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Perchlorate reduction by a mixed culture in an up-flow anaerobic fixed bed reactor

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***Wolinella succinogenes* HAP-1 is a Gram-negative microaerophile which reduces perchlorate to chloride by the proposed pathway ClO_4 to ClO_3 to ClO_2 to $Cl + O_2$. A cost-effective perchlorate treatment process has been established using a consortium of facultative anaerobic organisms and *W. succinogenes* HAP-1. The mixed anaerobic bacterial culture containing *W. succinogenes* HAP-1 was examined for the ability to form a biofilm capable of perchlorate reduction. An up-flow anaerobic fixed bed reactor (UAFBR) was packed with diatomaceous earth pellets and operated at residence times of 1.17 and 0.46 h to insure a viable biofilm had attached to the diatomaceous earth pellets. Reduction rates of perchlorate to chloride in the UAFBR could be maintained at 1 g of perchlorate reduced h^{-1} L^{-1}. Studies with the same bacterial consortium in continuously stirred tank reactors (CSTR) generally reduced 0.5–0.7 g of perchlorate h^{-1}. Viable cell counts were performed periodically on the diatomaceous earth pellets and demonstrated that the *W. succinogenes* HAP-1 population was maintained at 28–47% of the total microbial population. Scanning electron micrographs demonstrated that the external and internal surfaces of the diatomaceous pellets were densely colonized with microbial cells of multiple cell types. This is the first report of an anaerobic mixed culture forming a biofilm capable of perchlorate reduction.**

Keywords: up-flow anaerobic fixed bed reactor; *Wolinella succinogenes* HAP-1; ammonium perchlorate; biofilm; suspected endocrine disruptor

Introduction

Widespread use of perchlorate salts in propellants, explosives and pyrotechnic devices by the chemical, aerospace and defense industries has led to their release into the environment. Class 1.1 and class 1.3 rocket motor propellants contain 14% and 70% ammonium perchlorate, respectively [23]. The large solid rocket motor disposal inventory currently has 55 million pounds of propellant ready for treatment. Over the next 8 years this amount is expected to increase to 164 million pounds of solid propellant targeted for disposal [15]. The manufacture, refurbishment and maintenance of rocket motors for the last 40 years have resulted in contaminated soil and ground water [5]. Ammonium perchlorate is very soluble in water, (20 g 100 ml^{-1} at 25°C) and is stable in ground water.

Recent studies with Sprague–Dawley rats have demonstrated that the perchlorate ion competitively inhibits the uptake of iodine by the thyroid gland [11]. Iodine deficiencies, especially in pregnant women, are particularly detrimental to fetal development [21]. Perchlorate's interference with the normal function of the thyroid gland identifies it as a suspected endocrine disrupter [18]. The California Department of Health Services has established a provisional action level for perchlorate at 18 ppb for drinking water sources [5].

Vibrio dechloraticans Cuznesove B-1168 has been shown to reduce 70 mg perchlorate h^{-1} g^{-1} dry weight during anaerobic growth on ethanol or acetate [19]. A facultative aerobic bacterium GR-1 belonging to the B subgroup of *Proteobacteria* has been shown to link the oxidation of acetate to the reduction of perchlorate [17]. Various heterotrophic bacteria have been identified which reduce perchlorate to chloride at low rates [7,8,10]. Perchlorate reduction was associated with the nitrate reductase activity in these organisms [7,8,19].

Wolinella succinogenes HAP-1 is a Gram-negative microaerophile which has been shown to reduce perchlorate to chloride at a rate of 107 mg perchlorate h^{-1} g^{-1} dry weight cells [1,9]. *W. succinogenes* HAP-1 reduced perchlorate at concentrations of 7750 ppm and could survive at 30 000 ppm perchlorate [1,24]. Mixed substrate studies with nitrate and perchlorate demonstrated that strain HAP-1 preferentially utilized perchlorate [24]. Inhibition and pH profiles were different for nitrate and perchlorate reduction by *W. succinogenes* HAP-1 [24,25]. *W. succinogenes* HAP-1's high rate of perchlorate reduction has allowed us to design a treatment process for rocket propellant washout waste streams [2]. The current system contains a consortium of microorganisms from a sewage enrichment culture with up to 12 unknown facultative anaerobic bacteria and *W. succinogenes*. The consortium removes oxygen, nitrate, nitrite and sulfate from the waste stream [3,4]. The perchlorate reduction reaction was found to require an oxidation reduction potential of at least −110 mV which was provided by the fermentative growth of the mixed culture [1,2]. The fermentation of brewer's yeast by the mixed culture may also provide nutrients necessary for *W. succinogenes* to reduce perchlorate [1,4]. Aeration resulted in the complete and immediate inhibition of perchlorate reduction [1].

