Effect of carrier fill ratio on biofilm properties and performance of a hybrid fixed-film bioreactor treating coal gasification wastewater for the removal of COD, phenols and ammonia-nitrogen

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Abstract

The purpose of this study was to determine the effect different biofilm carrier filling ratios would have on biofilm morphology and activity and bacterial diversity in a hybrid fixed-film bioreactor treating high strength coal gasification wastewater (CGWW) for the removal of chemical oxygen demand (COD), phenols and ammonia-nitrogen. Results showed that a carrier fill of 70% formed a 'compact' biofilm, a 50% fill formed a 'rippling' biofilm and a 30% fill formed a 'porous' biofilm. The highest microbial activity was obtained with a 50% carrier fill supporting a relatively thin biofilm. The highest level of biofilm bound metals were aluminium, silicon, calcium and iron in the 'compact' biofilm; nitrogen, magnesium, chloride, sodium and potassium in the 'rippling' biofilm, and copper in the 'porous' biofilm. The bioreactor improved the quality of the CGWW by removing 49% and 78% of the COD and phenols, respectively. However, no significant amount of ammonia-nitrogen was removed since nitrification did not take place due to heterotrophic bacteria out-competing autotrophic nitrifying bacteria in the biofilm. The dominant heterotrophic genera identified for all three carrier filling ratios were *Thauera, Pseudaminobacter, Pseudomonas* and *Diaphorobacter*.

Key words : Anox-Kaldness, biofilm, next-generation sequencing, nitrification, pollutants, *Thauera*

Introduction

Coal gasification wastewater (CGWW) is generated in the process of coal gas purification and contains large quantities of very complex organic and inorganic pollutants. Main pollutants in the wastewater are various phenolic compounds, poly-nuclear aromatic hydrocarbons, ammonia, thiocyanate, cyanide (Jia *et al.* 2016), mono- and polycyclic nitrogen-containing aromatics, oxygen- and sulphur-containing heterocyclic compounds, sulphides, fatty acids and carbon dioxide (Gai *et al.* 2008). Thus, CGWW is considered to be very toxic, mutative, carcinogenic, teratogenic and may produce long-term adverse effects in the environment and on human health (Wang *et al.* 2013). Phenols have been reported to be carcinogenic by the World Health Organization and a limit of 1 µg/L has been set to regulate phenol concentrations in drinking waters (Pishgar *et al.* 2012).

There are intensive driving forces towards sustainable treatment and utilisation of wastewater. Reclamation of wastewater for recycling is growing rapidly and the demand for higher effluent quality is also increasing in a number of industries generating CGWW, such as the petro-chemical, coking, iron and steel refineries. The treatment of CGWW can be sustainable if suitable technologies can be applied that operate using less energy, lower operating costs and lower investment (Ji *et al.* 2015).

Biological treatment such as conventional activated sludge, sequencing batch reactor, and anoxic–aerobic and anaerobic–anoxic–aerobic processes, have been widely used to treat CGWW after being pre-treated by the processes of ammonia stripping and phenol solvent extraction to reduce the concentration of toxic compounds (Li & Han 2013). These biological processes have been shown to be more cost-effective, practical and highly reliable since there is minimal formation of toxic by-products and complete mineralisation of organic contaminants to water and carbon dioxide (Pishgar *et al.* 2012). However, due to the

presence of refractory and toxic compounds, the growth of nitrifying bacteria and specialised microbes in the aeration basin is restrained and/or these micro-organisms are washed out since they have low specific growth rates (Wang *et al.* 2013). Thus, the formation of a biofilm on the carrier would prevent the nitrifiers being washed out of the bioreactor.

