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Copper Removal by Biofilms

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Abstract
Biofilm systems have been widely used in wastewater treatment plants. However, little information is available on the impact of toxic chemicals on the performance of fixed film systems. This study was aimed at evaluating the impact of copper on a biofilm system by examining a variety of parameters, including reactor pH, DO, substrate concentrations, secretion of extracellular polymeric substances (EPS), and copper removal and accumulation. The microbial communities in the biofilms were also examined using automated ribosomal intergenic spacer analysis (ARISA). Four rotating drum biofilm reactors were used to produce biofilms. One reactor was used to produce biofilms under copper free conditions; while the others were used to produce biofilms grown under three different copper contamination levels, namely 100 ppb, 200 ppb, and 500 ppb, for a prolonged period. The following results were obtained: (1) biofilm reactor performance was not significantly impacted as demonstrated by the pH, DO, substrate removal, and total solids in the effluent; (2) however, copper contamination inhibited EPS production in the biofilms; (3) copper removal efficiencies of 25-31% were obtained for the three copper contamination levels studied; (4) fixed films functionalized as a reservoir to accumulate more copper over time; and (5) copper contamination selected for specific species that were able to tolerate this stress and that may contribute to its remediation.

Problems and Research Objectives
Heavy metal contamination is of growing concern nationwide because of the numerous health risks to animals and humans. Shock loads of metals can lead to complete failure of biological processes (Bagby and Sherrad, 1981, Battistoni et al., 1993). Copper was selected as a representative heavy metal for study because copper is one of the most commonly used heavy metals and one of the most widespread heavy metal contaminants of the environment. As a trace element, copper is essential to hemoglobin synthesis and as a cofactor of enzymes in metabolic processes. However, high levels of copper can be extremely toxic to living organisms and metabolic reactions can be inhibited (Donmez and Aksu, 2001).

While physical/chemical treatment methods tend to be expensive for metal removal, biological methods offer a more economical treatment option. Both activated sludge and fixed film systems have been used for wastewater treatment for over a century. Despite the importance of fixed film systems in wastewater treatment, little research has been done to evaluate the impact of toxic chemicals, such as heavy metals, on the activity and performance of fixed film systems.

The extracellular polymeric substances (EPS) represent the construction material in the biofilms that allows the cells to maintain stable micro consortia and establish synergistic relationships (Wingender et al., 1999). The production of EPS is a general property of *Bacteria* and *Archaea*, as well as algae and fungi (Wingender et al., 1999). Secreted in part by microorganisms during growth or cell lysis, EPS consist of organic substances, such as polysaccharides, uronic acids, proteins, nucleic acids, lipids, and humic substances (Christensen, 1989, Wingender et al., 1999). After water, polysaccharides and proteins are the major component of EPS and vary widely in their composition, structure and properties (Lazarova and Manem, 1995).

The majority of EPS polysaccharides are polyanionic due to the presence of either uronic acids or ketal-linked pyruvate (Sutherland, 2001a). In extracellular proteins, a variety of amino acids are negative charged. Other functional groups, such as carboxyls, phosphates, and sulfates, are also present (Wingender et al., 1999). The unique chemical structures of these components make the surface of EPS negatively charged, which subsequently allow EPS to protect the microorganisms from toxic environmental substances (Huang et al., 2000) and to be used as a pollutant removal strategy (Chipasa, 2003, Mittelman and Geesey, 1985, Gadd and C., 1993, Puranik and Paknikar, 1999).

EPS are not only a structural component, but also a functional component of biofilm that contribute significantly to biofilm activity and performance, maintaining stability (Mayer et al., 1999), providing shear force resistance (Sutherland, 2001a), and protecting cells from changes in pH, water quality, salt content and hydraulic pressure (Sutherland, 2001a, Urbain et al., 1993). Polysaccharides are found to play a key role in biofilm attachment (Tsuneda et al., 2003, Kachlany et al., 2001). Nonenzymatic lectins in the EPS were found to be directly involved in the bioflocculation process (Higgins and Novak, 1997). Another function of extracellular proteins is as enzymes that hydrolyze macromolecular organic matter to smaller units that can be taken up by bacterial cells (Marxsen and Fiebig, 1993). Large variations in exoenzymatic activities were demonstrated and speculations were made that this might be related to toxic compounds (Frolund et al., 1995).

