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Inhibition of perchlorate reduction by nitrate in a fixed biofilm reactor

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ABSTRACT

Perchlorate and nitrate were reduced simultaneously in fixed biofilm reactors. Reduction of 1000 μg L^{-1} perchlorate decreased slightly with the addition of 10–16 mg L^{-1} NO_3–N when excess acetate was supplied while denitrification was complete. When influent acetate was reduced by 50% to well below the stoichiometric requirement, perchlorate reduction decreased by 70% while denitrification decreased by only 20%, suggesting that competition for electrons by nitrate was a factor in inhibition. Reduction of nitrate was favored over perchlorate, even though reactor biofilm had been enriched under perchlorate-reducing conditions for 10 months. When excess acetate was restored, perchlorate and nitrate returned to initial levels. The average most probable numbers of perchlorate- and nitrate-reducing bacteria during excess substrate operation were not significantly different and ranged between 2.0×10^5 and 7.9×10^5 cells cm^{-2} media surface area.

The effect of nitrate on chloride generation by suspensions of perchlorate-reducing populations was studied using a chloride ion probe. The rate of reduction of 2 mM perchlorate decreased by 30% in the presence of 2 mM nitrate when excess acetate was added. When acetate was limited, perchlorate reduction decreased by 70% in the presence of equi-molar nitrate.

1. Introduction

Bacteria reduce perchlorate (ClO_4^-) to chloride (Cl^-) in a dissimilatory respiration process and perchlorate-reducing populations enriched from environmental media have been used in a variety of treatment process configurations [1,2]. However, other electron acceptors: oxygen, nitrate and sulfate, which are often present in perchlorate contaminated waters may limit the effectiveness of microbial perchlorate reduction processes. Of 228 sites in California contaminated with perchlorate, nitrate was found at 215 with concentrations ranging from 5 to as high as 27 mg L^{-1} NO_3–N. Perchlorate measured at those sites was significantly lower: 10–70 μg L^{-1} ClO_4^- [3]. Inhibition of perchlorate reduction by oxygen has been widely reported, and generally attributed to higher energy yields from reduction of oxygen compared with perchlorate [1,4].

The effect of nitrate on (per)chlorate respiration appears to be more complex than that of oxygen. Malmqvist and Welander [5] isolated four strains of chlorate reducing bacteria from Kraft waste which also grew with nitrate as the electron acceptor. Reduction of chlorate in activated sludge was suppressed immediately when nitrate was introduced into the growth medium; whereas ferric iron, Mn(IV), and sulfate had no effect on chlorate reduction [4]. Strains of denitrifying bacteria have been reported to reduce perchlorate to chloride using a dissimilatory nitrate reductase as well as specialized (per)chlorate reductases [2,6]. Herman and Frankenberger [7] reported that perchlorate and nitrate were reduced simultaneously by a bacteria isolated from biosolids, *perclace*, but the perchlorate reduction rate decreased slightly in the presence of nitrate. Two different reductases, nitrate reductase and perchlorate reductase, were found in *perclace* cultures in different locations in the cell indicating that both enzyme systems could be operative in a single organism allowing parallel reduction of the two oxyanions [8]. Xu et al. [9] observed lag times for induction of perchlorate, chlorate, and nitrate reductases in aerobically grown cultures of *Dechlorosoma* sp. KJ. However, there was little denitrification activity by strain KJ when grown on chlorate or perchlorate, and cultures enriched with nitrate as the electron acceptor did not reduce perchlorate immediately. It was concluded that there were two substrate-induced enzyme systems for nitrate and chlorate in strain KJ, consistent with the *perclace* study [9]. However, dual enzyme systems for nitrate and (per)chlorate are not universal. Strains of chlorate-reducing bacteria, *Pseudomonas chloritidismutans*, sp. nov and *Pseudomonas* sp. PDA, were isolated that did not reduce nitrate, indicating the existence of diverse (per)chlorate reduction pathways [9,10]. In a review of denitrification enzyme research, Hochstein and Tomlinson [11] cited a finding that type A nitrate reductases also reduce chlorate and bromate.

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The presence of two reductase enzymes does not necessarily mean that both reduction processes will occur simultaneously or in sequence. Complete dissimilatory reduction of nitrate (denitrification) involves four reductases for nitrate, nitrite, nitric oxide, and nitrous oxide, to produce dinitrogen gas. However, complete denitrification can be disrupted by many environmental factors such as oxygen, substrate availability, and even light [12,13]. van Rijn et al. [14] observed that certain substrates caused preferential flow of electrons to nitrate reductase resulting in accumulation of nitrite in cultures of *Pseudomonas stutzeri*. Stoichiometric accumulation of nitrite occurred in suspended mixed cultures at high pH (9.3) even though sufficient substrate was available for complete denitrification [15]. However, nitrite accumulation during denitrification under substrate-limited conditions was thought to be the result of competition for electrons between nitrate and nitrite reductases since complete denitrification resumed when additional substrate was supplied [16].

