Microbial community analysis of a full-scale membrane bioreactor treating industrial wastewater

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ABSTRACT

A Kubota™ submerged membrane bio-reactor was applied to treat wastewater from a sugar manufacturing industry. To achieve optimal results, fundamental and extended understanding of the microbiology is important. Fluorescence *in situ* hybridization was used to evaluate the microbial community present. The majority of cells visualized in the sludge flocs by staining with the DNA fluorochrome DAPI, hybridized strongly with a bacterial probe. Probes specific for the alpha-, beta-, and gamma-subclasses of proteobacteria and high G + C Gram positive bacteria were used to characterize the community structures by *in situ* hybridization. Sampling was carried out over 12 weeks and samples were fixed with 4% paraformaldehyde for gram positive organisms and ice cold ethanol for gram negative organisms. The activated sludge population usually constitutes about 80 to 90% of proteobacteria. However, in this study it was found that a relatively small amount of proteobacteria was present within the system. No positive hybridization signal was observed with any of the applied eubacterial family- level probes.

Key words | fluorescent *in situ* hybridisation, membrane bioreactor, population dynamics

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INTRODUCTION

Membrane systems have attracted much attention in wastewater and effluent treatment. However, only a few membrane systems can treat biological and solid loading in the effluent in a single process (Churchouse 1997). The Kubota™ submerged membrane bioreactor (SMBR) was developed by the Kubota Corporation in Japan. This was done to meet an increasing demand for a low maintenance treatment system capable of treating a wide range of effluents and subsequently allowing for on-site water reclamation with the effluent quality passing stringent discharge standards (Churchouse 1997). An industrious producer of sugar and prominent manufacturer of down-stream products in South Africa, introduced a Kubota™ Submerged Membrane Bio-Reactor to address their wastewater treatment problems. Sugar manufacturing requires large volumes of water for raw material cleaning, sugar extraction, furfural extraction, cooling and cleaning equipment. Thus the recycling of process wastewater is essential and the application of an SMBR proved feasible.

Some progress has been accomplished with regard to the process engineering. However for optimization and greater efficiency of the plant, fundamental and comprehensive knowledge of the microbial communities present is imperative. Thus far, such knowledge is limited. The elucidation of the bacterial community and their associated roles within the SMBR will allow for increased optimization of the plant. Fluorescent *in situ* hybridization is a unique molecular method that may be used for the identification of microorganisms within the SMBR. The problem the company faces with identification of the microorganisms in SMBR, is the inadequacy of microbiological equipment and expertise. FISH allows for the quick, simple and accurate detection of bacterial species without the need to culture. The application of the FISH technique is useful to

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determine the abundance of respective populations in microbial community samples.

FISH allows for the detection of three times more bacteria than plate counts (Amann 1995). At least 60%–90% of all cells present in activated sludge can be detected with DAPI, a fluorescence stain that binds strongly to DNA (Li *et al.* 2006). A molecular approach was selected since conventional analysis of microbial identification, of a plant of such a large scale is a tedious and daunting task. In addition, cultivation of bacteria from natural samples results in qualitative and quantitative discrepancies (Biol 2002). Bacteria, fungi and protozoa are the main role players in activated sludge. The species of microbes that exist within a system is directly related to the environmental conditions, process design and the mode of the plant operation.

The aim of this project was to identify the pre-dominant bacterial populations in the SMBR using FISH, to evaluate the different families of bacteria generated by the system and their impact on the operation of the plant.

MATERIALS AND METHODS

SMBR

A full-scale Kubota™ SMBR was employed to treat wastewater emerging from a sugar manufacturing industry. The SMBR was filled to capacity (5,000 m^3) with effluent from an Azeotrophe column and influent that diverted from a conventional activated sludge plant. The Azeotrophe column in the plant was responsible for the separation of furfural from the furfural condensate which comprised of 3% furfural, 1% acetic acid, sugar-cane wax and formic acid which are all by-products of sugar manufacturing. The conventional plant treated sewage and waste from the entire factory together with the sewage from the surrounding residential area. Previously the SMBR plant was operated using the Azeotrophe column as a primary source of influent. The extreme temperature range within the SMBR gives rise to thermophilic microorganisms. During a plant shutdown the Azeotrophe column failed to supply effluent to the SMBR. Consequently, the temperature decreased and mesophilic microorganisms dominated and the plant was unstable and thus resulted in poor performance. In order to regain maximum efficiency a period of 4–5 days was required. Thus, effluent from both the Azeotrophe and the conventional treatment plant were introduced to maintain favourable conditions. The Kubota™ SMBR plant has been in continuous operation since 2005. The SMBR was governed by a range of process parameters. It operated at an influent COD range of 50–6,000 mg L^{-1}. The MLSS of the SMBR was maintained between 9,323 and 15,662 mg L^{-1}.