In biofilm processes, such as hybrid fixed-film bioreactors, the microbial biomass is attached as a film onto the surface of an inert and solid medium called the carrier. These carriers provide a large surface area for the growth of the micro-organisms. Removal of the contaminants is achieved when the CGWW is brought into contact with the biofilm (Pal & Kumar 2014). Biofilm processes have been proven to be one of the most effective and competitive alternatives for the treatment of high strength CGWW without the problems associated with conventional activated sludge processes, such as low process efficiency, poor sludge settle ability and unstable system performance (Zhou *et al.* 2014). Hybrid fixed-film bioreactors have been shown to be more stable during fluctuating loading conditions and fixed micro-organisms are more resistant to changes in pH, nutrients and toxic substances (Wang *et al.* 2013).

The main objective of this study was to evaluate the effect of different carrier media fill ratios on the biofilm morphology and activities and bacterial diversity as well as the removal of chemical oxygen demand (COD), phenols and ammonia-nitrogen using a pilot plant (1,000 L) situated at a refinery. The hybrid fixed-film bioreactor was fed continuously with real-time CGWW. It was hypothesised that the biofilm formed on the carriers would contain nitrifying bacteria for nitrification and the suspended biomass would contain heterotrophic bacteria for the removal of organic carbon (COD and phenols). The hybrid fixed-film bioreactor was operated at a relatively high oxygen concentration (5–6 mg/L) and the CGWW had a relatively high chemical oxygen demand/ammonium-nitrogen (COD/NH$_4$-N) ratio.

Materials and methods

Pilot plant design and operating parameters

A stainless steel hybrid fixed-film pilot bioreactor (1,000 L) with three in series aeration compartments (Zone 1, Zone 2, and Zone 3) containing acclimatised biomass (>36 months) was used in this study. The bioreactor was linked directly to a continuous real-time feed stream from the plant. The CGWW was diluted to 33% to reduce the toxicity towards the biomass and to maintain an influent COD concentration around 2,000 mg/L. Dilution was one part CGWW to two parts recycled sludge. The reactor was operated at 36 °C ± 1 °C and pH controlled at 7.5 ± 0.5. Polyethylene biofilm carrier (density = 0.95 g/mL) (Anox-Kaldnes™ K1, Veolia Water Technologies, Sweden) filling ratio (based on volume) for each aeration zone was approximately 70, 50 and 30%, respectively. Food to mass ratio (kg COD/kg MLSS.d; MLSS: mixed liquor suspended solids) was maintained at ≤0.45 of the biodegradable COD fraction, the organic loading rate was maintained at the design capacity of ≤3.5 (kg COD/m^3.d), sludge retention time was approximately 18 days and the hydraulic retention time was approximately 33 h. The dissolved oxygen level was maintained between 5 and 6 mg/L using air diffusers fixed at the bottom of the respective aeration zones. Aeration also provided for hydraulic circulation. The clarifier up-flow velocity was controlled at ≤0.3 m/h.

Biomass activity

The oxygen uptake rate (OUR) of the biofilm was measured on-site using a Hach model HQ40d multimeter with an LDO 101 probe. A biochemical oxygen demand sample bottle (300 mL) was filled with 150 mL effluent and topped up with 150 mL AnoxKaldnes K1 carrier media taken from the respective zones, to simulate a 50% media fill fraction (by volume) for comparative biofilm activities.

Biomass weight

The carriers (200 per aeration zone) were gently rinsed with water (3×), then dried at 105 °C for 24 h. The dried carriers were cooled in a desiccator before weighing. The attached biomass was then removed by soaking the carriers in 0.25 N NaOH for 24 h. The alkali wash was repeated with sonication for 90 min. The carriers were then rinsed very well with water and dried for 24 h at 105 °C and re-weighed. The difference in weight was used to determine the amount of biomass on the carriers.

Biomass thickness and morphology

For scanning electron microscopy (SEM), the carrier samples were allowed to air dry. Pieces of the biofilm were removed and mounted with adhesive carbon tape on aluminium stubs in the upright position. These were viewed in a Zeiss Ultra Plus field emission scanning electron microscope (FE-SEM) (Zeiss, Germany) at 10 kV. A point to point

measurement was used to determine biofilm thickness using SmartSEM (Zeiss, Germany) software which was calibrated.

For scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDX), the samples were mounted with adhesive carbon tape on aluminium stubs. These were viewed with a Zeiss Ultra Plus field emission gun-scanning electron microscope (Zeiss, Germany) at 20 kV. Images were digitally captured using the calibrated SmartSEM software (Zeiss, Germany). EDX analyses were performed using the AZtecEnergy) software (Oxford Instruments, UK) linked to an Oxford detector (Oxford Instruments, UK) with an 80 mm^2 detection window. Imaging was performed using an in-lens detector for surface structure and an SE2 (Everhart-Thornley) detector for topography (Zeiss, Germany).

Chemical analyses

All samples were filtered through a 0.45 μm filter before testing. Soluble COD, phenols, nitrates, nitrites and ammonia-nitrogen were determined using *Standard Methods* (APHA 2012).

High throughput Illumina next-generation sequencing

Bacterial isolates were characterised by sequencing the 16S rDNA using the ZR Fungal/Bacterial DNA MiniPrep™ test kit (Zymo Research Corp, USA). The universal primers 16S-27F (target 16S rDNA sequence (5′ to 3′) AGAGTTTGATCMTGGCTCAG) and 16S 1492R (target 16S rDNA sequence (5′ to 3′) CGGTTACCTTGTTACGATT) were used to amplify the 16S target region using DreamTaq™ DNA polymerase (Thermo Scientific™, USA) and the primers were gel extracted using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) and sequenced in the forward and reverse directions on the ABI PRISM 3500xl Genetic Analyser (Applied Biosystems®, USA). For sequencing analyses, polymerase chain reaction products were purified using the ZR-96 DNA Sequencing Clean-up Kit™ and the purified products were analysed using the CLC Main Genomics Workbench v.7.0.3 bioinformatics software (CLCBio, Qiangen, Denmark) followed by a BLAST genome search on the most recent NCBI GenBank® database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sampling

Grab samples of the carriers and suspended biomass were taken from each zone on a weekly basis over a 6 month period and analysed on-site. Composite samples of the feed and effluent were taken on a daily basis, at a rate of 50 mL/h over a 24 h period, and analysed on-site. Samples for biofilm studies and next-generation sequencing (NGS) analyses were chilled to 4 °C and transported in a coolerbox to the designated laboratory for analysis within 24 h.

Statistical analysis

All samples for analysis were obtained at steady state conditions. Microsoft 7 Excel™ was used to determine the average values, standard deviations and *P*-values. Significance level: $\alpha = 0.05$. If $P \leq 0.05$, then difference was significant.

Results and Discussion

Removal of phenols, COD and ammonia-nitrogen

Phenol removal

The phenols in the feed ranged between 38 and 90 mg/L with an average of 70 ± 20 mg/L and the phenols in the effluent ranged between 6 and 34 mg/L with an average of 15 ± 8 mg/L. The removal of phenols ranged between 62 and 93% with an average of 78 ± 9%.

COD removal

The COD in the feed ranged between 1,400 and 1,980 mg/L with an average of $1,750 \pm 170$ mg/L and the COD in the effluent ranged between 480 and 1,170 mg/L with an average of 870 ± 80 mg/L. The removal of COD ranged between 37 and 62% with an average of 49 ± 10%.

Ammonia-nitrogen removal

The ammonia-nitrogen in the feed ranged between 166 and 684 mg/L with an average of 384 ± 100 mg/L and the ammonia-nitrogen in the effluent ranged between 200 and 730 mg/L with an average of 377 ± 95 mg/L. The removal of ammonia-nitrogen ranged between 2 and 46% with an average of 15 ± 14%. The reduction in ammonium-nitrogen was due to evaporative 'stripping' during aeration and not due to nitrification since there was no significant difference ($P \leq 0.05$) between the average influent nitrate-nitrogen (NO$_3$-N) (5.61 mg/L) and the average effluent NO$_3$-N (5.67 mg/L).

