Monitoring of the microbial community of a sequencing batch reactor bioaugmented to improve its phosphorus removal capabilities

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Abstract The acclimatisation of an activated sludge to enhanced biological phosphorus removal conditions was followed after and without bioaugmentation with a low amount of phosphorus-accumulating sludge. Phosphorus removal yields were monitored by conventional analytical methods and microbial communities evolutions were followed by a finger printing molecular technique (PCR-SSCP). While the benefit of the bioaugmentation seems real at the level of the reactor parameters, bioaugmentation speeded up the installation of good and stable phosphorus removal yield, the establishment of the inoculated microbial community in the bioaugmented reactor is still unclear. Both the bioaugmented and the control microbial communities evolved in a similar way to end up with apparently comparable populations. At the time of the experiment, the results suggest that the microbial community inoculated for the bioaugmentation did not establish in the reactor but compensated for phosphorus accumulation until the acclimatisation of an endogenous microbial community arose.

Keywords Biodiversity; microbial ecology; single strand conformation polymorphism; 16S rRNA

Water Science and Technology **Vol 43 No 3 pp 1–8** © IWA Publishing 2001

Introduction

The biological removal of phosphorus from wastewater is obtained by the acclimatisation of activated sludge to specific process parameters. These include alternate anaerobic-aerobic sequences and the presence of readily biodegradable carbon sources in the anaerobic zone only (van Loosdrecht *et al.*, 1997a). This acclimatisation, which seems to be possible from any activated sludge, requires a period of 40 to 100 days for the installation of good and stable phosphorus removal yields (Kortstee *et al.*, 1994). During this time there is an enrichment of the sludge in so far as uncharacterised microorganisms are able to accumulate the soluble phosphate from the effluent into internal polyphosphate granules (van Loosdrecht *et al.*, 1997b). Recently, it has been shown that the start up period of a sequencing batch reactor (SBR) could be considerably reduced by the bioaugmentation of the reactor with a small amount of acclimatised sludge or pure culture (Belia and Smith, 1997).

The inoculation of previously adapted microorganisms into non adapted activated sludge to improve plant capabilities, a practice called bioaugmentation, has been tested for different purposes: improvement of nitrification reliability (Rittmann and Whiteman, 1994), removal of toxic compounds (Wilderer *et al.*, 1991), or reduction of processes start up period (Stevens, 1989; Belia and Smith, 1997). The results have been controversial, the efficiency of bioaugmentation being often uncertain and the interpretation of the experiments being limited by the lack of appropriate tools to monitor the fate of the introduced microorganisms (Stephenson and Stephenson, 1992).

Nowadays, molecular techniques have been developed which allow the identification and monitoring of microorganisms without isolation and cultivation (for a review see Amann *et al.*, 1995). These techniques are essentially based on the detection and sequence comparison of the ribosomal RNA genes which are present in all living organisms (Hugenholtz *et al.*, 1998). The objective of this work was to use one of these techniques, the PCR-SSCP (for

Polymerase Chain Reaction and Single Strand Conformation Polymorphism) (Hebenbrock *et al.*, 1995; Lee *et al.*, 1996), to monitor the impact of the inoculation of a microbial community acclimatised to phosphorus removal conditions on the microbial community of a non-acclimatised sludge during its adaptation to conventional anaerobic-aerobic SBR sequences.

Materials and method

Sequencing batch reactors operations

The aerobic SBR of 3 litres working volume was run with four culture cycles per day. Each cycle consisted in a feeding of 1500 ml in 20 min., an aerobic stage of 270 min., a settling of 45 min. and a drawing off of 1500 ml in 25 min. Aeration and agitation were obtained by an air pump controlled with a vertical flow rate meter.

The reactors used for enhanced biological phosphorus removal (EBPR) were Biolafitte reactors of 1.5 litre working volume. Four culture cycles were applied per day. Each cycle consisted in a feeding of 750 ml in 3 min., an anaerobic stage of 150 min., an aerobic stage of 153 min., a settling of 45 min. and a drawing off of 750 ml of effluent for 9 min. Aeration was obtained by an air pump and rate was controlled with a vertical flow rate meter. Agitation was regulated at 200 rpm.