Sampling

The SMBR was sampled weekly throughout a 12 week period. The samples were immediately fixed in 4% para-formaldehyde (w/v) and 95% ethanol for FISH analysis (Amann 1995). All other analyses were performed subsequent to sampling.

Parameter measurement

On the day of sampling, various parameters were measured (pH, chemical oxygen demand {COD}, Phosphorous {P}, Total Nitrogen {TKN} and Ammonia). Temperature, pH and dissolved oxygen readings within the SMBR were obtained using the automated Human machine interface (HMI) equipped with a PT 1,000 probe.

Oligonucleotide probes

Probes were selected to cover the major subclasses of eubacteria that are found to be pre-dominant in activated sludge and SMBR's. The oligonucleotide probes were synthesized according to specification and were labeled with fluorescein at the 5′ end (Roche' Products (Pty) Ltd, South Africa). Heavy Teflon coated slides were pre-treated with a 10% v/v solution of poly-L-lysine for 15 minutes. The samples were then immobilised onto the slides. The probes (Table 1) were applied to all samples.

In situ hybridization

Hybridisation was carried out in 50-ml polypropylene tubes that served as chambers for whole-cell hybridization. The tubes were iso-tonically equilibrated with hybridization

Table 1 | Probes and stringencies

Probe name	Probe sequence (5′–3′)	Specificity	% FA*	NaCl (mol L^{-1})†	Reference
EUB338	GCT GCC TCC CGT AGG AGT	Bacteria	20	0.19	Daims *et al.* (1999)
EUB338-II	GCA GCC ACC CGT AGG TGT	Planctomycetes	20	0.19	Daims *et al.* (1999)
EUB338-III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	20	0.19	Daims *et al.* (1999)
NONEUB	ACT CCT ACG GGA GGC AGC	Control probe	20	0.19	Wallner *et al.* (1993)
BET42a	GCC TTC CCA CTT CGT TT	β-proteobacteria	35	0.08	Yeates *et al.* (2003)
GAM42a	GCC TTC CCA CAT CGT TT	γ-proteobacteria	35	0.08	Yeates (2003)
HGC69a	TAT AGT TAC CAC CGC CGT	Actinobacteria	25	0.15	Roller *et al.* (1994)
LGC354A	TGG AAG ATT CCC TAC TGC	Firmicutes	35	0.08	Meier *et al.* (1999)
LGC354B	CGG AAG ATT CCC TAC TGC	Firmicutes	35	0.08	Meier *et al.* (1999)
ALF1b	GGT AAG GTT CTG CGC GTT	α-proteobacteria	20	0.19	Wagner *et al.* (1993)

*Percentage of formamide (%$^{v}/_{v}$) in the hybridization buffer.
†Molarity of Sodium chloride in the wash buffer.

buffer as outlined by Amann (1995). A 10 μl mix of hybridization buffer {0.9 mol^{-1} NaCl, 0.01% SDS, 20 mmol l^{-1} Tris/HCl, pH 7.2 and X% formamide (v/v)} and probe (50 ng) was applied to each dehydrated spot. The concentration of formamide in the hybridization buffers, for each probe is listed in Table 1. The slides were hybridized at 46°C for 2 hours. Probes EUB338, EUB338II and EUB III were used in a mixture (Yeates *et al.* 2003). The probes BET42a and GAM42a were hybridized simultaneously to increase the specificity due to a mismatch between the target sequences of these probes at position 1,033 (Yeates *et al.* 2003). After hybridization the unbound or excess probe was washed off with a buffer (20 mmol l^{-1} Tris/HCl, 0.01% SDS, 5 mmol^{-1} EDTA and Y M NaCl) pre-warmed to 48°C. The slides were then transferred to a polypropylene tube filled with pre-warmed wash buffer and incubated at 48°C for 20 minutes. The buffer was washed off with deionized water and air dried. The concentration of sodium chloride for each probe is listed in Table 1. The slides were then stained with 10 μl of DAPI (0.25 μg mL^{-1}) for 10 minutes, then rinsed with deionised water and air dried. VECTASHIELD® was then added as an anti-fading agent.

