Comparison of chemo-, hetero- and mixotrophic denitrification in laboratory-scale UASBs

R. Sierra-Alvarez*, F. Guerrero**, P. Rowlette*, S. Freeman* and J.A. Field*

*Department of Chemical and Environmental Engineering, University of Arizona, P.O. Box 210011, Tucson, Arizona 85721-0011, USA
**Department of Biotechnology, Universidad Autónoma de México, México D.F., Mexico
(E-mail: rsierra@email.arizona.edu)

Abstract This study investigated removal of sulfide and \( p \)-cresol linked to denitrification in laboratory-scale upflow anaerobic granular sludge bed (UASB) bioreactors. Three parallel denitrification bioreactors were run for nine months, which were operated under chemolithoautotrophic conditions (i.e., using sulfide as electron donor - \( e \)-donor and bicarbonate as C source); heterotrophic conditions (with \( p \)-cresol as e-donor and C source), and mixotrophic conditions (utilizing both sulfide and \( p \)-cresol as electron donors), respectively. The average hydraulic retention time and nitrate load applied to the bioreactors was 13.4 h and 1,240 mg N-NO\(_3\)/l/day, respectively. The nitrate removal efficiency was 89, 95 and 99%, respectively, for the chemo-, hetero- and mixotrophic reactors. The mixotrophic UASB removed both sulfide and \( p \)-cresol almost completely, indicating that simultaneous removal of the inorganic and organic e-donors occurred. Nitrite was seldom observed as an intermediate. \( N_2O \) gas and methane concentrations in the biogas were also negligible. These results indicate that mixotrophic denitrification with phenols and sulfide is feasible in high rate UASB reactors.

Keywords Denitrification; microbial sulfoxidation; chemolithoautotrophic; mixotrophic; heterotrophic; \( p \)-cresol; biological wastewater treatment

Introduction Sour condensate effluents of petroleum refineries contain phenols, sulfide and ammonium (Berné and Cordonnier 1995). Complete biological removal of nitrogen would require nitrification followed by denitrification. A treatment scheme is proposed involving a sequence of anaerobic and nitrification reactors (Figure 1). A large fraction of the nitrified effluent is recycled to the anaerobic reactor to encourage denitrification. Removal of sulfide and phenols could potentially be linked to denitrification. The goal of this study was to compare denitrification with a mixture of inorganic (sulfide) and organic (\( \text{para} \)-cresol) electron donors. Three parallel laboratory-scale UASB denitrification reactors were run for nine months. The first one was operated under chemolithoautotrophic conditions with sulfide as electron donor (\( e \)-donor) and bicarbonate as the carbon source. The second one was operated under heterotrophic conditions with \( p \)-cresol as e-donor and C source. The denitrification achieved in these two reactors was compared with that of the third reactor, a mixotrophic reactor, utilizing both sulfide and \( p \)-cresol as e-donors.

Materials and methods

Microbial inocula Biomass cultivated in a laboratory-scale chemolithoautotrophic bioreactor fed with thiosulfate and nitrate was used to inoculate the bioreactors in this study. Methanogenic granular sludge (Eerbeek) from a commercial upward anaerobic sludge bed (UASB) reactor treating recycled paper mill effluents was also utilized in the batch biodegradation assays. The volatile suspended solids (VSS) concentration of the chemolithotrophic and Eerbeek sludge was 7.5\% and 12.9\%, respectively.
Batch bioassays