Biofilms have been shown to be effective for heavy metal removal and recovery from wastewater stream (Costley and Wallis, 2000). However, only limited information is available on the long-term impact of heavy metals on biofilm EPS secretion. A stimulated EPS production was reported by White and Gadd (White and Gadd, 2000) when anaerobic sulfate-reducing biofilms (SRB) were exposed to 13 mg/L copper. However, the impact of copper on EPS secretion of aerobic heterotrophic biofilms used for municipal wastewater treatment has not been well studied. Therefore, the objective of this study was to evaluate the impact of chemical stress induced by copper on an aerobic heterotrophic biofilm system.

Molecular fingerprinting techniques are useful tools that allow researchers to determine the diversity of microbial communities and phylogenetic groups in both engineered and natural systems without the need for cultivation (Dabert et al., 2002). These methods typically involve the extraction of DNA or RNA from (environmental/microbial community) samples and PCR amplification of sequences of interest. High population diversity has been discovered in both activated sludge and biofilms with molecular fingerprinting tools (Boon et al., 2002, Canstein et al., 2002). Specific microbial populations were found to be selected after exposure to toxic compounds by examining activated sludge (Xie et al., 2002).

Methodology

Laboratory Biofilm Reactors. Four rotating drum biofilm reactors (RDBRs) were used to generate biofilms for study (Figure 1). Each reactor consists of a rotating inner drum enclosed within a stationary outer cylinder forming an annular space in between. The inner rotating drum consists of 20 removable polycarbonate sampling slides to facilitate biofilm sampling. The reactors were seeded with the activated sludge from Clinton Wastewater Treatment Plant in Massachusetts. Each reactor had a 900 mL of liquid volume and the hydraulic retention time was

maintained at 110 minutes. The detailed reactor running conditions and flow diagram were previously published by Ramasamy and Zhang (2005). Each reactor was receiving a synthetic wastewater with a known chemical oxygen demand (COD; 150 mg/L) and copper concentration. One reactor was used to produce biofilms grown under copper-free conditions (i.e. control reactor); while the others were used to produce biofilms grown under 100ppb, 200 ppb, and 500 ppb copper concentrations for a prolonged period (more than 30 days).

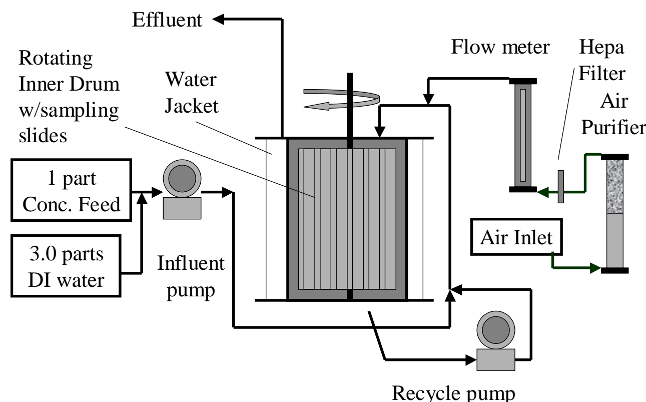

Figure 1. Experimental Flow Chart of Biofilm Reactor.

COD concentration, pH, and dissolved oxygen (DO) concentration are commonly used to determine the performance of the biological reactors; these three parameters (APHA et al., 1998) were measured three times a week. COD is also a good indicator of the biological activity of the microbial community. Once the reactors reached a quasi-steady state (constant effluent COD concentration, i.e. 20 mg/L), biofilms were sampled periodically to determine the total biomass content (represented by total solids [TS]) (APHA et al., 1998) and EPS concentrations (Zhang et al., 1999); total copper and free copper were measured using Porphyrin Method (HACH, 2002) for samples of influent, effluent, biofilms, and suspended biomass. In addition, molecular analysis of bacterial communities was conducted for the copper-free and copper-exposed biofilms (200 ppb) to reveal the potential differences in their community compositions.

Quantify EPS Secretion. Recovery of the EPS that surrounds the biofilms requires some preliminary extraction steps. Polysaccharides and protein are uniformly found as the major EPS components, having a protein to polysaccharides ratio between 0.2 to 5 (w/w) (Frolund et al., 1996). In this study, the EPS was extracted by the steaming method published by Zhang et al (1999).

