria were identified and reported on the basis of classical morphological features as described by Jenkins *et al*., 2003.The accurate identification and quantification of foaming and bulking-causative organisms may guide future activated sludge modeling and the development of rational control measures in the activated sludge units of the sewage treatment plant in Dubai.This investigation aimed at monitoring the bacterial community in the activated sludge system of a full-scale wastewater treatment plant(WTP) in Dubai over a period of one year beginning May 2010 using previously published 16S and 23S ribosomal RNA (rRNA) targeted oligonucleotide probes specific for established phylogenetic groups of bacteria. Fluorescence *in situ* hybridization (FISH) was carried on the activated sludge mixed liquor samples. A series of previously reported probes were used to detect the presence of the different groups and subgroups of bacteria directly within the sludge samples. The abundance of these groups was obtained numerically through computer analysis of the images taken when the hybridized samples were examined under the microscope.

MATERIALS & METHODS

250 ml of mixed liquor sludge samples were collected from aeration tanks of the activated sludge system of a full-scale WTP located at the Al Aweer area in Dubai. Samples were taken on a fortnightly basis spanning over a one year period. Samples were stored at 4°C and fixed within 24 hrs. The samples were fixed both in ethanol and 4% (w/v) paraformaldehyde by procedures described earlier (Schuppler *et al*., 1998). The fluorescence *in situ* hybridization technique was performed on the activate sludge mixed liquor samples using the methods described earlier (Amman, 1995; Daims *et al*., 2005). In order to find out the total area occupied by all bacteria present in the biomass sample, staining with DAPI (4,6-diamino-2-phenylindole) dye was employed. DAPI would stain the entire DNA presented in the sample. Once they were stained, a visual signal would be emitted and that would be captured by the imaging system (Daims *et al*.,2005).Hybridization with sludge samples was performed by domain, group/class, genus and species specific probes.The abundance of the various bacterial groups was obtained numerically through computer analysis of the images taken when the hybridized samples were examined under the microscope.The list of oligonucleotide probes used in this study and their specificity are given in Table 1. All oligonucleotide probes were labeled at their 5' end by tetramethyl rhodamine isothiocyanate (TRITC).

The slides were visualized by epifluorescence microscopy at 1,000X magnification on a Nikon Eclipse 80i (Japan) microscope equipped with a 100-W mercury lamp and filter sets G-2E/C for TRITC, B-2E/C for FITC and N UV-2E/C for DAPI. Images were captured and analyzed using the ProgResC5 digital camera system (JENOPTIK, Germany). Pictures were processed as tagged-image file format (TIFF) files on a personal computer running Image-J software, version 1.42 and analyzed by the help of Metamorph version 4.6r8 program (Universal Imaging Corp). The program would calculate the area from which the fluorescence signal was recorded. The final result was presented in terms of the percentage calculated when this area was divided by the total area of the field of observation (Daims *et al*., 2005).

RESULTS & DISCUSSION

The detailed profile of various bacterial groups and their changes in the activated sludge mixed liquor samples over the study period is shown in figure 1. Activated sludge physiological active bacterial populations were observed with respect to 4',6-diamidino-2-phenylindole (DAPI) staining as described earlier (Manz *et al*., 1992; Amann *et al*., 2001; Daims *et al*., 2005). During the study period, approximately 79.1- 92.9 % of the microbes stained by DAPI were targeted by Eub338 mix (I-III) ensuring adequate bacterial 16S rRNA.

It is evident from FISH analysis, that the percentages of bacteria belonging to several groups like alpha, beta, gamma class of proteobacteria including gram positive population of Low G+C and High G+C group remained almost constant irrespective of the treatment plant conditions. A total of 24 activated sludge mixed liquor samples were used for hybridization by six higher subclass specific oligonucleotide probesin FISH analysis. The major bacterial groups identified (on the overall average basis during whole study period)in descending order of their frequency of occurrence were: gamma subclass of proteobacteria(25%),gram positive bacteria with high G+C content (15%),gram positive bacteria with low G+C content (9%), beta-proteobacteria (8%) and alpha-proteobacteria (5 %). In addition, a more specific MNP1 probe (Schuppler et al. 1998) targeted at the majority of nocardioform actinomycetes group members was applied to the mixed liquor samples.