For all reactors, temperature was 19ºC±2ºC. Feed addition and effluent discharge were accomplished using peristaltic pumps. All equipment was run on timers. Hydraulic retention times were 12 hours. Once they had reached 4000 mg/l, the mixed liquor suspended solids (MLSS) were maintained around this concentration by automatic withdrawal of mixed liquor at the end of the aerobic stage three times a week.

All the reactors were fed with a synthetic wastewater composed of: (Kuba *et al.*, 1993): acetic acid, 375 mg/l; K_2HPO_4, 49 mg/l; KH_2PO_4, 28 mg/l; $MgSO_4 \cdot 7H_2O$, 600 mg/l; $CaCl_2 \cdot 2H_2O$, 70 mg/l; NH_4Cl, 227 mg/l; EDTA, 10 mg/l; mineral solution 2 ml/l.

The activated sludge without phosphorus removal capabilities was collected from a low-load municipal wastewater treatment plant which performs aerobic carbon removal (Mirepeisset, France). The acclimatised sludge with good phosphorus removal capabilities was collected in a SBR which had been running for three months with good and stable phosphorus removal parameters. It had been seeded by an activated sludge originating from a municipal wastewater treatment plant which performs EBPR (Roanne, France).

Analytical measurements

Analytical measurements were performed as described before (Rustrian *et al.*, 1997). Ammonium was determined using a Büchi 320 apparatus. Nitrate, nitrite and phosphate were measured by an ion exchange chromatography system using conductivity detection (IonPacAS4A Analytical Column, DIONEX-100, Toulouse, France). Acetate concentration was measured by gas chromatography with a Shimadzu GC-8A apparatus with argon as a carrier gas, using a katharometer detector. MLSS were measured by drying 15 ml of mixed liquor for 24 hours at 100ºC.

Microbial 16S rDNA PCR-SSCP analysis

Ten ml samples of mixed liquor were collected in the middle of the reactors at the end of the aerobic stage and centrifuged at 17500×g and 4ºC for 10 min. Cell pellets were suspended within 1 ml Guanidine Thiocyanate 4M-Tris-Cl 0.1M Ph 7.5 and 0.15 ml of N-Lauroyl sarcosine 10%, split up in four microtubes and immediately stored at –80ºC. Total genomic DNA were extracted from the frozen pellets as previously described with omission of the initial homogenisation step (Godon *et al.*, 1997).

For the single-strand-conformation polymorphism analysis (SSCP), the V3 variable region of the 16S rRNA genes were amplified by PCR with the Bacterial primers: W49:

ACGGTCCAGACTCCTACGGG (forward, *E. coli* position 330 (Brosius *et al.*, 1981)) and W34 TTACCGCGGCTGCTGGCAC (reverse, *E.coli* position 533). The primer W34 was labelled with the 5'-fluorescein phosphoramidite TET (Applied Biosystems, Perkin-Elmer). PCR reaction mix were as follows: 100 ng of total DNA, *Pfu* turbo supplied buffer 1X, dNTP 0.2 mM each, primers 3 ng/µl each, H2O up to 50 µl and 1.25 U of *Pfu* turbo DNA polymerase (Stratagene). After an initial denaturation step of 45 sec. at 94ºC, 25 cycles were applied with 30 sec. at 94ºC, 30 sec. at 57ºC and 30 sec. at 72ºC. Reactions were ended by a 10 min. elongation at 72ºC and cooling at 4ºC. One µl of the PCR mixture was added to 3.5 µl of loading buffer (50% formamide, 7M urea, 3mg/ml blue dextran) and 0.5 µl of the internal standard Genescan-2500 TAMRA (Applied Biosystems). The sample were then denaturated for 2 min. at 94ºC and placed directly on ice before loading on the gel. SSCP electrophoresis parameters on the ABI model 373A sequencer stretch (Applied Biosystems, Perkin-Elmer) and SSCP gel composition were described elsewhere (Zumstein *et al.*, 2000). Data processing was performed with the Genescan software (Applied Biosystems).