Microbial Community Diversity using ARISA. Automated ribosomal intergenic spacer analysis (ARISA) was used to analyze the microbial community diversity in both copper-free and copper-exposed biofilms (200 ppb copper). ARISA uses the heterogeneity of the intergenic transcribed spacer (ITS) region between 16S and 23S rRNA genes to distinguish strains and closely related species (Jensen et al., 1993, Maes et al., 1997), and this tool has been used in natural microbial communities and biofilms (Fisher and Triplett, 1999).

Genomic DNA was extracted and purified from triplicate biofilm samples from each bioreactor using FastDNA SPIN Kits, as recommended by the manufacturer (Qbiogene, Irvine CA). Purified genomic DNAs were quantified using a Hoefer DyNA Quant 200 (Amersham Biosciences Inc.) fluorometric assay and 2 ng were used as template in subsequent 50 µl PCRs. The intergenic spacer regions (IGS) were amplified using primers 1406f (which targets universal

16S rRNA genes) and 23Sr (which targets *Bacteria* 23S rRNA genes), with previously described PCR conditions (Fisher and Triplett, 1999), except that primer 1406f was labeled with phosphoramidite-linked WellRED dye D4 (Proligo Inc., Boulder CO). PCR products were analyzed by agarose gel electrophoresis and were ethanol-precipitated, resuspended in sample loading solution (SLS, Beckman-Coulter, Fullerton CA), and analyzed by automated capillary electrophoresis using a Beckman-Coulter CEQ 2000XL. A 50-1000 bp a molecular size standard labeled with WellRED dye D1 (MapMarker 1000, BioVentures Inc., Murfreesboro TN) was analyzed in conjunction with each sample. Capillary electrophoresis conditions used were as follows: capillary temperature of 50˚ C, an initial denaturation step at 90˚ C for 120 sec, a 2.0 kV injection for 15 sec, and separation at 4.2 kV for 120 min.

Because the PCR primers used target regions within the 16S and 23S rRNA genes, about 125-140 bp of each rRNA gene was amplified in addition to the intergenic spacer region between them (Fisher and Triplett, 1999). Therefore, only peaks >390 bp were considered in the analysis (peaks <390 bp were considered PCR artifacts, such as primer-dimers). Further processing of ARISA data was done as described by Brown et al. (2005).

Principal Findings and Significance

Reactor Performance. The pH (7.2 ± 0.2) and dissolved oxygen concentrations (2.5-3.5 mg/L) in all the reactors remained constant throughout the experiment (Figure 2). Copper contamination did not significantly impact the substrate removal; COD removal was only reduced by 1-3% (Figure 3). On average, 84% COD removal was seen in the control reactor, and 81-83% COD removal was seen in the copper contaminated reactors. Total solids in the effluent were monitored and the results indicate that no major sloughing occurred under the three different copper contamination levels studied (Figure 4).