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This study was designed to examine whether the multi-membered consortium was capable of attaching and forming a biofilm, and at what rate the biofilm would reduce perchlorate. The advantages of biofilms for the treatment of waste streams are well established [13]. The biofilm provides the organism's protection against perturbations in temperature, pH, desiccation and other environmental factors often associated with bioreactor operation.

Materials and methods

Abiotic perchlorate stability study

Ammonium perchlorate, 1.17 g L^{-1} (1000 ppm) or 0.117 g L^{-1} (100 ppm), was added to tap water containing 5 g L^{-1} BYF-100 (BYF-100: Red Star, Milwaukee, WI, USA). BYF-100 contains 54% naturally occurring protein, peptides, free amino nitrogen, vitamins and trace elements. The solution was adjusted to pH 7.2 with dilute HCl and sterilized by autoclaving. Solutions were incubated anaerobically at 25°C and 1-ml samples removed aseptically at specific times for perchlorate measurement. Each ammonium perchlorate concentration was analyzed in triplicate.

Source and growth of organisms

The mixed culture containing *W. succinogenes* was obtained from a CSTR operating at feed concentrations of 6000 ppm perchlorate and 12 g L^{-1} of brewers yeast extract (BYF-100) at 40°C, with an 18-h residence time. The effluent perchlorate concentration was $<$300 ppm. The effluent line was connected to the UAFBR operating at a flow rate of 1 ml min^{-1} with a residence time of 23 h. The inoculation of the UAFBR was continued for 6 days.

Configuration and operation of the up-flow fixed bed anaerobic reactor

The ammonium perchlorate waste stream was from Minuteman III rocket motor washout (Aerojet, Propulsion Division, Sacramento, CA, USA) containing approximately 10% perchlorate. The concentrated perchlorate waste stream was diluted with tap water to the desired perchlorate concentration. Nutrient and waste-stream were pumped into a l-L mixing vessel prior to entering the fixed bed reactor. The mixing vessel was sparged with nitrogen, controlled for pH and adjusted to pH 7.0 with sodium hydroxide. The fixed bed reactor was manufactured using acrylic tube of 0.635 cm thickness and was 1.1684 m in length with a 7.62-cm inside diameter. The reactor was water jacketed and the inlet was designed to allow up-flow to minimize introduction of air into the system. The exclusion volume was 1.0 L when the reactor contained 2544 g (dry weight) of diatomaceous earth pellets (Celite Bio-catalyst carrier R-635, World Minerals Inc, Lompoc, CA, USA). The diatomaceous earth pellets had a mean pore diameter of 20 μm and a surface area of 0.27 m^2 g^{-1}. The nutrient was supplied from a 10-L glass bottle, sealed and stirred during reactor operation. The nutrient source was a 60 g L^{-1} solution of BYF-100 in tap water. The pH of the BYF-100 nutrient was adjusted to 4 with sulfuric acid to prevent microbial growth during the 7-day period required for its use. Perchlorate feed concentrations were maintained at 1500 ppm and 500 ppm with residence times of 1.17 h and 0.46 h, respectively. The feed concentrations were 1500 ppm perchlorate and 3 g L^{-1} BYF-100. When treating 500 ppm perchlorate, 1 g BYF-100 was used, maintaining a 2:1 ratio of nutrient to perchlorate.

A 55-gallon high density polyethylene tank reactor received the reactor effluent which entered at the bottom of the tank and exited the top. The tank reactor did not receive any nutrients, nitrogen, or regulation of temperature or pH.