Previous research has resulted in identification of the enzyme systems responsible for perchlorate and nitrate reduction and interactions between the two processes in both pure and mixed cultures. However, operation of mixed culture biological processes for treatment of many perchlorate-contaminated source waters will be enhanced by understanding the effects of inhibition and/or competition by nitrate, which is commonly found with perchlorate, often at significantly higher concentrations.

The primary objective of this research was to investigate inhibition of perchlorate reduction by nitrate in mixed cultures, evaluating the use of substrate supply and internal recirculation to improve a biofilm perchlorate reduction process when nitrate was present.

2. Materials and methods

2.1. Biofilm reactors

Activated sludge was collected from a local municipal wastewater treatment plant and acclimated to use perchlorate as the sole electron acceptor in a sequencing batch reactor (SBR) operated under anoxic conditions. SBR influent contained 150 mg L^{-1} perchlorate and 200 mg L^{-1} acetate. Ammonium and phosphate were added to achieve a C:N:P ratio of 100:12:3, along with trace minerals. After enrichment, SBR suspended solids were recirculated for 48 h to inoculate two identical biofilm reactors (0.7 m LW, 15 cm ID) packed with high-porosity plastic media (specific gravity 0.9, media porosity 0.92). After inoculation, both reactors were operated in upflow mode with influent flow rate of 37.5 L d^{-1} corresponding to an empty bed contact time (EBCT) of 8 h. Influent to one reactor was treated in a single-pass dispersed plug flow regime, while the second reactor had internal recirculation at a ratio of 20:1 recirculation:influent flow rate. Before nitrate addition began, the reactors had received influent containing 1000 μg L^{-1} perchlorate as the only electron acceptor with 7 mg L^{-1} acetate as the electron donor.

For the nitrate inhibition experiments, two concentrations of nitrate were added to the influent, 10 and 16 mg L^{-1} NO_3–N, along with 1000 μg L^{-1} perchlorate. Acetate was increased to 50 mg L^{-1} to achieve a C:N mass ratio of approximately 2:1 which has been reported to be sufficient for denitrification [16]. At the higher level of nitrate addition, acetate was increased to 100 mg L^{-1} for a C:N ratio of 2.5:1. With 16 mg L^{-1} NO_3–N in the influent, the molar ratio of nitrate to perchlorate was 114:1, so it was assumed that the stoichiometric requirement for acetate for perchlorate reduction would be met with the addition of excess acetate for denitrification. For the substrate limitation experiment, the influent acetate was reduced to 50 mg L^{-1} resulting in a C:N ratio of 1.3:1 at the higher nitrate influent concentration.

2.2. Monitoring perchlorate reduction by chloride generation

The effects of oxygen, nitrate, and substrate availability on perchlorate reduction were measured using mixed liquor that was transferred from SBR cultures. For preparation of chloride generation test, 240 mL of mixed liquor was rinsed to remove chloride, dewatered by centrifuge, and diluted into 250-mL flasks containing 200 mg L^{-1} (2 mM) perchlorate. Oxygen was added through a diffuser stone for a flask medium concentration of 7 mg L^{-1}. The initial flask nitrate concentration was 28 mg L^{-1} (2 mM). Anoxic tests were carried out with N_2 in the flask headspace. Phosphate buffer was added to flask media to maintain pH at 7.0. Perchlorate reduction was monitored during the 60-min tests using a chloride electrode (Denver Instrument Co., PN:300742.0) and the method described by Xu and Logan [17]. The high perchlorate concentration was used for two reasons. First was to achieve a rapid reaction rate, necessary because the chloride probe was prone to drifting out of calibration after 1–2 h of continuous operation. Second, the detection level of the chloride probe was relatively high, over 1 mg L^{-1}, necessitating a significant amount of perchlorate reduction. Flask media all contained approximately 5 g L^{-1} MLSS from the SBR.

2.3. Analytical methods and MPN technique

Three sampling ports were located at equal intervals at the base, middle, and top of the media bed in the biofilm reactors. Liquid samples were taken at each sampling port in the biofilm reactor columns by using 6-in. (15.24 cm) long needles. All samples were filtered through 0.2 μm syringe filters and stored at 4 °C before analysis.