Results and discussion

Experimental design

The experiment involved four SBRs (Figure 1). An aerobic SBR was seeded with an activated sludge which had no phosphorus removal capabilities and run for 20 days to adapt the ecosystem to SBR conditions and synthetic wastewater feeding. Then it was split at the beginning of a cycle between two reactors. One reactor was inoculated with 3% (dry weight/dry weight) of a sludge which had been acclimatised to anaerobic-aerobic (A/O) SBR process and had good phosphorus removal capabilities: it will be called the bioaugmented reactor. The biomass used for bioaugmentation was collected at the end of the aeration stage of the reactor, at the time where the polyphosphate reserves were completed. The other reactor was not inoculated and run as a control to follow the natural adaptation of the ecosystem to the running parameters. Both reactors were then run with alternate anaerobic-aerobic conditions to induce the phosphorus removal capabilities of the microbial communities. To keep the two reactors as comparable as possible, both reactors were run with the same pump supply, air supply, synthetic wastewater feeding and overall running parameters. Reactors were kept running until a 100% yield of phosphorus removal was reached for several days. Running parameters were measured twice a week. Sludge sample were withdrawn periodically at the end of the aerobic stage of the cycle and frozen for molecular analysis of the microbial communities.

Kinetics of the phosphorus removal development with and without bioaugmentation

Figure 2 shows the kinetics of soluble carbon, nitrogen and phosphorus concentrations within the parent reactors just before the bioaugmentation. The non P-accumulating parent sludge used the acetate completely, transformed the ammoniac into nitrate and did not

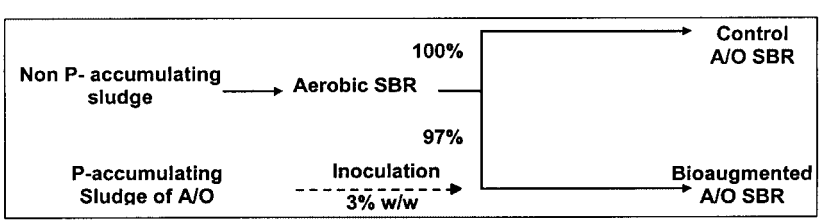

Figure 1 Experimental design

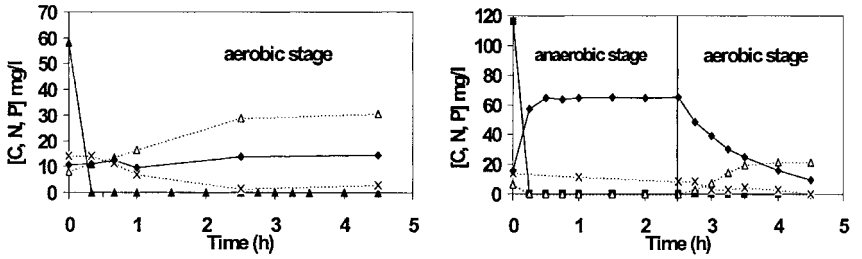

Figure 2 Soluble C, N and P concentrations during one cycle of the non P-accumulating (left) and the P-accumulating (right) parent reactors. ▲: AGV; X: N–NH_4; △: N–NO_3; ◆: P–PO_4

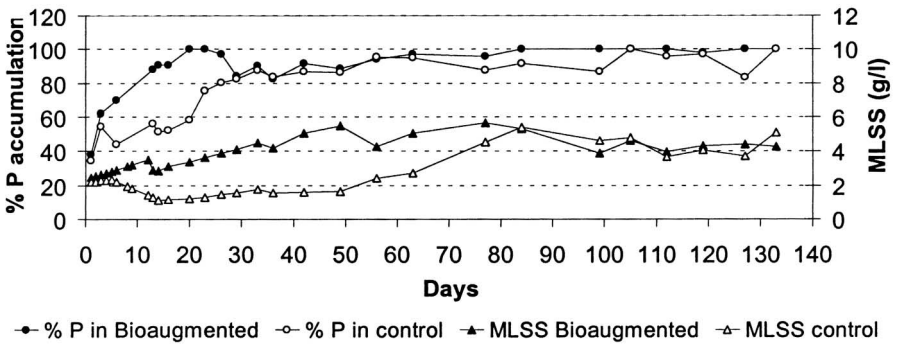

Figure 3 Evolution of the MLSS (mixed liquor suspended solids) and P accumulation in the bioaugmented and control reactors with time. T0 corresponds to the day of bioaugmentation