Viable cell counts

At 14-day intervals, diatomaceous earth pellets were aseptically removed from the top of the reactor. One gram (wet weight) samples were washed twice with sterile buffer and crushed by circular grinding for 5 min with a mortar and pestle in 10 ml ammonium perchlorate (AP) medium with the following composition (per liter): yeast extract 10 g; peptone 10 g; sodium formate 5 g; fumaric acid 5 g; ammonium perchlorate 1.17 g; and resazurin 1 mg. The medium was adjusted to pH 7.2 with dilute HCl. Dilutions were performed on this material and plated on AP agar under anaerobic conditions. Plates were examined after a 96-h anaerobic incubation at 40°C and colony forming units determined. *W. succinogenes* HAP-1 cells can be identified on AP media due to reduction of perchlorate and oxidation of resazurin resulting in pink halos around the colonies [1].

Electron microscopy

Diatomaceous earth pellets were aseptically removed from the top of the reactor on day 20 of operation and fixed with 1% glutaraldehyde (EM grade: Polysciences, Inc, Warrington, PA, USA) in 0.01 M Cacodylate buffer at pH 7.2. Increasing concentrations of ethanol in water (10, 30, 50, 70, 95 and 100%) were applied sequentially to the pellet (20 min each application) to achieve dehydration [16]. Critical point drying was performed as described by the manufacturers (Ladd Research Industries, Inc, Burlington, VT, USA). Sputter coating was performed with gold, as previously described [16].

Analytical methods

The measurement of perchlorate reduction was performed with a perchlorate ion-selective electrode (Phoenix Electrode Co, Houston, TX, USA) and model 901 pH/ion meter (Orion Research, Boston, MA, USA). Perchlorate concentrations were confirmed with a ion chromatograph (Model 4500i, Dionex Corp, Sunnyvale, CA, USA) equipped with a conductivity detector. The column was an Econosphere C18 5u HPLC column (Alltech Associates Inc, Deerfield, IL, USA).

Results

Abiotic studies indicated that perchlorate was stable in the presence of organic material with no measurable loss of perchlorate under anaerobic conditions during the 228 days for the samples containing 1000 ppm and no loss from the 100-ppm samples over 145 days (Figure 1).