Anions (ClO_4^-, NO_3^-, NO_2^-, Cl^-, and CH_3COO^-) were monitored by using an ion chromatograph (Dionex model 300 DX, Dionex, Sunnyvale, CA) with IonPac AS11 analytical and AG11 guard column. Perchlorate was measured with 100 mM NaOH eluent and a 1000 μL sample loop. Nitrite was also measured occasionally by colorimetric analysis (diazotization method, # 8507) using a spectrophotometer (Model DR2200, Hach Chemical Co., Loveland, CO).

Perchlorate and nitrate reducing bacteria in the biofilm were enumerated using the micro-plate MPN technique developed by Rowe et al. [18]. A redox dye, resazurin, was used in the MPN media to indicate consumption of perchlorate or nitrate [19]. MPN tests for perchlorate reducing bacteria and nitrate reducing bacteria were conducted separately by the control of growth medium (perchlorate or nitrate). For each MPN assay, four rings attached biofilm were mixed with 35 mL of extraction solution (0.3% sodium pyrophosphate, 0.22% sodium polyphosphate, 0.1% Tween 80, Na_2CO_3 buffer for pH 7) and placed in a 50-mL sterile centrifuge tube. Tubes were shaken on a rotary shaking table for 12 h. MPN incubations were done using 10-fold serial dilutions of eight replicates of placed into 0.32-mL wells of sterile tissue culture plates. After inoculation, MPN plates were placed in inflatable bags under N_2 gas and incubated in the dark for 5 weeks at 22 °C.

3. Results and discussion

3.1. Perchlorate reduction in the presence of nitrate

When excess acetate was added to perchlorate in the absence of nitrate, perchlorate was reduced to below detection level (4 μg L^{-1}) in both biofilm reactors with no benefit from recirculation observed. When 10 mg L^{-1} NO_3–N was added, effluent perchlorate increased to 14 ± 31 μg L^{-1} in the plug flow reactor and 1 ± 1 μg L^{-1} in the reactor with recirculation. Neither increase was significant at the 5% level. Relative progress of the two reduction

Table 1
Comparison of effluent perchlorate in column biofilm reactors receiving 1000 μg L^{-1} ClO_4^- and variable nitrate in the influent

	Control–No nitrate (μg L^{-1} ClO_4^-)	10 mg L^{-1} NO_3–N added (μg L^{-1} ClO_4^-)	16 mg L^{-1} NO_3–N added (μg L^{-1} ClO_4^-)
Plug flow reactor	3 ± 4	14 ± 31	19 ± 18
Recirculation reactor	BDL	1 ± 1	6 ± 4
Number (n)	9	10	7

BDL: below detection level for perchlorate, 4 μg L^{-1} ClO_4^-. Data are averages of n samples ±1S.D. Samples below detection level were considered as "zero" for averaging.

Table 2
MPN per unit media surface area for ClO_4^- and NO_3^- reducing bacteria in biofilm reactors with 1000 μg L^{-1} ClO_4^- and excess acetate addition

Influent NO_3–N (mg L^{-1})	ClO_4^- reducing bacteria (cells cm^{-2})		NO_3^- reducing bacteria (cells cm^{-2})	
	Plug flow	Recirculation	Plug flow	Recirculation
10	$1.03 \times 10^6 \pm 8.21 \times 10^5$	$7.81 \times 10^5 \pm 5.44 \times 10^5$	$5.95 \times 10^5 \pm 7.12 \times 10^5$	$5.59 \times 10^5 \pm 6.48 \times 10^5$
16	$2.02 \times 10^5 \pm 8.36 \times 10^4$	$7.88 \times 10^5 \pm 5.62 \times 10^5$	$5.55 \times 10^5 \pm 5.92 \times 10^5$	$4.03 \times 10^5 \pm 4.15 \times 10^5$

MPN values have been normalized per unit area of media surface. Data are averages of biofilm samples taken from three different reactor locations ±1S.D.

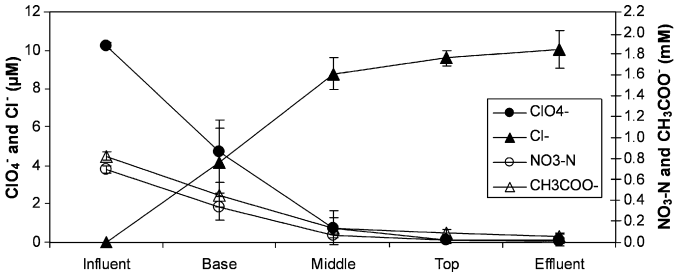

Fig. 1. ClO_4^-, NO_3^-, CH_3COO^-, and Cl^- concentration profiles in the plug flow biofilm reactor receiving 10 mg L^{-1} NO_3–N and 1000 μg L^{-1} ClO_4^-. Error bars are ±1S.D.

reactions in the plug flow reactor, is shown in Fig. 1, which contains profiles for ClO_4^-, NO_3^-, CH_3COO^-, and Cl^- with influent containing 10 mg L^{-1} NO_3–N, 1000 μg L^{-1} ClO_4^-, and 50 mg L^{-1} acetate. Chloride was produced in stoichiometric amounts confirming complete reduction of perchlorate in the biofilm column reactors.