All of the 24 mixed liquor samples successfully hybridized by EUBmix (I-III) probes with respect to DAPI (80-90% as shown in figure 1). This indicated the presence of highly physiologically active bacterial populations within the samples (Manz *et al*., 1994). Furthermore, morphological examination of mixed liquor sludge samples using methods described by Jenkins *et al*., 2003 also revealed a large number of filamentous

Table 1. Oligonucleotide probes used in this study

Probe	Sequence (5`-3`)	Specificity	Reference
Eub338	GCTGCCTCCCGTAGGAGT	Domain bacteria	Amann etal. 1990
Eub338II	GCAGCCACCCGTAGGTGT	Domain bacteria (Planctomycetales)	Daims etal. 1999
Eub338III	GCTGCCACCCGTAGGTGT	Domain bacteria (Verrucomicrobiales)	Daims etal. 1999
Alpha 1b	CGTTCGYTCTGAGCCAG	alpha-Proteobacteria	Manz etal. 1992
Beta42a	GCCTTCCCACTTCGTTT	beta-proteobacteria	Manz etal. 1992
Gamma42a	GCCTTCCCACATCGTTT	*gamma-Proteobacteria*	Manz etal. 1992
HGC 69a	TATAGTTACCACCGCCGT	gram positive high G+C content	Schuppler et al.1998
LGC354A	TGG AAG ATT CCC TAC TGC	gram positive low G+C content (firmicutes)	Meier et al. 1999
LGC354B	CGG AAG ATT CCC TAC TGC	gram positive low G+C content (firmicutes)	Meier et al. 1999
LGC354C	CCG AAG ATT CCC TAC TGC	gram positive low G+C content (firmicutes)	Meier et al. 1999
SNA	CATCCCCCTCTACCGTAC	*Sphaerotilus natans*, few *Leptothrix* spp., Eikelboom -170	Wagner et al. 1994
LDI	CTCTGCCGCACTCCAGCT	"*Leptothrix discophora*", *Aquaspirillum metamorphum*	Wagner et al. 1994
LMU	CCCCTCTCCCAAACTCTA	*Leucothrix mucor*	Wagner et al. 1994
HHY	GCCTACCTCAACCTGATT	genus *Haliscomenobacter*	Wagner et al. 1994
TNI	CTCCTCTCCCACATTCTA	*Thiothrix nivea*	Wagner et al. 1994
021N	TCCCTCTCCCAAATTCTA	Eikelboom type 021N	Wagner et al. 1994
S-G-Gor-0596-a-A-22	TGCAGAATTTCACAGACGACGC	genus *Gordona*	De Los Reyes et.al. 1997
S-S-G.am 0192-a-A-18	CACCCACCCCCATGCAGG	*Gordona amarae*	De Los Reyes et al. 1997
M*N*P1	TTAGACCCAGTTTCCCAGGCT	Nocardioform actinomycetes	Schuppler et al.1998

bacteria during the current study period and as indicated in our earlier study (Faheem and Khan, 2009). There were at least three distinct filamentous bacteria that were detected by GAM42a, HGC69a and Alpha1b probes. The filamentous bacteria targeted by GAM42a and HGC69a were always observed in all of the samples during the period of study. In all of the samples, the Gam42a probe identified long branched irregular filaments whose population remained constant. However, genus and species specific oligonucleotide probes for filamentous gram negative bacteria like *Sphaerotilus natans* (SNA), *Liptothrix discophora* (LD1), *Leucothrix mucor* (LMU), *Haliscomenobacter* (HHY), *Thothrix nivea* (TN1) and Eikelboom type 021N (021N) failed to hybridize in the samples. This might be due to low permeability of these bacterial populationstospecific oligonucleotide probes by employed fixation procedures as indicated in other studies (Manz *et al*., 1994; Schuppler *et al*., 1998; Davenport *et al*., 2000).The oligonucleotide probe Alpha1b identified small branched irregular filaments in at least 10 mixed liquor samples.