accumulate phosphate. The P-accumulating parent sludge showed the classical kinetics of carbon uptake and phosphorus release in the anaerobic stage, followed by the phosphorus accumulation in the aerobic stage, of conventional anaerobic-aerobic SBR sludge.

After bioaugmentation, the bioaugmented and control reactors were run with the same anaerobic-aerobic sequences as the parent P-accumulating SBR. The phosphorus removal yield of the reactors (Figure 3) was then estimated by calculating the percent of phosphorus accumulated during the aerobic stage of one cycle as follow: ((soluble P at the end of the anaerobic stage – soluble P at the end of the aerobic stage) * 100) / soluble P at the end of the anaerobic stage.

The bioaugmentation speeded up the start up of the reactor (Figure 3). The yield of phosphorus accumulation in the bioaugmented reactor increased regularly and reached 100% of the P present in the reactor after 20 days of running in anaerobic-aerobic conditions. Thereafter the yield stabilised rapidly and stayed above 85% until the end of the experiment. In this reactor, the carbon source was consumed in the anaerobic stage of the cycles from the beginning of the experiment and the biomass grew rapidly (Figure 3). The MLSS shift down from days 12 to 14 corresponds to the time where a complete consumption of carbon occurred in the anaerobic stage of the cycle (data not shown). At day 20, the reactor showed the classical C, N and P concentrations kinetics during a cycle (as in Figure 2 (right panel)).

In the control reactor however, the amount of phosphorus accumulated during a cycle increased slowly and was only of about 60% after 20 days (Figure 3). After 33 days, the yield stabilised between 80 and 95% of P accumulation and was comparable with the one of the bioaugmented reactor. The lag between both reactors can be explained by the decrease of the amount of MLSS in the control reactor during the first 14 days of the experiment (Figure 3). During this time the carbon source was consumed only during the aerobic stage

of the cycle. Days 14 and 29 correspond to the beginning and the total consumption of carbon during the anaerobic stage of the cycle, respectively. Classical C, N and P concentrations kinetics during a cycle were observed only at day 56 (data not shown).

We concluded that bioaugmentation speeded up the development of good phosphorus removal performances on the reactor by at least ten days. Moreover, the bioaugmented reactor always gave better and more stable phosphorus removal yields than the control reactor throughout the 130 days of experiment. These results are in agreement with the observations of Belia and Smith (1997) who speeded up the development of EBPR in a SBR from 58 days to 5 to 14 days by its bioaugmentation with 10% of previously acclimatised sludge or pure culture of *Acinetobacter*.

Evolution of the microbial communities of the reactors

The evolution of the microbial communities of the reactors was followed by PCR-SSCP. The PCR step allows a selective amplification of a highly variable region of the microorganisms' 16S rRNA genes which behave as a signature for the different microorganisms' species. From one reactor sample, it results in a mixture of DNA fragments which corresponds to the different species of microorganisms present in the reactor. These DNA fragments are then heat denaturated and separated by SSCP electrophoresis. By this technique single stranded DNA fragments are separated according to their secondary structure (conformation) which depends on their nucleotide sequence. Electrophoresis on an automated sequencer provides optimal band separation. The use of one fluorescently labelled PCR primer allows laser detection of only one single strand of the DNA and the use of an inner standard for reliable comparison of migration patterns between each sample. Results are given as electrophoregrams where any ecosystem can be represented by a profile of peaks where each peak corresponds to the major species of the ecosystem microorganisms.