When influent nitrate was increased to 16 mg L^{-1} NO_3–N and acetate to approximately 100 mg L^{-1}, effluent ClO_4^- increased to 19 μg L^{-1} in the plug flow reactor. The recirculation reactor performance was better with 6 μg L^{-1} ClO_4^- in the effluent. Perchlorate, nitrate, and acetate profiles in the plug flow reactor for the higher nitrate addition are shown in Fig. 2. The plug flow reactor profiles in Figs. 1 and 2 indicate that perchlorate and nitrate reduction were occurring simultaneously along the flow path. However, increasing the influent nitrate concentration from 10 to 16 mg L^{-1} NO_3–N did result in higher effluent perchlorate even when sufficient acetate was added to meet stoichiometric requirements. The fact that the profiles for the two electron acceptors virtually overlay one another suggests that when sufficient substrate is present, these processes can occur in parallel. Had nitrate reduction been favored over perchlorate reduction, a lag in perchlorate consumption would have

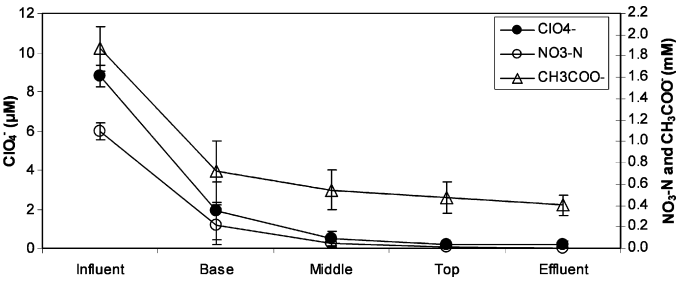

Fig. 2. ClO_4^-, NO_3^-, and CH_3COO^- concentration profiles in the plug flow biofilm reactor receiving 16 mg L^{-1} NO_3–N and 1000 μg L^{-1} ClO_4^-. Error bars are ±1S.D.

been expected. Complete denitrification was observed the day after nitrate addition began, and no nitrite was detected in any samples, indicating that induction of denitrification was very rapid in the mixed culture biofilm. Other researchers observed transient nitrite accumulation after nitrate was added to *perclace* cultures grown on perchlorate [7].

Table 1 has effluent perchlorate concentration data with 0 (control), 10, and 16 mg L^{-1} NO_3–N added. In the plug flow reactor, addition of nitrate at concentrations of both 10 and 16 mg L^{-1} NO_3–N resulted in an increase in perchlorate from an average of 3 to 14 μg L^{-1} and 19 μg L^{-1}, respectively. The effluent perchlorate for the reactor with internal recirculation increased from consistently below the detection level to averages of 1 and 6 μg L^{-1} ClO_4^- after addition of 10 and 16 mg L^{-1} NO_3–N, respectively. The increase in effluent perchlorate when 16 mg L^{-1} NO_3–N was added to the recirculation reactors was significant ($p \leq 0.05$); the increase in effluent perchlorate from the plug flow reactor was greater, but not significant because of the high variance ($p = 0.053$).

The most probable numbers of perchlorate and nitrate nitrate-reducing bacteria are shown in Table 2. The MPN values for perchlorate and nitrate-reducing populations were not significantly different, in spite the large difference in concentrations of perchlorate (0.01 mM) and nitrate (0.7–1.1 mM). One explanation is that the same cells could use either electron acceptor and grew in the MPN enrichment using whichever was present. Herman and Frankenberger [7] reported simultaneous nitrate and perchlorate reduction in batch and column studies using the isolate *perclace*. However, there was little nitrate reduction by cultures of *Dechlorosoma* sp. KJ enriched on perchlorate or chlorate media [9]. The rapid initiation of denitrification in the reactors which had received only perchlorate as the electron acceptor for over 10 months suggests that either the perchlorate reductase enzymes had the capability to reduce nitrate and nitrite, some biofilm cells were constitutive for denitrification enzymes, or that induction of denitrifying enzymes in perchlorate-reducing cells was very rapid.