Several large cocci in clusters were found in most of the samples targeted by Gamma 42a. Also, there were long and short rods targeted by Gam42a probe, probably *Enterobacteriacae*, observed in all of the samples. The oligonucleotide probe Alpha1b identified small cocci in tetrad arrangement as reported by Seviour, 2002 and single cell rods probably belonging to alpha-subclass of proteobacteria. However, tetrad

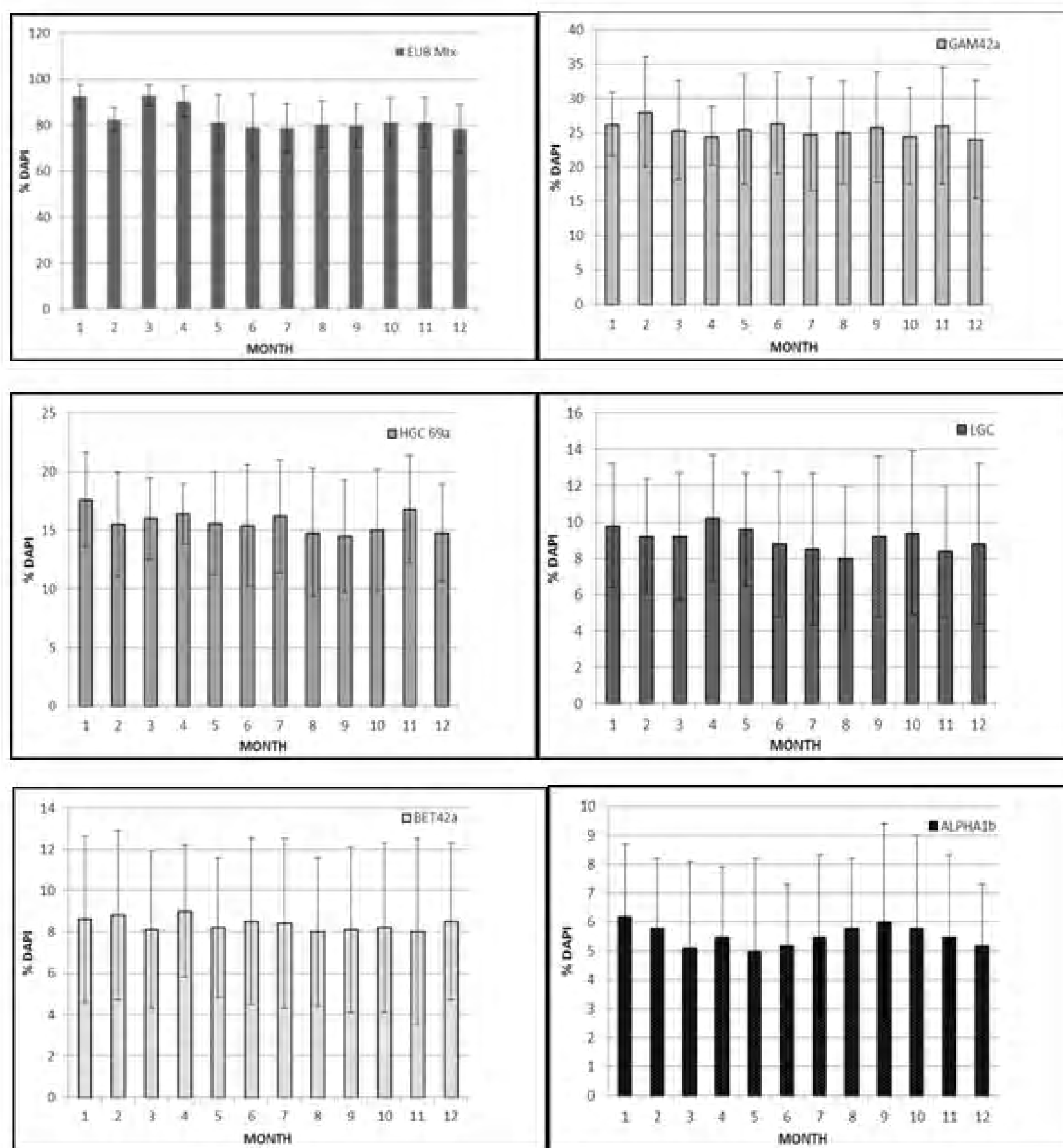

Fig.1. Profiles of various bacterial groups targeted by EUB338 mix, GAM42a, HGC69a, LGC, BET42a and ALPHA1bprobes obtained from FISH analysis over a 12 month period in DSTP (% with respect to DAPI). Error bars: standard deviation

cocci were consistently found in atleast 20 mixed liquorsamples.In a few samples, the Beta 42a probe detected small rods and small cocci of 1-2 µm size existing individually and in clusters.The gram positive bacteria with low G+C (LGC mix probe) targeted mostly spore bearing rods, similar to those described in earlier reports (Ajithkumar *et al*., 2001) and cocci with a size of 2-3 µm. In at least 20 samples, the LGC mix probe (Meier *et al*., 1999) identified long or small rods scattered throughout the sample. The cocci targeted by the LGC probe were found in 18 of the 24 samples. These cocci occurred in clusters or in diplococci/streptococci/ staphylococci arrangements. However, quantitatively the LGC probe targeted quite a small percentage of the bacterial population in comparison to GAM42a and HGC69a probes.

The HGC69a probe detecteda group offilamentous bacteria that was not targeted by Gamma 42a and Alpha1b. It was noted that the population of this group of gram positive bacteria with high G+C content remained dominant throughout the sampling periodas described earlier (Faheem and Khan, 2009). This was probably because of the frequent foaming incidencesobserved in the treatment plant throughout