The PCR-SSCP profiles of the microbial communities of the parent sludge are shown on the top of Figure 4. The microbial community of the non P-accumulating sludge at the time of its retrieval from the wastewater treatment plant (T_{-20}) was highly diverse since its profile presents an uninterrupted succession of peaks. However, during the 20 days of adaptation to the aerobic SBR and synthetic feeding conditions, this diversity seems to disappear to finally end up with only one extremely major peak at the time of the bioaugmentation (T_0). This observation can be explained by a simplification of the microbial community as a consequence of the running parameters and especially the unique carbon source of the synthetic feeding. It should be stressed however that this type of profile could result also from the outbreak of one species of the ecosystem. Other species of microorganisms would still be present in the reactor but at an undetectable level. They would be detected only when their relative proportion in the ecosystem increases (T_{29} to T_{105}).

The profile obtained from the microbial community of the parent P-accumulating reactor at the time of the bioaugmentation contains two major peaks and at least nine other distinguishable peaks (Figure 4).

Then the evolution of the control and bioaugmented reactor microbial communities were followed with time. A total of 22 profiles were analysed and a summary of the results is presented in Figure 4.

At T_0, both profiles of the control and the bioaugmented reactors are rigorously identical which indicates that the amount of microorganisms inoculated into the bioaugmented reactor was too low to be detectable. Then both communities evolved similarly toward an increase of the microorganisms' diversity. At T_{14}, the appearance of new peaks is detected on the left edge of the major parental peak. At T_{29}, both reactor profiles contain two major peaks which do not co-migrate with the initial parental peak which is not detectable any