3.2. Substrate limitation

The importance of competition for substrate electrons was investigated in the biofilm reactors receiving 1000 μg L^{-1} ClO_4^- and 16 mg L^{-1} NO_3–N while influent acetate was decreased from 100 to 50 mg L^{-1}. This resulted in a decrease in the C:N ratio from 2.5:1 to 1.3:1 and a significant decline in perchlorate reduction ($p \leq 0.05$). As shown in Fig. 3, in the plug flow reactor, effluent perchlorate rose from 19 to 200 μg L^{-1} in 2 days. After 10 days had risen to nearly 700 μg L^{-1}. Effluent perchlorate from the recirculation reactor increased more slowly, reaching to 700 μg L^{-1} after 21

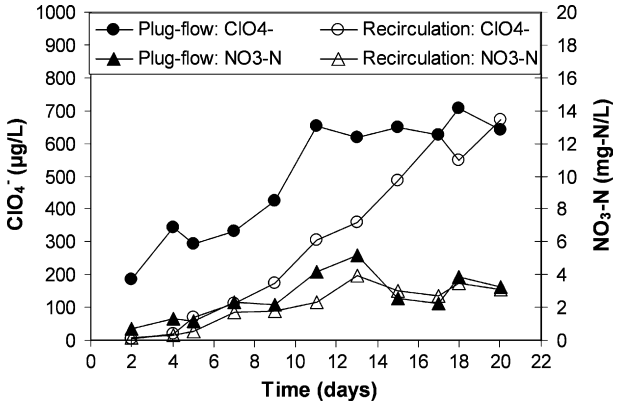

Fig. 3. Effluent perchlorate and nitrate in the plug flow and recirculation biofilm reactors when carbon was limited. Influent contained 1000 µg L^{-1} ClO_4^-, 16 mg L^{-1} NO_3–N and 52 mg L^{-1} acetate (C:N = 1.3:1). Time is days after acetate was decreased.

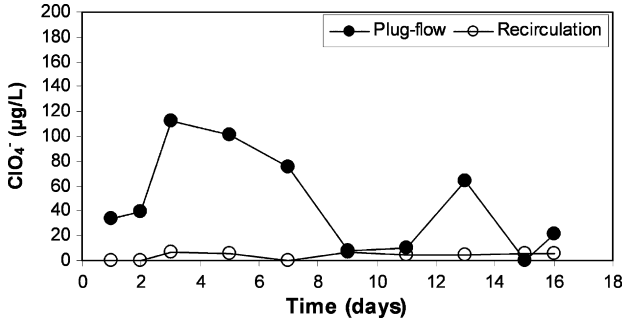

Fig. 4. Effluent ClO_4^- from the biofilm reactors after restoration of influent acetate to C:N = 2.5:1. Time is days after acetate addition was increased.

days. Effluent nitrate increased from detection level to an average of 3 mg L^{-1} NO_3–N in both biofilm reactors. No nitrite (NO_2^-) accumulation was observed in the biofilm reactors during substrate-limited operation, which was unexpected since nitrite accumulation during substrate-limited denitrification has been reported by other investigators [16].

Influent acetate was restored to initial levels (C:N = 2.5:1) after 3 weeks to evaluate the recovery of perchlorate reduction and denitrification in the plug flow and recirculation reactors. Fig. 4 has effluent perchlorate data after acetate was restored to the initial (excess) level. Complete perchlorate and nitrate reduction was observed in the recirculation reactor within 1 day after acetate was restored. However, it was more than a week before comparable effluent perchlorate and nitrate concentrations were seen in the plug flow reactor effluent. (Nitrate data not shown.) Nine days after restoration of influent acetate, average effluent perchlorate concentrations from the plug flow and recirculation reactors were 62 and 3 µg L^{-1}, respectively.

MPN data for perchlorate and nitrate reducing bacteria reactors are compared in Table 3 for the plug flow and recirculation reactors, respectively. Biofilm samples were collected from three locations

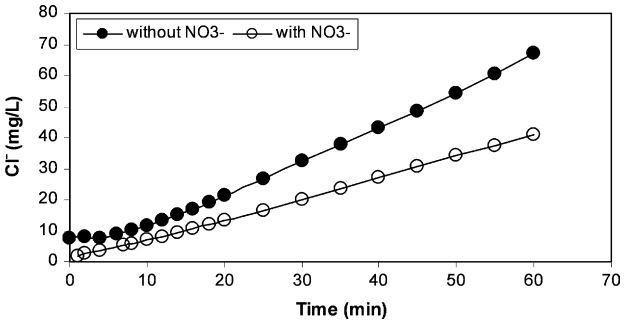

Fig. 5. Effect of 2 mM nitrate on reduction of 2 mM perchlorate in flasks inoculated with perchlorate-reducing suspension, average MLSS in flasks was 5 g L^{-1}. Excess acetate (6.7 mM) was added to both flasks.