Biodegradation of p-cresol (2.35 mM) under methanogenic, denitrifying and sulfate-reducing conditions was investigated in shaken batch bioassays. Denitrifying and sulfate-reducing assays were supplied with stoichiometric concentrations of the e-donor and electron accepting substrate (e-acceptor), i.e., 6 mM NO_3^--/mM p-cresol and 4.75 mM SO_4^{2--}/mM p-cresol, respectively. The basal media used in the methanogenic and denitrifying batch bioassays (BM1) consisted of (in mg/l): K_2HPO_4 (250), NH_4Cl (280); MgSO_4·7 H_2O (10), CaCl_2·2 H_2O (10); MgCl_2·6 H_2O (100); NaHCO_3 (5,000); yeast extract (25), and trace element solution (1 ml/l). The basal media used in the sulfate-reducing assays (BM2) contained (in mg/l): K_2HPO_4 (600), Na_2HPO_4·2 H_2O (795); NH_4Cl (280); MgCl_2·6 H_2O (83), Na_2SO_4 (1500); CaCl_2·2 H_2O (10); NaHCO_3 (4,000); 2-bromoethane sulfonate (6,330), yeast extract (25), and trace element solution (2 ml/l). The trace element solution contained (in mg/l): H_3BO_3 (50), FeCl_2·4 H_2O (2000), ZnCl_2 (50), MnCl_2·4H_2O (50), (NH_4)_6Mo_7O_24·4H_2O (50), AlCl_3·6 H_2O (90), CuCl_2·6 H_2O (2000), NiCl_2·6 H_2O (50), CuCl_2·2 H_2O (30), NaSeO_3·5 H_2O (100), EDTA (1000), Resazurin (200) and 36% HCl (1 ml/l). The pH of the medium was adjusted to 7.2 with HCl or NaOH, as required. The culture medium (50–100 ml) and microbial inoculum (0.5 g VSS/l) was added to 160-ml vials. Flasks were sealed and flushed as indicated above. Abiotic controls (without inoculum) were run in parallel to monitor the possible abiotic degradation of p-cresol. All bioassays were conducted at 30°C utilizing triplicate cultures. Liquid samples were taken periodically to determine substrate or/e-acceptor utilization and product formation. Headspace samples in methanogenic assays were analyzed for methane at regular intervals throughout the experiment.

Continuous bioreactor experiments

Continuous experiments comparing denitrification under chemolithotrophic-, mixotrophic- and heterotrophic conditions were conducted at 30 ± 2°C utilizing three 500-ml Upflow Anaerobic Sludge Bed (UASB) reactors, coded as R1, R2 and R3, respectively. The reactors were inoculated with chemolithoautotrophic sludge (10 g VSS/l). The reactor media were prepared using a basal medium that contained (in mg/l): K_2HPO_4 (800), KH_2PO_4 (300); NH_4Cl (400); MgSO_4·7 H_2O (400), NaHCO_3 (2,000), and trace element solution (2 ml/l). The trace element solution consisted of (in mg/l): EDTA (500); ZnSO_4 (22.0); CaCl_2 (55.0); MnCl_2·4 H_2O (50.6); (NH_4)_6MoO_24·4 H_2O (11.0); CuSO_4·5 H_2O...
The average concentration of nitrate and e-donating substrate(s) in each reactor and period of operation is shown in Table 1. Nitrate, nitrite, sulfide, sulfate, thiosulfate, p-cresol, phenol, acetate and pH value were monitored daily in the reactor influent and effluent. Influent flow rate and biogas production were also monitored regularly. Biogas composition (methane, nitrous gas, N₂) was determined occasionally. Sludge samples were collected periodically for characterization of specific metabolic activities.

**Chemical analysis**

Nitrate (NO₃⁻), nitrite (NO₂⁻), sulfate (SO₄²⁻), thiosulfate (S₂O₃²⁻) were determined by suppressed conductivity – ion chromatography using a AS11-HC4 Dionex column and 30 mM KOH as eluent at a flow rate of 1.2 ml/min. Dinitrogen gas (N₂) and nitrous gas (N₂O) in biogas samples were analyzed by gas chromatography (GC) with thermal conductivity detection. The methane content in gas samples as well as p-cresol, phenol and volatile fatty acid (VFA) concentrations in liquid samples was determined by GC with flame ionization detection. Sulfide in culture media was quantified colorimetrically by the methylene blue method (Trüper and Schlegel, 1964). Other characteristics of the culture medium and microbial inoculum (e.g., pH, VSS) were determined according to *Standard Methods* (APHA 1998). All liquid samples, with the exception of those used for sulfide analysis, were filtered (0.20 μm membrane filter) prior to analysis.