the study period. Most of the bacteria targeted by HGC69a were either branched filaments or long, medium or small size curved rods. These filamentous morphotypes were found to be dominant in all of the mixed liquor samples. All 24 samples gave positive hybridization with the HGC69a probe indicating that the gram positive bacteria with high G+C content, resembling "nocardia amarae like organism" (Stainsby *et al.*, 2002), was the most significant microbial community in DSTP. This observation suggests that this group significantly influenced the activated sludge process. *In situ* hybridization using the nocardioformspecific MNP1 probe (Schuppler *et al.*, 1998) was performed on the samples previously hybridized with HGC69a probe. MNP1 probe was able to detect two morphotypes. One branched filament type representingtypical nocardioform actinomycetes (Fig. 2A)and the other one comprising of short irregular rods (Figure 2B). This observation supports an earlier study (Schuppler *et al.*, 1998), where MNP1 probe detected similar populations with different morphologies.

The samples containing nocardioform populations detected by probe MNP1 and HGC69a were further analyzed by hybridization with the *Gordona amarae* and genus Gordona specific probes (Table 1) previously reported by De Los Reyes *et al.*, 1997. These two probes failed to detect bacterial populations in WTP samples indicating that Gordona genus members were not dominant in DSTP. However, it is difficult to draw an early conclusion on the absence of this particular bacterial community in the DSTP, as the oligonucleotide probes used in this study were designed for specific studies in other countries were probably not suitable to detect the populations of Gordona genus in DSTP. The failure to detect targeted bacterial populations in activated sludge mixed liquor samples in this study by genus and species specific oligonucleotide probes (SNA, LD1), LMU, HHY, TN1 and 021N) might also be explained by this limitation. It is possible that the bacterial species found in DSTP might be different due to the different geographical distribution affected bylocal environmental conditions in the UAE.