in each reactor after 35 days of operation at 2.5:1 C:N ratio, after 24 days at 1.3:1 C:N ratio; and 21 days after acetate was restored to 2.5:1 C:N. After 24 days of substrate-limited operation, the MPN of perchlorate-reducing bacteria decreased by over two log units in both the plug flow and recirculation reactors. Decrease in the denitrifying population MPN in both reactors was less, approximately 1.5 log units. After restoration of acetate addition, the MPN's for perchlorate and denitrifying bacteria increased more rapidly in the recirculation reactor, although the numbers had not reached the pre-substrate limitation values in either reactor after 21 days.

It was hypothesized that one benefit of recirculation was dilution of competing electron acceptors, slowing diffusion of nitrate into the biofilm. Published values of the diffusion coefficient, D, for NO_3^- and ClO_4^- at infinite dilution in water are 1.792 and 1.902×10^{-5} cm^2 s^{-1} [20]. The biofilm diffusion coefficients for nitrate and perchlorate were calculated to be 1.3 and 1.2×10^{-5} cm^2 s^{-1}, respectively using a biofilm retardation factor (porosity = 0.879) reported by other researchers [21] based on biofilm thickness value (50–60 µm) which was obtained in the previous research [22]. With similar diffusion transport coefficients, dilution of influent nitrate by recirculation could have reduced the effect of substrate competition on perchlorate reduction, especially at the inlet region of the biofilm reactor.

3.3. Chloride electrode test

Direct measurement of inhibition of perchlorate reduction by nitrate and oxygen were conducted using suspended cultures to minimize transport effects associated with the biofilm reactors. Profiles of chloride generation in flasks containing 2 mM perchlorate (200 mg L^{-1}) either alone or with 2 mM nitrate (28 mg L^{-1} NO_3–N) are shown in Fig. 5. Excess acetate was added at a concentration of 6.67 mM (400 mg L^{-1}). Perchlorate-reducing suspension concentrations in the flasks were between 4.8 and 5.7 g L^{-1} MLSS. The slopes of the chloride accumulation profiles were calculated by linear regression. The specific chloride generation rate for the suspended culture with perchlorate as the only electron acceptor was 0.21 mg-Cl g-MLSS^{-1} min^{-1}. When nitrate was present, the chloride generation rate dropped by one-third to

Table 3
MPN of ClO_4^- and NO_3^- reducing bacteria per unit media surface area throughout combined perchlorate reduction and denitrification operation in the plug flow and recirculation biofilm reactors

Carbon addition	ClO_4^- reducing bacteria (cells cm^{-2})		NO_3^- reducing bacteria (cells cm^{-2})	
	Plug flow	Recirculation	Plug flow	Recirculation
Excess	$2.02 \times 10^5 \pm 8.36 \times 10^4$	$7.88 \times 10^5 \pm 5.62 \times 10^5$	$5.55 \times 10^5 \pm 5.92 \times 10^5$	$4.03 \times 10^5 \pm 4.15 \times 10^5$
Limitation	$1.28 \times 10^3 \pm 5.07 \times 10^2$	$1.11 \times 10^3 \pm 2.65 \times 10^1$	$9.80 \times 10^3 \pm 8.51 \times 10^3$	$1.23 \times 10^4 \pm 5.01 \times 10^3$
Restore	$1.05 \times 10^3 \pm 7.66 \times 10^1$	$1.55 \times 10^4 \pm 5.99 \times 10^3$	$6.12 \times 10^3 \pm 4.50 \times 10^3$	$4.81 \times 10^4 \pm 5.94 \times 10^4$

Influent C:N mass ratio was 2.5:1 during "Excess" and "Restore" sample periods, and C:N = 1.3:1 during "Limitation".

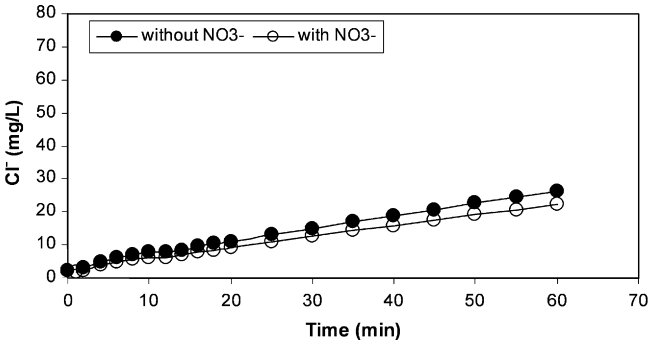

Fig. 6. Effect of 2 mM nitrate on reduction of 2 mM perchlorate in flasks inoculated with perchlorate-reducing suspension, average MLSS in flasks was 5 g L^{-1}. Acetate added to control flask was 0.17 mM, and 0.42 mM acetate was added to the flask medium containing both nitrate and perchlorate.