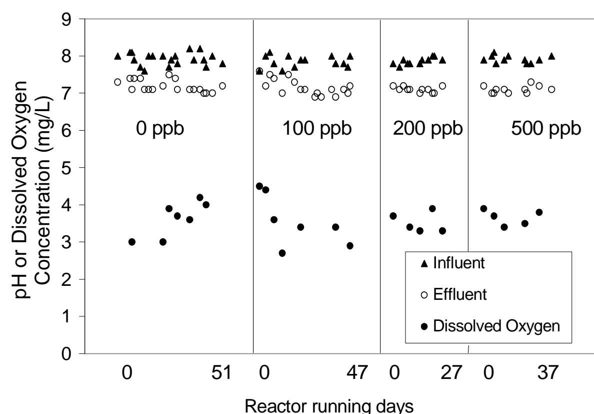

Figure 2. Reactor pH and dissolved oxygen

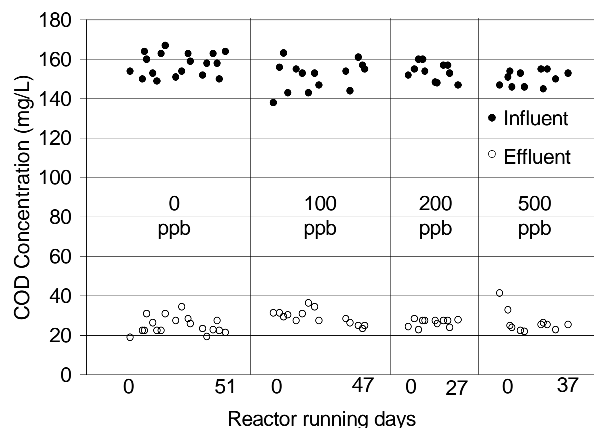

Figure 3. Substrate removal

Impact on EPS production: EPS production was found to be inhibited by copper under all the copper contamination levels studied (100 ppb, 200 ppb, and 500 ppb) comparing to the EPS concentrations in the control biofilms (Figure 5). EPS-polysaccharides were inhibited by 30-54%, EPS-protein was inhibited by 11-39%, and the total EPS inhibition was between 28-39%. This finding presents an example of the interesting dynamic process of EPS production, suggesting a complex stress response mechanism that has not been completely understood.

Further study is warranted to better understand the factors that govern EPS's stimulation and inhibition and the extent to which EPS protect microorganisms from toxic environmental substances during this process. The knowledge gained will allow us to come up with a strategy to minimize the inhibition effect.

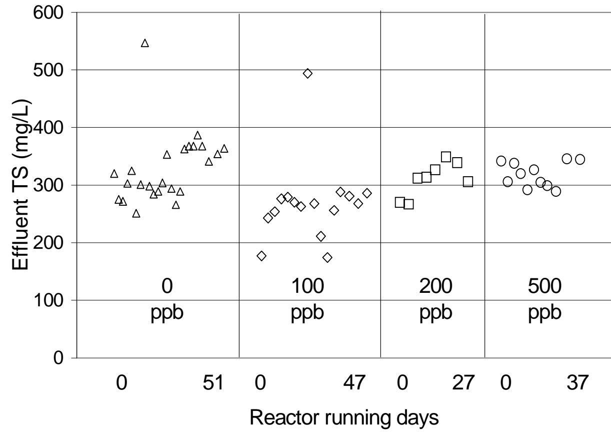

Figure 4. Total solids in the effluent

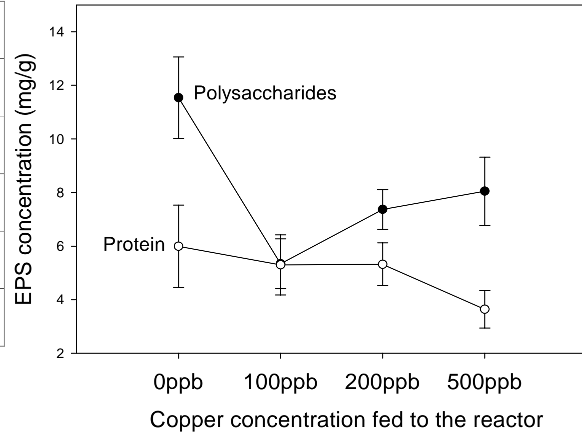

Figure 5. Impact on EPS production

Copper Removal: The removal efficiency of free copper remained relatively constant (31% ±7%, 25%±4%, and 26%±6, respectively) for all the copper contamination levels studied. The removal of both total copper and free copper (in μg/L) increased with the increase of copper contamination (Figure 6). In terms of copper accumulation, more copper was found in the fixed films than that in the suspended biomass (Figure 7). At 100 ppb copper contamination level, fixed films accumulated 126 times more copper than did the suspended biomass; the difference in accumulating copper between the fixed films and suspended biomass dropped when more copper contamination was introduced to the reactor (i.e., 21 times at copper contamination level