Previous studies examining sand as a matrix for biofilm

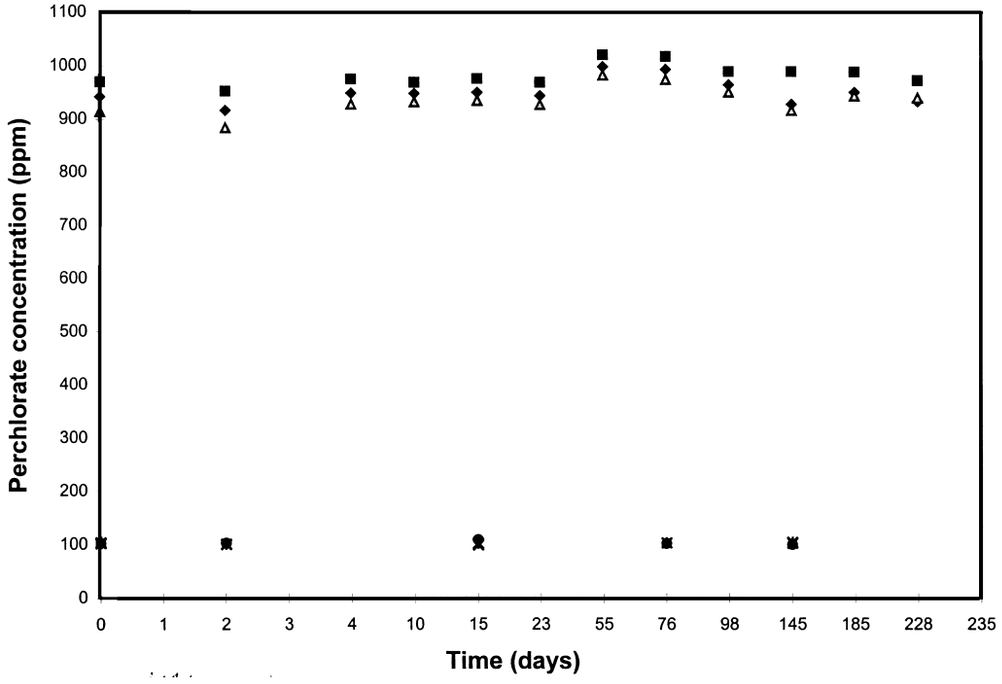

Figure 1 Abiotic decomposition of perchlorate. ◆, Sample 1000 C; ■, sample 1000 B; △, sample 1000 A; X, sample 100 C; *, sample 100 B; ●, sample 100 A. Samples 1000 A,B and C are triplicates of a 1000 ppm perchlorate stock. Samples 100 A,B and C are triplicates of a 100 ppm perchlorate stock. Slight fluctuations in readings were experienced in the Dionex system as eluents and suppressor liquids were remade for long periods of operation. These are demonstrated by increases in perchlorate concentrations on days 55, 76 and 98.

formation by the perchlorate-reducing bacterial consortium were unsuccessful. This study re-examined biofilm formation with calcined diatomaceous earth pellets. The cell concentrations in the reactor averaged 5.5×10^8 *W. succinogenes* HAP-1 cells g^{-1} pellets and 3.4×10^9 other cell types g^{-1} pellets. Bacterial cell concentrations were determined on day 15 for the tank reactor, which was found to contain 7.0×10^7 *W. succinogenes* HAP-1 cells ml^{-1} and 1.6×10^8 other cells types ml^{-1}. The significant cell numbers found in the tank reactor suggest carryover of microorganisms from the fixed bed reactor.

The perchlorate effluent concentrations were monitored (Figure 2). Perchlorate reduction by the UAFBR to $<$300 ppm was maintained during the entire operation and to $<$100 ppm during 95% of the operational period. The system was not sensitive to loss of nitrogen to the reactor and it is possible that nitrogen may only be necessary during start-up periods. This was demonstrated by the interruption of nitrogen feed on day 15 with loss for approximately 11 h and again on day 33 for 14 h. The rate of perchlorate reduction was not affected. It would thus appear that once the microbial culture has reached sufficient numbers the oxygen in the system is removed by the facultative anaerobic organisms. In the UAFBR the rate of 1–1.2 g of perchlorate reduced h^{-1} L^{-1} was maintained for 40 of 42 days of operation until the experiment was terminated (Figure 3). The decrease in perchlorate reduction on days 25 and 26 was due to malfunction in the nutrient pump, resulting in loss of nutrient. Once the nutrient was restored the perchlorate reduction rate recovered. Rates of perchlorate reduction were stable during minor perturbations in perchlorate concentrations, fluctuations in the range of pH 6.2 to 7.5, and nitrogen flow from 0 to 1.0 mg L^{-1}. Perchlorate was reduced in the tank reactor from levels as high as 125 ppm perchlorate to non-detectable levels (Figure 2). The tank reactor had a residence time of approximately 46 h compared to 0.46 h for the UAFBR. We propose that the bacterial population sloughed off from the biofilm and unused nutrients from the reactor allowed the further reduction of perchlorate in the tank reactor, but because perchlorate was reduced to non-detectable concentrations it was impossible to determine an accurate rate of perchlorate reduction.

W. succinogenes HAP-1 cell numbers ranged from 2.0×10^8 to 9.1×10^8 cells g^{-1} of pellet (Table 1). Cell numbers for other bacteria ranged from 5.8×10^8 to 1.05×10^9 g^{-1} of pellet (Table 1). Bacterial populations remained stable during the operating period even though minor perturbations in medium, pH, nitrogen addition, and reactor temperature were experienced. *W. succinogenes* HAP-1 comprised approximately 29–48% of the total bacterial population during the 42-day operation period.