0.14 mg-Cl g-MLSS^{-1} min^{-1}. When acetate was limited (0.17 mM) and perchlorate (2 mM) the only electron acceptor, the chloride generation rate decreased to 0.07 mg-Cl g-MLSS^{-1} min^{-1}. When nitrate was added with perchlorate in a flask with limited acetate, the chloride generation rate decreased 14% over the flask with only perchlorate added, to 0.06 mg-Cl g-MLSS^{-1} min^{-1} (Fig. 6). As a comparison to the effect of nitrate, chloride generation also was measured for an aerated flask and an anoxic control. Chloride generation stopped after 5 min when 7 mg L^{-1} dissolved oxygen was present even with 8 mM acetate added (data not shown), confirming that the perchlorate reducing bacteria in the enriched cultures were facultative anaerobes, and that oxygen was not used simultaneously with perchlorate in the mixed cultures.

Statistical comparison of the rates using a *t*-test confirmed that the specific perchlorate reduction (chloride production) rate decreased significantly in the presence of nitrate at equal molarity ($p \leq 0.05$) although the flask suspensions had never been exposed to nitrate-reducing conditions. Acetate limitation also resulted in a significant decrease in the specific perchlorate reduction rate, with or without nitrate present ($p \leq 0.05$). The complete inhibition of perchlorate reduction by oxygen suggests a different respiration control mechanism for oxygen than that of nitrate. The effect of oxygen was so rapid that preferential shunting of electrons to aerobic electron transfer enzymes may have occurred, bypassing perchlorate reductases. A different mechanism was suggested by Stouthamer [23] who found that oxygen inhibited nitrate reduction by *Paracoccus denitrificans* by preventing cellular uptake of nitrate. Perchlorate reduction continued in the presence of nitrate, although at a reduced rate. The effect of nitrate was also immediate which might suggest the recruitment of the perchlorate reducing enzymes already present in the cells to use nitrate as the electron acceptor, rather than induction of separate nitrate reductases, which might have delayed inhibition.

4. Conclusions

This research investigated the nitrate effect on perchlorate reduction in a fixed biofilm reactor with two different flow regimes. Based on the observed data, the following conclusions were drawn as below:

(1) Reduction of influent nitrate at concentrations of 10 and 16 mg L^{-1} NO_3–N occurred simultaneously with reduction of 1000 μg L^{-1} perchlorate when acetate was added in a mass C:N ratio of 2:1. No acclimation period for denitrification in the columns was required, although the biofilm populations had not been exposed to nitrate during 10 months of operation.

(2) For the plug-flow reactor, effluent ClO_4^- increased from 3 ± 4 to 19 ± 18 μg L^{-1} with the higher nitrate addition. In the recirculation reactor, effluent ClO_4^- increased from uniformly below the detection level to 6 ± 4 μg L^{-1}.

(3) Populations of perchlorate and nitrate reducing bacteria measured by the MPN method were statistically the same in both the plug flow and recirculation reactors suggesting that the perchlorate-acclimated biomass was capable of reducing nitrate as well. This result was consistent with simultaneous perchlorate and nitrate reduction measured in the column liquid samples.

(4) When influent acetate was decreased by 50%, ClO_4^- and NO_3^- reduction both decreased significantly: ClO_4^- reduction declined from 100 to 30% and NO_3^- reduction decreased from 100 to 80%, suggesting that nitrate reduction was favored under substrate limited conditions. The recirculation reactor was more resistant to inhibition during the first few days of substrate-limited operation, but after 3 weeks, both flow regimes exhibited the same loss of performance. MPN's of both perchlorate bacteria decreased by over two logs after 21 days of substrate limitation.

(5) After influent acetate was restored to the 2.5:1 ratio, effluent ClO_4^- and NO_3^- decreased to previous levels in the reactor with flow recirculation within 1 day. Complete recovery in the plug flow reactor was not observed until 10 days after acetate was restored. MPN's for perchlorate and nitrate-reducing populations had not returned to previous levels after 16 days of recovery.

(6) Chloride evolution assays showed that perchlorate reduction was completely inhibited by oxygen at 7 mg L^{-1}, regardless of substrate availability suggesting that oxygen suppresses activity of the perchlorate reductase system. The perchlorate reduction rate decreased by 30% in the presence of an equimolar concentration of nitrate even when excess acetate was added, indicating that denitrification resulted in a decrease in the perchlorate reduction rate, but not complete inhibition.