Biofilm properties

The OUR for Zone 1 was higher than in Zone 2 and Zone 3 (Table 1), However, the oxygen requirement in terms of biomass (mg O_2/mm^2) was 2.18, 15.43 and 2.66 for the respective zones, which implies the biofilm microbial consortium was more active in Zone 2 than in Zone 1 and Zone 3. The oxygen requirement in terms of biofilm thickness (mg O_2/µm) was 1.22, 5.46 and 5.03, respectively which implies the biofilm microbial consortium was more active in 'thinner' biofilms (Zone 2 and Zone 3) than in thicker biofilms (Zone 1). This was due to the higher number of active cells in relation to the biofilm extracellular polymeric substance (EPS) (Andersson 2009).

The biofilm of Zone 1 differed significantly ($P \leq 0.05$) from Zone 2 and Zone 3. The biofilm in Zone 1 was thicker than in Zone 2 and Zone 3 due to the higher COD loading rate (Karizmeh *et al.* 2014). Higher electrolyte (conductivity, osmolarity) and nutrient concentrations promote deeper substrate penetration into the biofilm as well as greater cell reproduction and thus thicker biofilm (Gerardi 2002). The biomass volume in Zone 1 was higher than in Zone 2

and Zone 3. Shear stresses on the biofilm surface will also affect biofilm thickness and structure.

Biofilm morphology and effect of metals and ions

Biofilm morphology

SEM analyses indicated the biofilm morphology to be relatively 'compact' for Zone 1 (Figure 1), 'rippling' for Zone 2 (Figure 2) and 'porous' for Zone 3 (Figure 3). EDX analyses of the biofilm layers indicated the presence of carbon, oxygen, sodium, magnesium, aluminium, silicon, phosphorus, sulphur, chloride, potassium, calcium, nitrogen, copper and iron in all three aeration zones. The levels of carbon, oxygen, phosphorus and sulphur present in the biofilm layers were very similar. Levels of aluminium, silicon, calcium and iron were highest in Zone 1 with nitrogen, magnesium, chloride, sodium and potassium highest in Zone 2, and, copper highest in Zone 3. Therefore, the cell clusters, interstitial voids and channels, and the mass transfer properties of nutrients will differ between the respective biofilms (Picioreanu *et al.* 2001).

Effect of metals and ions on biofilm morphology

EPS are anionic and act as an ion exchange resin where divalent bridges can be formed or broken due to relative concentrations of monovalent and divalent cations competing for negative sites. The presence of divalent cations influences biofilm formation directly through its effect on electrostatic interactions, and indirectly via physiologically

Table 1 | Biofilm properties measured at different carrier filling ratios

Parameters measured	Zone 1	Zone 2	Zone 3
Biomass carrier filling ratio (%)	70	50	30
OUR (mg O_2/L·h)	62.6	33.8	33.7
Average biofilm thickness (µm)	51.5	6.19	6.61
Average biomass (mg/mm^2) dry basis	29.8	2.19	6.89

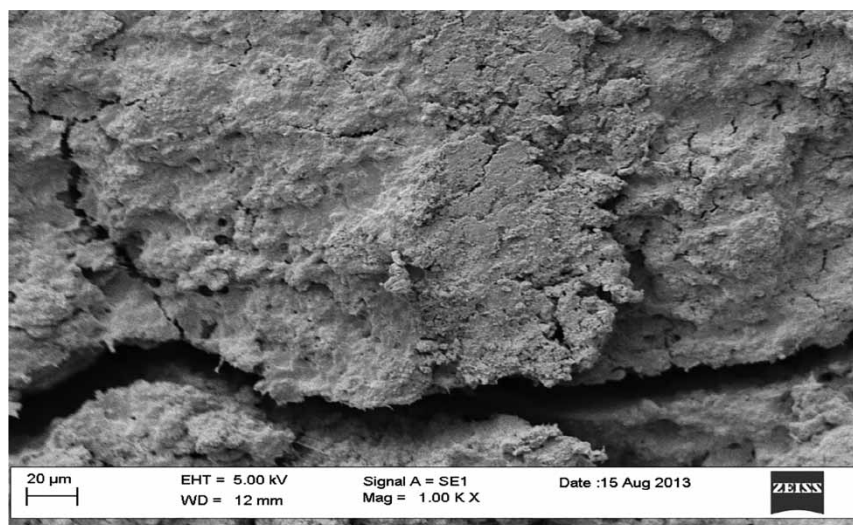| 20 µm	EHT = 5.00 kV	Signal A = SE1	Date :15 Aug 2013	ZEISS
	WD = 12 mm	Mag = 1.00 K X		