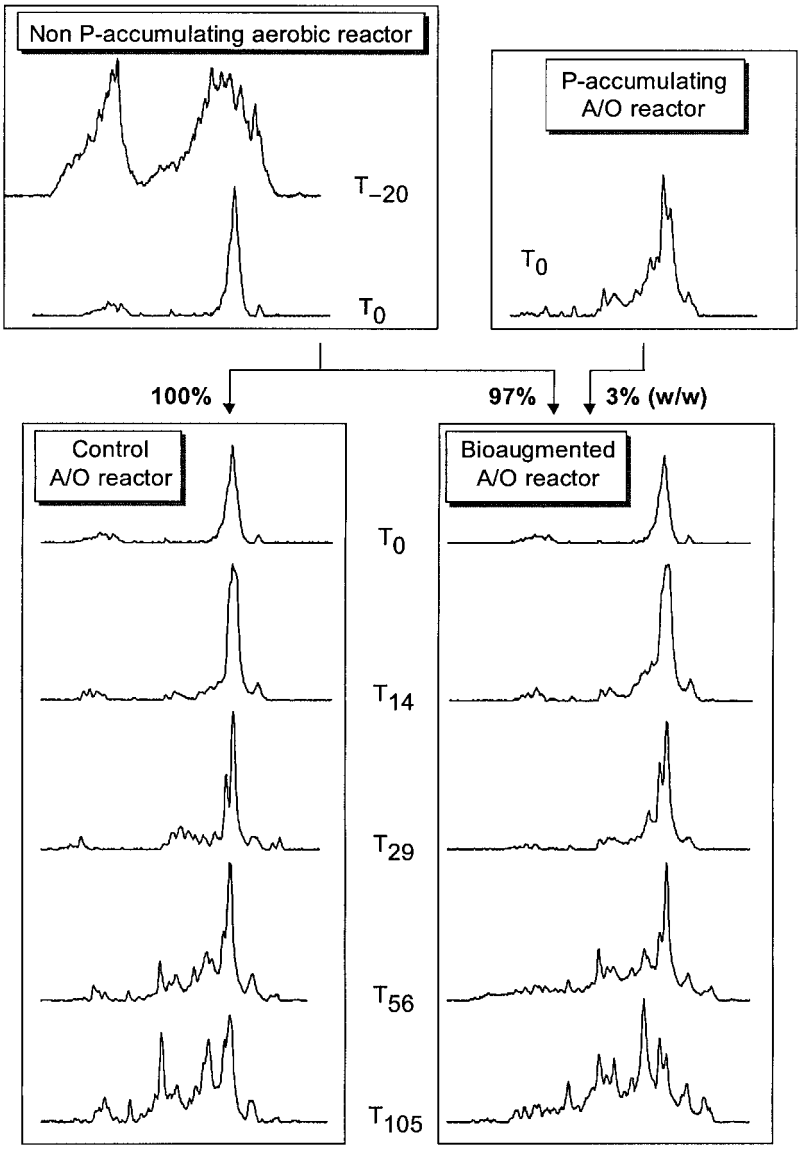

Figure 4 Microbial communities dynamics of the reactors throughout the experiment

more. Then between time T_{29} and the end of the experiment, the number of peaks increases constantly to end up with about 20 major distinguishable peaks.

The alignment of the profiles of the parent ecosystems with the P-accumulating ecosystems of the control and bioaugmented reactors at T_{105} is shown in Figure 5. It shows that six out of the eleven peaks of the inoculated P-accumulating parent ecosystem co-migrate with peaks detectable within the bioaugmented reactor ecosystem profile. It would suggest that the inoculated microorganisms established in the reactor. However, all these peaks are also found in the control reactor profile and a total of 12 peaks out of about 20 seems to be common between the bioaugmented and the control reactor profiles. These results are in contradiction to the previous one and suggest that indeed the P-accumulating communities which developed in the control and bioaugmented reactors are very similar. The major peak of the parent non P-accumulating sludge does not co-migrate with any of the other reactors peaks (Figure 5).

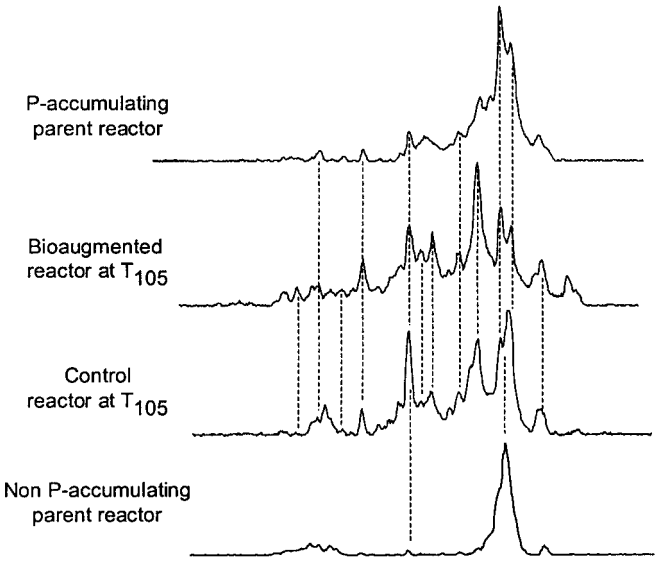

Figure 5 Comparison of the three P-accumulating ecosystems and non P-accumulating ecosystem

According to these results, the fate of the microorganisms inoculated during the bioaugmentation is still unclear. The fact that the three P-accumulating ecosystems (parent, control and bioaugmented reactors) seem to exhibit similar microbial communities, while they have all been seeded with different ecosystems, suggests that the inoculated microorganisms did not established in the bioaugmented reactor. In this case, bioaugmentation would have not resulted in a true seeding of the reactor, but would have been a transient state which compensated for the slow acclimatisation of the endogenous P-accumulating microbial community. This latter hypothesis seems supported by the shape of the yield curves shown in Figure 3. In general, it would suggest that the success of bioremediation might depend more on our ability to drive the endogenous ecosystems than on the isolation of very specialised microorganisms. A similar conclusion had been proposed by Wilderer *et al.* (1991) after the bioaugmentation of a SBR with a *Pseudomonas putida* strain able to degrade 3-Chlorobenzoate.