Image analysis

The slides were then viewed using a Zeiss (Germany) Axiolab HBO50/AC microscope (Carl Zeiss, Göttingen, Germany) fitted for epifluorescence and Zeiss filter sets (Filter set number: 61 for tetramethylrhodamine 5-isothiocyanate {TRITC}and 45 for 4′,6′-diamidino-2-phenylindole {DAPI}). Images were captured using a Zeiss AxioCam MRc camera. Image analysis was carried out using the Zeiss AxioVision Release 4.6 (12-2006) imaging system. Thirty random fields were selected for each hybridisation.

RESULTS AND DISCUSSION

The microbial community structure of the SMBR was analysed using FISH with oligonucleotide probes (Amann 1995). Prior to hybridisation, pre-treatment of the samples was optimised to ensure cell dispersion for efficient probe hybridisation. These probes were selected based on published literature which states that these subclasses of bacteria are present in conventional activated sludge processes and membrane bioreactors (Li *et al.* 2006).

All samples were initially stained with DAPI to analyze the activated sludge. DAPI binds to anything containing DNA, thus providing an idea of the content of organisms within the activated sludge. It was observed that the sludge was very compact and impermeable for hybridization with the probes. Thus additional pre-treatment was required. Sonication proved to be an efficient method for dispersing sludge flocs to expose the cells for hybridization. This method was optimised and the ideal sonication parameters were concluded to be 8 watts for 8 minutes.

Following hybridisation with the Eubacterial family level probes, the images were analysed. Very few bacteria

were observed when the samples were stained with DAPI (Figure 4a). In some samples no bacteria was observed. Additionally, the Eubacterial population, specifically the proteobacteria, was observed in small numbers (Figure 4b). When compared to activated sludge where eubacteria particularly α, β and γ proteobacteria predominate, the current findings showed a comparatively low eubacterial population (Akiyama *et al.* 2000). These findings do not support literature that states that α, β and γ proteobacteria predominate in MBR systems (Li *et al.* 2006). Very poor and in some instances no positive hybridization signals could observed and thus no quantification of cells could be conducted. These findings were also observed under DAPI. Large ovoid cells were observed to be relatively abundant. Microscopic analysis further revealed that these cells resembled *Chroococcus sp.*, a genus of Cyanobacteria. There was evidence of non-specific binding (Figure 4b circled region).

Despite the probes being specific for eubacteria, binding occurred to an unknown substance within the sludge exhibiting fluorescence. A very small bacterial population was observed using DAPI however no positive hybridization signal was obtained with any of the applied probes. This could be attributed to the possibility that the population observed did not belong to the domain eubacteria.

The pH of the SMBR dropped to 5.2 during week two (Figure 1). During this time, industrial sugar washings were being performed and this may have fermented which resulted in a lower pH. Caustic soda was added to increase the alkalinity which stabilized the pH. The temperature and DO had little effect on the overall microbial population. During week three there was a drastic decrease in the total nitrogen (Figure 2). During this period an abundance of the filamentous organism was observed. Fungi are often associated with low nutrient levels (Guest & Smith 2002). The organism presumed to be the Cyanobacterium, *Chroococcus sp.*, is known to proliferate in the presence of high phosphorus concentrations. The phosphorus levels within the SMBR were very high (Figure 2). It was also observed that ammonia and TKN were variable (Figure 2). These variations can be associated with the high MLSS in the SMBR (Figure 3) (Churchouse 1997). To ensure efficient operation of SMBR's, the bioreactors are usually operated with high biomass concentrations of 8–15 g MLVSS L^{-1} (Sridang *et al.* 2008). These high concentrations induce low food to microorganism ratios (low F/M ratio) and are thus able to reduce sludge production and allow for high solid retention times (Ben Aim & Semmens 2002). These factors favour organisms that have poor settling characteristics,

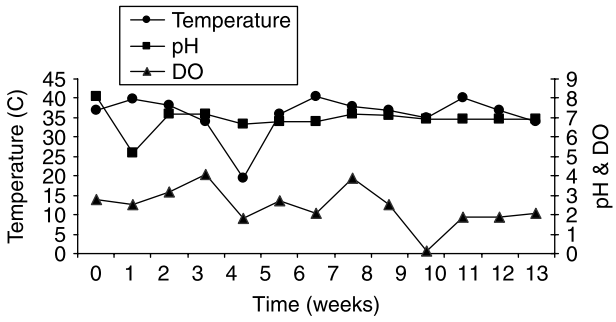

Figure 1 | Graphical representation of pH, temperature and dissolved oxygen (DO) in the SMBR.