Confirmation of the presence of a bacterial biofilm and evidence that bacteria had penetrated the pellets and attached to external and internal surfaces were provided by scanning electron microscopy of the pellet material. Electron micrographs of diatomaceous earth pellets are shown in Figure 4. The electron micrographs confirm the formation of a biofilm with several bacterial cell morphologies from the mixed population consortium. Micrographs of the external and internal surfaces of pellet material from the reactor allowed comparison of cell numbers internally and

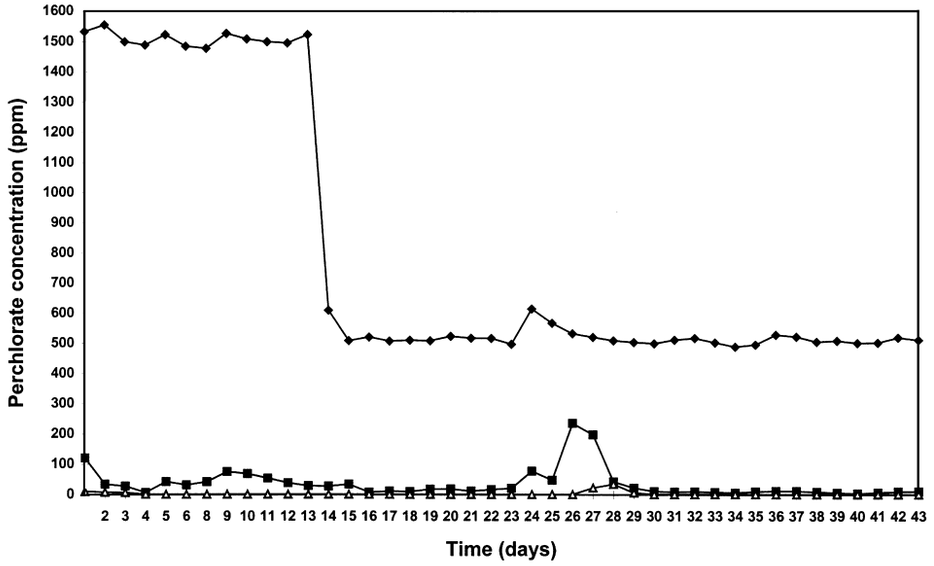

Figure 2 Perchlorate concentrations in the feed stream, effluent of UAFBR and tank reactor during the 42 days of operation. —◆—, Reactor feed; —■—, reactor effluent; —△—, tank reactor effluent.

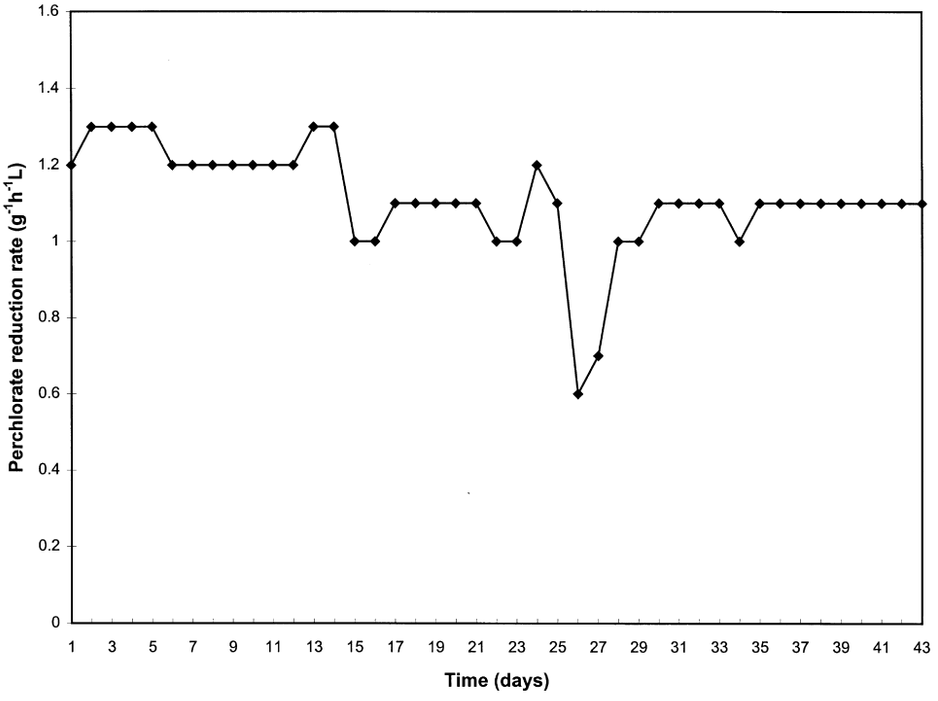

Figure 3 Rates of perchlorate reduction in the UAFBR (g perchlorate reduced h^{-1} L^{-1}).