CONCLUSION

This study evaluated, for the first time, the bacterial community structure in the activated sludge samples of a full-scale wastewater treatment plant in Dubai. The population changes of the major higher taxonomic groups such as proteobacteria (alpha, beta and gamma), gram positive bacteria with high G+C and low G+C content was evaluated by FISH technique. The major bacterial groups identified in descending order of their frequency of occurrence were: gamma subclass of proteobacteria (25%), gram positive bacteria with high G+C content (16%), gram positive bacteria with low G+C content (9 %), beta-proteobacteria (8%) and alpha-proteobacteria (5 %). Previously published genus and species specific oligonucleotide probes targeted at bacteria such as*Sphaerotilus natans*, *Leptothrix*, *Leucothrix*, *Haliscomenobacter*, *Thiothrix*, Eikelboom 021N (Type 021N) and *Gordona amarae*failed to hybridize in the sludge samples. Although a few of these filamentous bacteriawith similar morphologies were successfully detected in the samples by higher subclass specific oligonucleotide probes. A specific nocardioform actinomycete group member was found to be dominating in the system throughout the period of study. This bacterium belonged to the high G+C group of gram positive bacteria that was targeted by both HGC69a and MNP1 oligoucleotide probe. The nocardioform actinomycetes group members exhibited both branched and single cell morphotype in most of the samples. Further work based on clone library based approaches is recommended. With the 16S rRNA sequences known, new specific probes should be designed for FISH analysis to further the investigation.

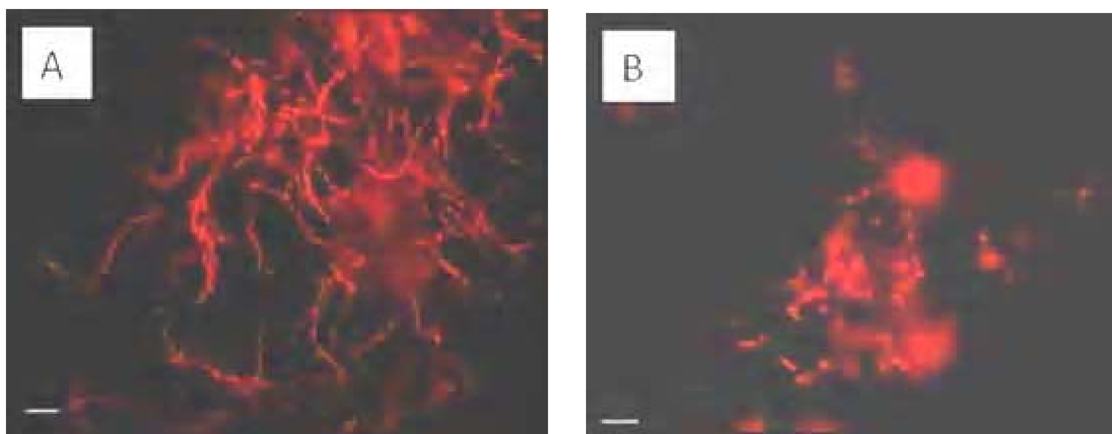

Fig. 2. *In situ* hybridization of nocardioform actinomycetes group in activated sludge samples from DSTP. A, B: sludge samples hybridized by TRITC–labeled MNP1 probe. Bar = 10µm and applies to all photomicrographs

These new probes, together with those presently available, will help to reveal the dynamics of the bacterial community in wastewater treatment plants in the United Arab Emirates.

ACKNOWLEDGEMENT

This work was financially supported by Zayed University Research incentive fund (RIF) (activity code: R10003) awarded to the first author. We thank Dr. Carol Campbell for critical reading of the manuscript. We are grateful to the personnel at the Dubai sewage treatment plant for providing activated sludge samples.

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