Figure 1 | Scanning electron microscopy of the biofilm in Aeration Zone 1.
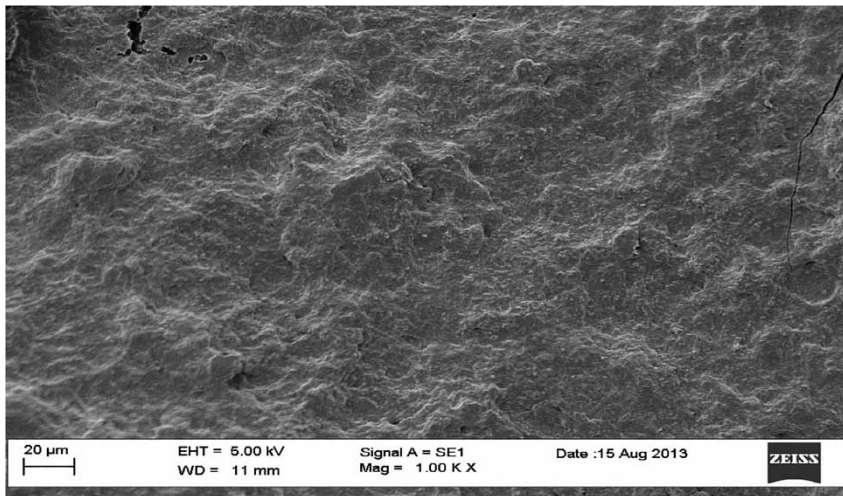
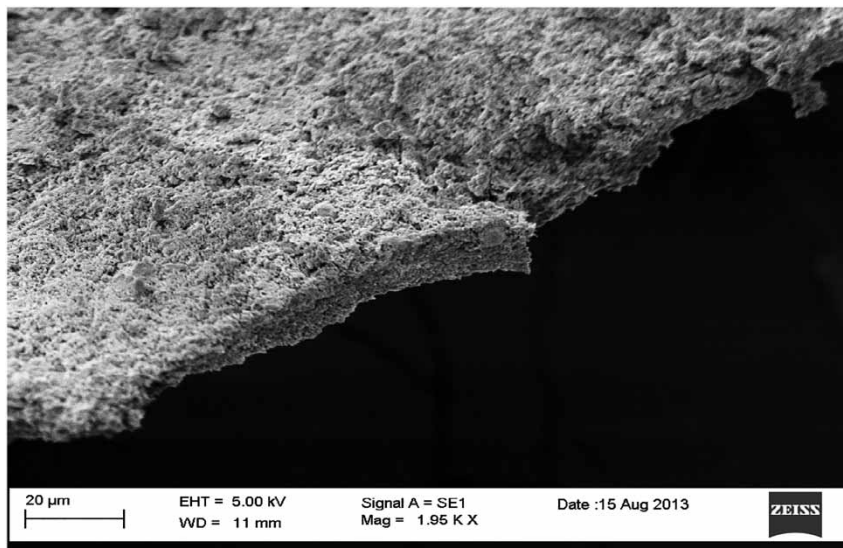**Figure 2** | Scanning electron microscopy of the biofilm in Aeration Zone 2.