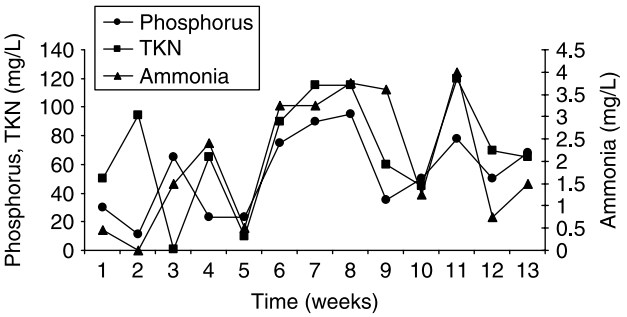

Figure 2 | Graphical representation of phosphorus, TKN and ammonia in the SMBR.

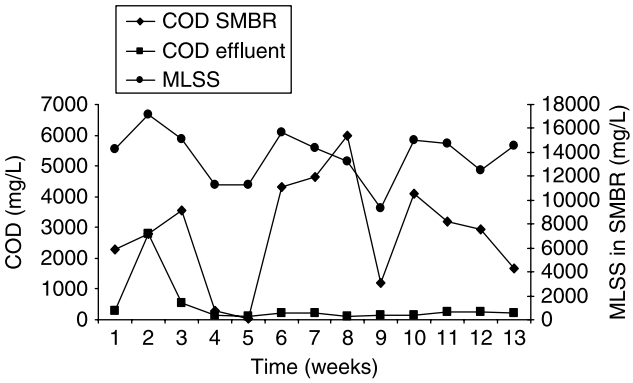

Figure 3 | Graphical representation of COD and MLSS in the SMBR.

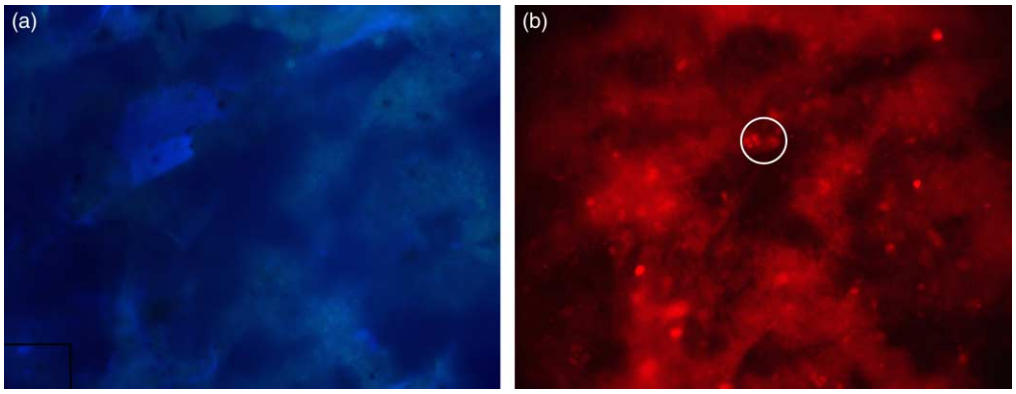

Figure 4 | (a) and (b): Micrographs of SMBR samples stained with DAPI (a) and hybridized with tetramethylrhodamine 5-isothiocyanate (b).

longer generation times and those that are able to degrade complex substrates (Guest & Smith 2002; Rosenberger *et al.* 2002). As s result of this, higher organisms proliferate and may feed on lower organisms as was evident in this study. The overall COD removal efficiency of the plant was good (Figure 3). This cannot be attributed to the proteobacteria, as they were not the dominant organisms in the SMBR. The results of our findings suggest that Cyanobacteria and fungi were playing a greater role than previously believed (Figure 4).

CONCLUSIONS

The SMBR plant demonstrated good COD removal capacities. These removal efficiencies are usually attributed to Eubacteria but more specifically proteobacteria. However, the results of this study contradict these theories, since these respective populations were present in very minute quantities. Fungi have been implicated in higher degradation rates and have a greater ability to degrade cellulose, hemicellulose and lignin materials which are major constituents of sugar cane (Guest & Smith 2002). The composition and heterogeneity of the dominant microbial population and their influence on process performance seem to be dependent on a variety of factors particularly influent composition. Thus the efficient removal capacities could be attributed to the abundance of higher organisms, which are the Cyanobacteria and filamentous fungi. However, a smaller population of bacteria was observed under DAPI but was not proteobacteria. This population could possibly belong to the domain Archae. Further research using a more comprehensive set of oligonucleotide probes needs to be conducted. Additionally the use of PCR-DGGE would allow for the construction of a profile to monitor any possible population shifts and subsequent RT-PCR with specific primers to quantify the pre-dominant population.

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