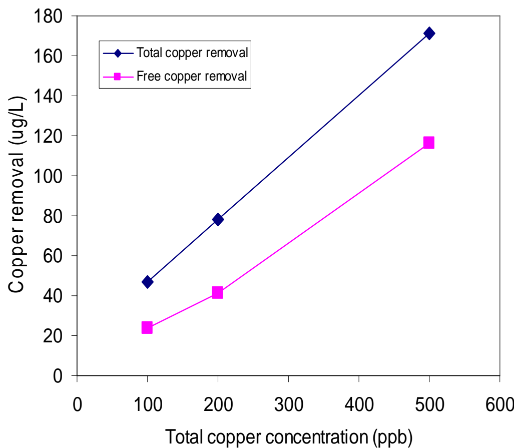

Figure 6. Copper removal

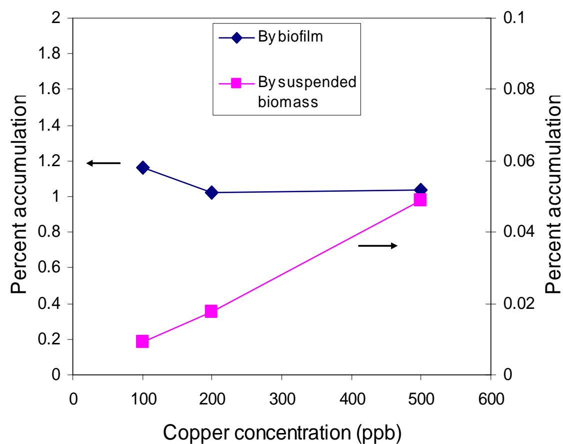

Figure 7. Copper accumulation

of 500 ppb). Two factors could contribute to this significant difference in copper accumulation between the fixed films and suspended biomass; one is that fixed films stayed in the system for a much longer period of time, versus a 110 minutes retention time for the suspended biomass, second, suspended biomass mostly came from the detached biofilms, which explains why later the difference became smaller at 500 ppb copper contamination level (Figure 7).

In general, copper accumulation in the fixed films was quite low for all three copper contamination levels studied (~1%, Figure 7). This finding seems to correlate with the inhibited EPS production suggesting the importance of EPS for copper removal.

Microbial Community Diversity: ARISA profiles of the bacterial communities in both copper-free and 200 ppb copper-exposed biofilms revealed qualitative and semi-quantitative differences in their community compositions (Figure 8). Each ARISA peak represents a distinct IGS length polymorphism and, in general, a distinct bacterial strain or species. Comparison of ARISA profiles from copper-free and copper-exposed (200 ppb copper) biofilms revealed several differences in microbial community composition. In particular, bacteria represented by the ARISA peak at 584 bp were not detectable in the copper-free biofilms, but were significant in copper-exposed samples. These results indicate that exposure to 200 ppb copper selects for specific microbial populations that are able to tolerate this stress and that may contribute to its remediation.

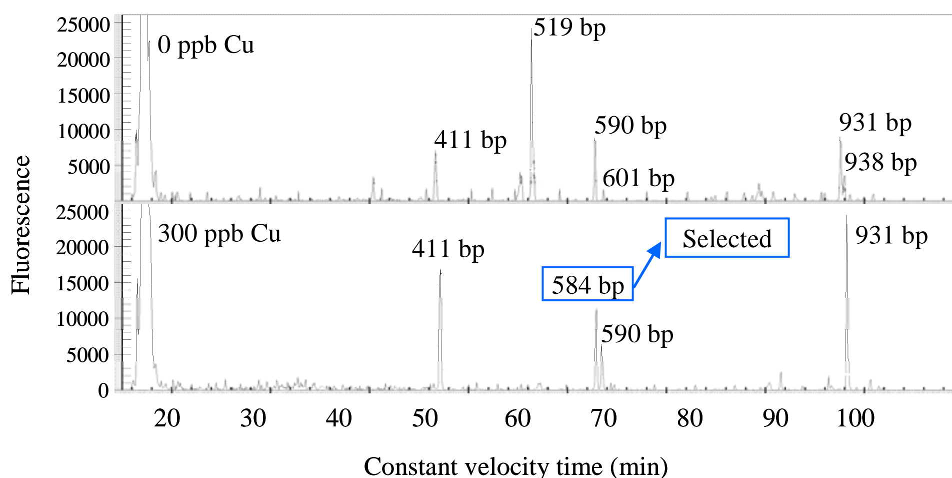

Figure 8. ARISA profiles of biofilm microbial communities. (Triplicate analyses of each sample showed similar results, data not shown)

Conclusions

The major conclusions based on the results collected are: (1) biofilm reactor performance was not significantly impacted as demonstrated by the pH, DO, substrate removal, and total solids in the effluent; (2) however, copper addition inhibited EPS production which could be the reason for low copper accumulation in the fixed films; (3) copper removal efficiencies of 25-31% were obtained for the three copper contamination levels studied; (4) fixed films functionalized as a

reservoir to accumulate more copper over time; and (5) copper contamination selected for specific species that were able to tolerate this stress and that may contribute to its remediation.

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