Figure 3 | Scanning electron microscopy of the biofilm in Aeration Zone 3.

dependent attachment processes by acting as cellular cations and enzyme cofactors (Song & Leff 2006; Todar 2006). Calcium and phosphorus absorb and accumulate in the biofilm as insoluble compounds, rendering the biofilm highly resistant to detachment. Calcium ions act as cross-linkers and make the EPS more proteinaceous (thinner) (Revanuru & Mishra 2011). Sulphur is required for protein synthesis, phosphorus for nucleic acids and metabolic cofactors, and nitrogen for proteins, some sugars and nucleic acids. Silicon and aluminium increase biofilm resistance to toxic shocks, and the presence of iron and sulphur contribute to the aggregation of biomass. Potassium, magnesium,

sodium, calcium, iron and chlorides are macronutrients required for membrane stabilisation (Hu *et al.* 2013).

Nitrification and bacterial identifications

Nitrification bacteria

Autotrophic bacteria (family of *Nitrobacteraceae*) such as ammonia-oxidising bacteria (AOB) (*Nitrosomonas, Nitrosospira, Nitrosococcus*) and nitrite-oxidising bacteria (NOB) (*Nitrospira, Nitrobacter, Nitrospina*) are required for nitrification to take place (Andersson 2009). Zhang

et al. (3201) reported *Nitrosomonas* to be dominant in high ammonia environments, *Nitrosospira* to be dominant in low ammonia environments, *Nitrobacter* to be dominant in high substrate availability conditions and *Nitrospira* to be dominant during starved conditions. Optimal temperature for AOB ranges between 30 °C and 35 °C, and 35 °C t o 40 °C for NOB (Shore *et al.* 2012). This indicates that *Nitro-somonas* and *Nitrobacter* should be present within the deeper sections of the biofilm formed on the carriers.

Bacterial identifications (biofilm)

NGS indicated that the dominant kingdom classification for all the biofilms was *Bacteria*. The dominant phyla were *Proteobacteria*, *Firmicutes* and *Bacteriodetes*. These phyla were also reported by Meng *et al.* (5201) as being present in coking wastewater. The dominant classes were *Beta-proteo-bacteria*, *Alpha-proteobacteria*, *Gamma-proteobacteria;* the dominant orders were *Rhizobiales, Rhodocyclales and Burkholeriales*; the dominant families were *Rhodocyclaceae*, *Alcaligenaceae* and *Phyllobacteriaceae*. The closest genera and species match hits, using the BLASTN genome (≥99% homology) search on the most recent NCBI GenBank® data-base (http://blast.ncbi.nlm.nih.gov/Blast.cgi), were *Thauera butanivorans* (NR_040797.1), *Pseudaminobacter salicycla-toxidans* (NR_028710.1)*, Pseudomonas aeruginosa* (AF440523) and *Diaphorobacter nitroreducens* (AB07856.1). The biofilm morphology and activity for each aeration zone differed; however, the population remained fairly simi-lar. No autotrophic nitrifying bacteria developed in the deep dense sections of the respective biofilms. The biofilms can thus be regarded as consisting of heterotrophic bacteria possibly having nitrification capabilities under ideal bio-reactor conditions since Ge *et al.* (5201) reported *Diaphorobacter* sp. PD-7 strain as being capable of phenol degradation, heterotrophic nitrification and aerobic denitri-fication in activated sludge from a coking wastewater plant.

Conclusions

The main conclusions are as follows:

- Bioreactor hydrodynamics and biofilm characteristics such as activity, density, diversity and structure affected the performance of a hybrid fixed-film bioreactor. Thus, control of the biofilm structure is an important require-ment for stable bioreactor performance since the biofilm controls the rate of mass transfer of nutrients

and substrate to the microbial community within the bio-film (autotrophic nitrifiers) and on the surface of the biofilm (heterotrophs).
- Nitrification and carbon removal did not take place under high oxygen concentration since the autotrophic nitrifying bacteria in the heterogeneous biofilm were out-competed by the heterotrophic bacteria. The biofilm thickness was not sufficient (thin) to support autotrophic nitrifiers. However, the biofilm did support possible het-erotrophic nitrifying bacteria.
- The use of a culture-independent method such as high-throughput Illumina NGS allowed for the identification of bacteria that would not have been isolated/identified using traditional culturing methods.

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