Conclusions

The bioaugmentation of a non P-accumulating SBR sludge by 3% w/w of a previously acclimatised P-accumulating sludge speeded up the reactor start up by at least ten days. The effect on the phosphorus removal yield of the reactor was clearly visible. However, the fate of the inoculated community in the bioaugmented reactor is still unclear. The observation that the P-accumulating sludge of the parent, control and bioaugmented reactors are very similar suggests that the microorganisms inoculated for the bioaugmentation did not truly established in the reactor. They probably stayed in the reactor and performed phosphorus removal until they were overgrown by the endogenous P-accumulating microbial community. The identification of the microorganisms corresponding to the peaks of the parent and final ecosystems profiles is underway.

Acknowledgements

The authors wish to acknowledge the contributions made by Dominique Patureau, Emmanuelle Zumstein, Marion Leclerc, Céline Delbès and Théodore Bouchez for useful discussion of this work.

References

Amann, R. I., Ludwig, W. and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143–169.

Belia, E. and Smith, P.G. (1997). The bioaugmentation of sequencing batch reactor sludges for biological phosphorus removal. *Wat. Sci.Tech.*, **35** (1), 19–26.

Brosius, J., Dull, T., Sleeter, D. and Noller, H. (1981). Gene organisation and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.*, **148**, 107–127.

Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997). Molecular microbial diversity of an anaerobic digester determined by small-subunit rRNA sequences analysis. *Appl. Environ. Microbiol.*, **63**, 2802–2813.

Hebenbrock, K., Williams, P. and Karger, B. (1995). Single strand conformational polymorphism using capillary electrophoresis with two-dye laser -induced fluorescence detection. *Electrophoresis*, **16**, 1429–1436.

Hugenholtz, P, Goebel, B. and Pace, N. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.*, **180**(18), 4765–4774.

Kuba, T., Smolders, G., van Loosdrecht, M. and Heijnen, J.J. (1993). Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. *Wat. Sci. Tech.*, **27**(5–6), 241–252.

Kortstee, G., Appeldoorn, K., Bonting, C., van Niel, E and van Veen, H. (1994). Biology of polyphosphate-accumulating bacteria involved in enhanced biological phosphorus removal. *FEMS Microbiology Reviews*, **15**, 137–153.

Lee, D.H., Zo, Y.G. and Kim, S.J. (1996). Non-radioactive method to study genetic profiles of natural bacterial communities by PCR-Single-Strand-Conformation Polymorphism. *Appl. Environ. Microbiol.*, **62**, 3112–3120.

Rittmann, B. and Whiteman, R. (1994). Bioaugmentation: a coming of age. *Water Quality International*, **1**, 12–16.

Rustrian, E., Delgenes. J.P., Bernet, N. and Moletta, R. (1997). Nitrate reduction in acidogenic reactor: influence of wastewater COD/N–NO_3 ratio on denitrification and acidogenic activity. *Environ. Technology*, **18**, 309–315.

Stephenson, D. and Stephenson,T. (1992). Bioaugmentation for enhancing biological wastewater treatment. *Biotech. Adv.*, **10**, 549–559.

Stevens, N.C. (1989) The application of bioaugmentation to waste water treatment. *Int. Biodeterioration*, **25**, 87–95.

van Loosdrecht, M.C.M., Hooijmans, C.M., Brdjanovic, D. and Heijnen, J.J. (1997a). Biological phosphate removal processes. *Appl. Microbiol. Biotechnol.*, **48**, 289–296.

van Loosdrecht, M.C.M., Smolders, G.J., Kuba, T. and Heijnen, J.J. (1997b). Metabolism of micro-organisms responsible for enhanced biological phosphorus removal from wastewater. *Ant. van Leeuwenhoek*, **71**, 109–116.

Wilderer, P., Rubio, M. and Davids, L. (1991). Impact of the addition of pure cultures on the performances of mixed culture reactors. *Wat. Res.*, **25**(11), 1307–1313.

Zumstein, E., Godon, J.J. and Moletta, R. (2000).Examination of two years of community dynamics in an anaerobic bioreactor using fluorescence polymerase chain reaction (PCR) single-strand-conformation polymorphism analysis. *Environmental Microbiology*, **2**(1), 69–78.