**Results and discussion**

**Biodegradation of p-cresol in batch assays under various redox conditions**

The microbial degradation of p-cresol (2.35 mM) under methanogenic, denitrifying, and sulfate-reducing conditions, was investigated in shaken batch bioassays. The ability of two different microbial consortia, *i.e.*, chemo-lithoautotrophic denitrifying sludge (CDS) and granular methanogenic sludge (GMS), for p-cresol degradation was compared (Figure 2). Para-cresol was readily degraded by both consortia under nitrate-reducing conditions. Degradation of the phenolic compound was accompanied by a stoichiometric decrease in the concentration of nitrate. Para-cresol degradation rates under denitrifying conditions were 1.41 and 1.13 mmol/g VSS/day for the GMS and CDS consortia, respectively. A lag phase of 10 days was observed in GMS prior to the onset of degradation. Degradation of p-cresol under methanogenic and sulfate reducing conditions was only observed in GMS after a lag period of 44 days. Para-cresol degradation resulted in the concomitant formation of methane in nearly stoichiometric quantities. These results suggest that p-cresol is more susceptible to degradation under denitrifying conditions than under methanogenic- or sulfate reducing conditions. Several literature studies

<table>
<thead>
<tr>
<th>Reactor and experimental period</th>
<th>Duration (days)</th>
<th>NO₃⁻ conc. (mg NO₃⁻/l)</th>
<th>H₂S conc. (mg S₂⁻/l)</th>
<th>p-cresol conc. (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor 1</td>
<td>0–292</td>
<td>372.9 ± 52.9</td>
<td>103.9 ± 23.7</td>
<td>NA</td>
</tr>
<tr>
<td>Reactor 2&lt;sup&gt;§&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period I</td>
<td>0–163</td>
<td>382.3 ± 78.8</td>
<td>101.9 ± 30.0</td>
<td>86.8 ± 20.7</td>
</tr>
<tr>
<td>Period II</td>
<td>164–198</td>
<td>483.8 ± 77.1</td>
<td>147.5 ± 33.5</td>
<td>148.0 ± 17.5</td>
</tr>
<tr>
<td>Period III</td>
<td>199–220</td>
<td>727.7 ± 99.1</td>
<td>218.9 ± 57.2</td>
<td>189.2 ± 19.6</td>
</tr>
<tr>
<td>Period IV</td>
<td>221–292</td>
<td>382.1 ± 30.7</td>
<td>124.3 ± 20.4</td>
<td>95.3 ± 13.5</td>
</tr>
<tr>
<td>Reactor 3&lt;sup&gt;§&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period A</td>
<td>0–146</td>
<td>379.9 ± 54.2</td>
<td>NA</td>
<td>82.6 ± 23.2</td>
</tr>
<tr>
<td>Period B</td>
<td>147–292</td>
<td>724.5 ± 129.4</td>
<td>NA</td>
<td>171.8 ± 24.2</td>
</tr>
</tbody>
</table>

<sup>§</sup>All reactors were operated with a constant hydraulic retention time of 13.4 ± 2.0 hours
confirm that simple phenols are readily biodegraded under denitrifying conditions (Khoury et al. 1992; Schie and Young 1998; Fang and Zhou, 1999). Therefore, phenols in sour condensate wastewaters should be expected to be amenable to degradation in denitrifying bioreactors.

Comparison of chemo-, hetero- and mixotrophic denitrification in laboratory-scale UASBs

Three 500-ml denitrifying laboratory-scale reactors were operated under either chemolitho-autotrophic, mixotrophic or heterotrophic conditions. The chemolithoautotrophic reactor (R1) utilized sulfide as an e-donor and bicarbonate as a carbon source. The mixotrophic reactor (R2) received a mixture of sulfide and p-cresol as e-donors. The heterotrophic reactor (R3) was supplied with p-cresol as the electron donating substrate. All reactors were operated with a constant hydraulic retention time (HRT) of 13.4 ± 2.0 hours.

Table 1 provides a schedule of operational periods for the various reactors and lists the average concentration of nitrate and electron donating substrate(s) in the influent for each reactor and period of operation. The average nitrate concentrations in the feed of the various reactors varied between 373 and 728 mg NO$_3^-$/l. In each case, the concentration of nitrate added was in stoichiometric relation with the sum of the electron donating capacity of sulfide and/or p-cresol.

Table 2 shows the average volumetric nitrate loading as well as the removal efficiency determined for nitrate, sulfide and p-cresol for each reactor period of operation. The bioreactors were operated at an average HRT of 13.4 ± 1.9 hours throughout the experiment.
The average nitrate loading applied to the various reactors varied between 157 and 280 mg N-NO$_3$ per litre of reactor and per day.