externally on the pellets. Approximately three times the cells were found on the external surface compared to the internal surface.

Discussion

Recent reports of large-scale contamination of ground water with perchlorate [5,6], combined with perchlorate being a suspected endocrine disruptor [18], have led to interest in perchlorate removal from large volumes of water. Perchlorate is very stable in ground water and would not have an appreciable chemical decomposition rate (Figure 1). To our knowledge, this is the first time the abiotic decomposition of perchlorate has been examined in the presence of organic material under anaerobic conditions. Anaerobic conditions were selected to closely represent the environment of perchlorate in groundwater.

Reactor design for perchlorate degradation initially focused on CSTRs which were effective for waste streams with high concentrations of perchlorate and low liquid vol-

Table 1 Bacterial numbers (colony forming units, CFU) for the up-flow anaerobic fixed bed reactor

Time (days)	*W. succinogenes* HAP-1	Other bacteria
2	10[a] (11)[b]	26 (3)
16	21 (7)	53 (0.7)
30	91 (5)	103 (2)
42	77 (18)	95 (6)

[a]Numbers reported as 10^7 CFU g^{-1} wet weight diatomaceous earth pellet. Pellets were removed from the reactor, plated anaerobically on AP agar and incubated at 40°C for 48 h before counting.
[b]Standard deviation given in parentheses.

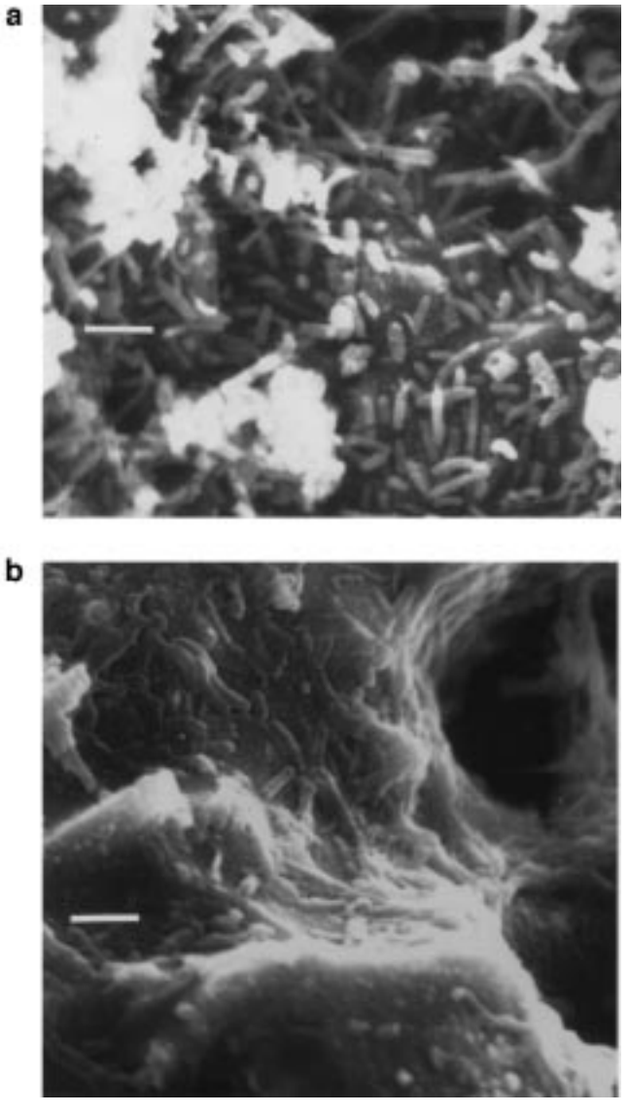

Figure 4 Scanning Electron Micrographs (SEM) of external and internal surfaces of diatomaceous earth pellets from the UAFBR. The sample was taken on day 20 with the reactor feed at 509 ppm perchlorate, effluent concentration 18 ppm perchlorate and a residence time of 0.46 h. Bar = 2 μm. (a) External surface of diatomaceous earth pellet. (b) Internal surface of diatomaceous earth pellet.

