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## ORIGINAL ARTICLE

# Modelling salinity inhibition effects during biodegradation of perchlorate

C. Park and E.A. Marchand

Department of Civil and Environmental Engineering, University of Nevada at Reno, Reno, NV, USA

**Keywords**

acclimation, biological reduction, high salinity, kinetic model, perchlorate.

**Correspondence**

Eric A. Marchand, University of Nevada at Reno, Department of Civil and Environmental Engineering, Mail Stop 258, Reno, NV 89557-0152, USA. E-mail: marchand@unr.edu

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**Abstract**

**Aims:** To determine the mathematical kinetic rates and mechanisms of acclimated perchlorate ( $\text{ClO}_4^-$ )-reducing microbial cultures by incorporating a term to relate the inhibitory effect of high salinity during biological reduction of concentrated perchlorate solutions.

**Methods and Results:** Salt toxicity associated with the biodegradation of concentrated perchlorate (200, 500, 1100, 1700 and 2400  $\text{mg l}^{-1}$  as  $\text{ClO}_4^-$ ) was investigated using two microbial cultures isolated from a domestic wastewater treatment plant [return activated sludge (RAS) and anaerobic digester sludge (ADS)]. Experiments were performed in wastewaters containing various sodium chloride concentrations, ranging from 0% to 4.0% (w/v) NaCl (ionic strength: 0.14–0.82  $\text{mol l}^{-1}$ , total dissolved solids: 5.3–42.6  $\text{g l}^{-1}$ ) at near-neutral values of pH (6.7–7.8). Perchlorate biodegradation was stimulated through stepwise acclimation to high salinity. The ADS culture was capable of reducing perchlorate at salinities up to 4% NaCl, while the RAS culture exhibited complete inhibition of perchlorate degradation at 4% NaCl, probably resulting from either a toxic effect or enzyme inactivation of the perchlorate-reducing microbes. Further, a kinetic growth model was developed based on experimental data in order to express an inhibition function to relate specific growth rate and salinity.

**Conclusions:** Biological reduction of concentrated perchlorate wastewaters using either acclimated RAS or ADS cultures is feasible up to 3% or 4% NaCl, respectively. In addition, the kinetic model including a salinity inhibition term should be effective in many practical applications such as improving reactor design and management, furthering the understanding of high salinity inhibition, and enhancing bioremediation under high salinity loading conditions.

**Significance and Impact of the Study:** Applications of these findings in water treatment practice where ion exchange or membrane technologies are used to remove perchlorate from water can have the potential to increase the overall attractiveness of these processes by eliminating the need to dispose of a concentrated perchlorate solution.

**Introduction**

Ammonium perchlorate (AP,  $\text{NH}_4\text{ClO}_4$ ) has been used extensively as the energetic oxidizer and principle ingredient (>75%) of propellants in solid fuel for rockets, missiles and other projectiles. AP has been produced at full-scale since the early 1950s in Nevada, CA, and other states

(United State Environmental Protection Agency: US EPA 2002; Hogue 2003). Large amounts of AP and other explosive-related compounds have been released at locations where rocket fuel is produced, refurbished, maintained and disposed, or at sites where rockets are tested and fired. Consequently, perchlorate has been detected in a variety of drinking water resources, both ground and surface water,

particularly in the south-western US (Schumacher 1960; Batista *et al.* 2002; Hogue 2003). Currently, the American Pacific Corporation (<http://www.apfc.com/>) is the largest producer of AP in the US and has estimated that the AP production will average about 7257–9072 metric tonnes per year between 1999 and 2008.

The contamination of groundwater by perchlorate is becoming an increasing concern for the water treatment industry throughout the US, as the highly soluble perchlorate anion (solubility of  $249 \text{ g l}^{-1}$  for  $\text{NH}_4\text{ClO}_4$ ) has been released in at least 20 states (Schilt 1979; US EPA 2002). The primary health concerns associated with perchlorate in drinking water are related to its inhibition of the normal function of the thyroid gland. Specifically, perchlorate interferes with the ability of the thyroid to process iodine (US EPA 2002). Furthermore, perchlorate is also believed to adversely affect the proper development of the nervous system in young children (Crump *et al.* 2000). Although perchlorate is not yet regulated at the federal level, the California Department of Health Services (CDHS: <http://www.dhs.cahwnet.gov/>) has recently lowered its provisional action level of  $18 \mu\text{g l}^{-1}$  for drinking water sources to  $6 \mu\text{g l}^{-1}$  to protect their drinking water consumers. The US EPA placed the perchlorate on the contaminant candidate list in 1998 (Perciasepe 1998) and the new CDHS recommendations may influence the promulgation of a National Primary Drinking Water Regulation for perchlorate.