Chemolithooautotrophic reactor (R1). The average nitrate removal in the reactor was 89%. Conversion of nitrate to elemental nitrogen (N$_2$) was not quantitative and varying concentrations of nitrite were detected in the effluent. The recovery of nitrite-N as a percent of the influent nitrate-N was initially around 40–55% but slowly declined to values between 5–30%. Most of the rest of the nitrogen was accounted for by N$_2$-gas. Incomplete denitrification was probably due to e-donor limitations. Although hydrogen sulfide was added in stoichiometric concentrations and the influent was freshly prepared every 2–3 days and stored refrigerated under anoxic conditions, partial oxidation of sulfide occurred during storage due to the relatively unstable character of this reduced sulfur compound. Sulfide was removed almost completely (>99%) throughout the experiment. Initially, almost all of the influent sulfide-S was recovered in the effluent as sulfate-S. However, conversion of sulfide to sulfate declined dramatically between days 100 to 210, probably due to reactor overloading. The decrease conversion to sulfate coincided with accumulation of yellowish elemental sulfur (S$_0$) in the sludge bed. The hydrophobic nature of elemental sulfur caused the sludge bed to float. High recoveries of sulfate-S (59%) were attained again after day 225 in response to lowering the load.

Mixotrophic reactor (R2). Table 2 shows the average efficiencies for the 4 periods of R2 operation. With the exception of the overloaded period III, nitrate removal exceeded 91%, p-cresol removal exceeded 92% and sulfide removal was 98% or higher. Nitrite was not recovered as a major intermediate of nitrate reduction. Gas production from the reactor fluctuated however averaged data of the gas production indicated that a large fraction of the nitrate removal was recovered as N$_2$ assuming most of the CO$_2$-scrubbed gas was N$_2$, a fact corroborated by a methane composition of less than 1%. The sulfide removal was consistently near 100% even in period III. Initially sulfate-S recovery was relatively high, however, after 100 days of operation the values declined and were very sporadic. The sporadic nature remained until the end of the experiment with a slight tendency towards greater sulfate-S recoveries after day 225.

Heterotrophic reactor (R3). Table 2 shows the average efficiencies for the two periods of R3 operation. In period A and B when the p-cresol concentration was approximately 80 and 170 mg/l, respectively; the p-cresol removal efficiency was consistently near 100% with only a few exceptions. The nitrate removal efficiency was generally near 100% except for periods directly following increases in the p-cresol concentration.
For example, an increment of the p-cresol concentration from 50 to 125 mg/l at the beginning of the experiment was associated with a decline in the nitrate removal efficiency from 100% to 85%. The efficiency quickly was restored to 100%, indicating a population of p-cresol degrading denitrifying bacteria becoming enriched in the reactor biomass. A similar pattern was again observed as period A was shifted to period B of higher p-cresol concentrations. For the first 150 days of operation, nitrite was not observed as an intermediate of denitrification. The gas production indicated that a large fraction of the nitrate removal was recovered as N2, assuming that most of the CO2-scrubbed gas was N2 (methane composition was also less than 1%). Following the dramatic increase in p-cresol concentration after day 150, nitrite started to accumulate as product of nitrate removal, accounting most of the time for 1 to 20% of the influent nitrate-N. The p-cresol removal efficiencies attained by R3 were 100 and 97% in periods A and B, respectively.

Conclusions
These results indicate that mixotrophic denitrification with phenols and sulfide is feasible in high rate UASB reactors. The laboratory-scale bioreactor operated in this study could accommodate volumetric nitrate loading rates of up to 210 mg NO3-N/l/day with nitrate removal efficiencies exceeding 99%. Under these conditions, sulfide and p-cresol were almost completely removed, indicating that simultaneous removal of the inorganic and organic electron donating substrates occurred. Ongoing work is aimed at the characterization of the dominant microbial populations in the chemolithotrophic reactor using culture-based and molecular ecology techniques.

Acknowledgements
This research was funded by a grant from the National Science Foundation (NSF-0115851 award). NSF support to Dr. R.SA. is also acknowledged (NSF-0137368 award). Participation of various undergraduate research students (P.R. and S.F.) was partially funded by a NSF REU grant and by grants from the University of Arizona/NASA Undergraduate Research Internship Program and/or the Undergraduate Biology Research Program (UBRP). F.G. was supported by a CONACyT grant.

References