umes [1,2,12]. This study utilized large volumes of liquid contaminated with low perchlorate concentrations to simulate a ground water contamination situation. A fixed bed reactor allows treatment of large volumes of dilute liquid waste while maintaining the bacterial population. Previous studies in our laboratory using a sand matrix resulted in limited perchlorate reduction due to poor colonization of the sand matrix. Calcined diatomaceous earth was selected because of its internal pores and positive surface charge at neutral pH. It was reasoned that the negatively charged bacteria should attach and colonize effectively. Effective perchlorate removal with reactor residence times of 0.46 h demonstrated that a functional biofilm had been established in the reactor since anaerobic cultures growing at 25°C would be expected to have generation times well over 2 h [1,2]. Viable counts of bacteria attached to the matrix suggested that a stable bacterial population existed in the reactor under high flow conditions and demonstrated that *W. succinogenes* HAP-1 was maintained at 29–48% of the bacterial population during the operation period. Previous results from the CSTR have indicated rates for perchlorate reduction are optimal when the *W. succinogenes* HAP-1 organism comprises 30–50% of the bacterial population [2]. Electron micrographs demonstrated that cells were densely colonizing both external and internal surfaces of the diatomaceous earth pellets by day 20 of reactor operation.

The ability to establish a functional biofilm of a mixed culture which degrades perchlorate at rates of 1 g h^{-1} L^{-1} broadens the application of various treatment regimes for perchlorate waste water. The highest perchlorate reduction rates obtained to date with the CSTR were 0.5–0.7 g h^{-1} L^{-1}. Nutrients must be added to CSTR systems to maintain a biomass, regardless of the perchlorate concentration, resulting in very high nutrient ratios lb^{-1} of perchlorate destroyed. The use of a CSTR results in higher COD and BOD in the reactor effluent due to the discharge of nutrients and biomass. Nutrient costs for large-scale biotreatment of perchlorate wastewater potentially limits the process for industrial applications. A fixed film reactor which allows maintenance of biomass should reduce the nutrient cost of continual biomass replacement and significantly reduce the effluent COD and BOD.

Waste treatment bioreactors often experience fluctuating operating conditions. Bacterial cells within a biofilm are more resistant to perturbations in the system than free swimming organisms in a liquid matrix [14,22]. Studies with biofilms have demonstrated that immobilized cells are less susceptible to changes in temperature, pH, biocides, and oxygen [13,14]. The ability to maintain a perchlorate reduction rate of 1 g h^{-1} L^{-1} for 42 days demonstrates that the biofilm population was very stable. CSTRs achieved average perchlorate reduction rates of 0.22 to 0.41 g h^{-1} L^{-1}. Nutrient upsets rapidly reduced these rates to 0.087 g h^{-1} L^{-1} [20]. This wide range of rates suggests that the free living cells were more susceptible to system perturbations than organisms within a biofilm.

Biofilms also allow bacteria to associate with other microbes to enhance degradation processes [13]. The perchlorate molecule is reduced to chloride and oxygen by one bacterial organism, but perchlorate reduction in a waste stream requires several bacterial members [1,2,24,25]. Ten

to twelve different cell morphologies, including both Gram-positive and Gram-negative bacteria, have been identified as stable members of the bacterial consortium which reduces perchlorate. These results suggest a biofilm possibly facilitated interactive processes between consortium members resulting in enhanced rates of perchlorate reduction. This study demonstrates the efficiency of attached organisms for the reduction of perchlorate. The information generated in this study will allow us to consider other treatment designs which employ a biofilm to accomplish perchlorate reduction in various environmental settings.

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