In summary, this research demonstrated the simultaneous nitrate and perchlorate reduction in a fixed biofilm reactor with sufficient carbon addition and showed the fixed biofilm reactor with internal recirculation was more resistant to nitrate inhibition during carbon substrate limitation. The information obtained from this research will have application to the development of treatment processes to remove numerous other biodegradable contaminants that occur at low concentration in natural waters.

References

[1] J.D. Coates, U. Michaelidou, R.A. Bruce, S.M. O'connor, J.N. Crespi, L.A. Achenbach, Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria, Appl. Environ. Microbiol. 65 (12) (1999) 5234–5241.
[2] B.E. Logan, A review of chlorate- and perchlorate-respiring microorganisms, Biorem. J. 2 (2) (1998) 69–79.
[3] H. Wang, A. Eaton, B. Narloch, National assessment of perchlorate contamination occurrence: AwwaRF and AWWA, 2002.
[4] C.G. van Ginkel, C.M. Plugge, C.A. Stroo, Reduction of chlorate with various energy substrates and inocular under anaerobic conditions, Chemosphere 31 (9) (1995) 4057–4066.
[5] A. Malmqvist, T. Welander, Anaerobic removal of chlorate from bleach effluents, Water Sci. Technol. 25 (7) (1992) 237–242.
[6] G.B. Rikken, A.G.M. Kroon, C.G. van Ginkel, Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation, Appl. Microbiol. Biotechnol. 45 (3) (1996) 420–426.
[7] D.C. Herman, W.T. Frankenberger, Bacterial reduction of perchlorate and nitrate in water, J. Environ. Qual. 28 (1999) 1018–1024.
[8] T. Giblin, W.T. Frankenberger, Perchlorate and nitrate reductase activity in the perchlorate-respiring bacterium perclace, Microbiol. Res. 156 (4) (2001) 311–315.

[9] J. Xu, J.J. Trimble, L. Steinberg, B.E. Logan, Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA, Water Res. 38 (2004) 673–680.
[10] A.F.W.M. Wolterink, A.B. Jonker, S.W.M. Kengen, A.J.M. Stams, *Pseudomonas chloritidismutans* sp. nov., a non-denitrifying, chlorate-reducing bacterium, Int. J. Syst. Evol. Microbiol. 52 (2002) 2183–2190.
[11] L.I. Hochstein, G.A. Tomlinson, The enzymes associated with denitrification, Ann. Rev. Microbiol. 42 (1988) 231–261.
[12] Y. Barak, Y. Tal, J. van Rijn, Light-mediated nitrite accumulation during denitrification by *Pseudomonas* sp. Strain JR12, Appl. Environ. Microbiol. 64 (3) (1998) 813–817.
[13] S. Otte, N.G. Grobben, L.A. Robertson, M.S.M. Jetten, J.G. Kuenen, Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions, Appl. Environ. Microbiol. 62 (7) (1996) 2421–2426.
[14] J. van Rijn, Y. Tal, Y. Barak, Influence of volatile fatty acids on nitrite accumulation by a *Pseudomonas stutzeri* strain isolated from a denitrifying fluidized bed reactor, Appl. Environ. Microbiol. 62 (7) (1996) 2615–2620.
[15] C. Glass, J. Silverstein, Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation, Water Res. 32 (3) (1998) 831–839.
[16] J. Oh, J. Silverstein, Acetate limitation and nitrite accumulation during denitrification, J. Environ. Eng.-ASCE 125 (3) (1999) 234–242.
[17] J. Xu, B.E. Logan, Measurement of chlorite dismutase activities in perchlorate respiring bacteria, J. Microbiol. Methods 54 (2003) 239–247.
[18] R. Rowe, R. Todd, J. Waide, Microtechnique for most-probable-number analysis, Appl. Environ. Microbiol. 33 (3) (1977) 675–680.
[19] T.F. Guerin, M. Mondido, B. McClenn, B. Peasley, Application of resazurin for estimating abundance of contaminant-degrading micro-organisms, Lett. Appl. Microbiol. 32 (2001) 340–345.
[20] CRC Handbook of Chemistry and Physics, 74th ed., CRC.
[21] B.E. Logan, Environmental Transport Processes, Wiley-Interscience, 1999.
[22] H. Choi, J. Silverstein, Effluent recirculation to improve perchlorate reduction in a fixed biofilm reactor, Biotechnol. Bioeng. 98 (1) (2007) 132–140.
[23] A.H. Stouthamer, Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*, J. Bioenerg. Biomembr. 23 (2) (1991) 163–185.