At present, several types of water treatment systems have been tested and evaluated for their effectiveness at reducing perchlorate concentrations in water. Ion (anion) exchange systems and membrane treatment systems are promising technologies that have been identified for perchlorate treatment (Batista *et al.* 2000; Roquebert *et al.* 2000; Gu *et al.* 2001). However, these water treatment systems generate high concentrations of perchlorate ( $200\text{--}500 \text{ mg l}^{-1}$  as  $\text{ClO}_4^-$ ) and saline waste streams (i.e. due to the high affinity of perchlorate for anion exchange sites of ion exchange resins, high levels of regenerant brines with 6–12% NaCl are required), which require further treatment technologies before disposal (Gu *et al.* 2001; Batista *et al.* 2002; Gingras and Batista 2002). Biological treatment offers an attractive option for removing high concentrations of perchlorate from these waste streams, and can eliminate the needs for special perchlorate disposal of treatment system residuals.

During biological perchlorate treatment, perchlorate serves as the terminal electron acceptor, and ultimately becomes reduced to chloride and oxygen (Rikken *et al.* 1996). Several micro-organisms capable of reducing perchlorate as a terminal electron acceptor during the oxidation of organic compounds as an electron donor have been isolated from freshwater environments

(Malmqvist *et al.* 1994; Coates *et al.* 2000; Michaelidou *et al.* 2000). However, many of the freshwater perchlorate-reducing organisms isolated cannot thrive in high salinity environments such as those produced by ion exchange regenerant streams or membrane concentrate streams (Logan *et al.* 2001a). The growth of *Dechlorisoma suillus* strain PS and *Dechlorospirillum anomalous* strain WD, both isolated from swine waste lagoons, were completely inhibited at levels above 1% NaCl (Michaelidou *et al.* 2000). Another perchlorate-respiring microbe (*Dechlorimonas agitata* strain CKB) isolated from paper mill wastewaters grew well in environments with <2% NaCl (optimal growth in 1% NaCl), but no growth occurred at 4% NaCl (Coates *et al.* 2000). Furthermore, *Ideonella dechloratans* isolated from activated sludge could not grow at 3% NaCl (Malmqvist *et al.* 1994). A recent study reported that freshwater mixed cultures obtained from a municipal wastewater treatment plant were able to successfully degrade high concentrations of perchlorate ( $650 \text{ mg l}^{-1}$  as  $\text{ClO}_4^-$ ) in 3% NaCl wastewaters, through stepwise acclimation to high salinity (Park and Marchand 2002). We demonstrate here a model system to induce freshwater perchlorate-reducing microbes able to acclimate to high salinity environments within a practical range of salt tolerance, and this system is useful to solve current perchlorate problems under extreme conditions.

To date, most of the biological kinetic models (i.e. the Monod model or the Andrews model) have been developed solely on the basis of substrate concentration and the influence of the substrate concentration on the microbial growth rate (Monod 1942; Andrews 1968). However, in high salinity wastewaters, the concentration of NaCl exerts a marked influence in determining the overall growth kinetics when other growth factors such as pH, temperature, and required nutrients are not limiting (Coates *et al.* 2000; Logan *et al.* 2001a; Park and Marchand 2002). In spite of this fact, no one has described the mathematical kinetic relationship between the NaCl concentration and the specific microbial growth rate. Development of a growth rate model that incorporates the inhibitory effect of high salinity conditions would allow for better optimization of biological treatment processes operated under high salinity conditions.

The purpose of this study was to investigate the kinetic growth rates and mechanisms of acclimated perchlorate-reducing communities (return activated sludge, RAS and anaerobic digester sludge, ADS) growing in successively elevated NaCl concentrations (0–4%). In addition, the variation in growth rate was evaluated at different perchlorate concentrations (200, 500, 1100, 1700 and  $2400 \text{ mg l}^{-1}$   $\text{ClO}_4^-$ ). In all cases, the wastewater pH was maintained at near-neutral values (6.7–7.8) because it has been reported that the optimum growth of perchlorate-reducing

microbes occurred at pH values between 7.1 and 7.5 (Attaway and Smith 1993; Coates *et al.* 2000).

## Materials and methods

### Batch flask reactor (BFR)

The BFRs (1-l media bottle; Kimble Glass Inc., Vineland, NJ, USA) were used for both the preliminary and batch growth kinetic studies and had a liquid working volume of 750 ml with a headspace volume of approx. 250 ml to accommodate gas production. The BFRs were operated at room temperature (21–25°C) under anaerobic conditions on a shaker table at 150 rev min<sup>-1</sup> to maintain well-mixed conditions.

### Sequencing batch reactor (SBR)

The SBRs (Cytostir<sup>®</sup> Stirred Bioreactor; Kontes Glass Co., Vineland, NJ, USA) were used for the acclimated profile study and were operated to identify the perchlorate biodegradation capacities of RAS and ADS acclimated cultures at various salinity levels. The SBRs had a liquid working volume of 4 l with a headspace volume of approx. 1.5 l. Six ports were provided from two large side arms to accommodate the synthetic wastewater feed, acetate feed, nitrogen gas (N<sub>2</sub>) inlet and outlet, spent media removal (decant cycle) and sampling. Adjustable mixing was maintained by stirring blades which prevented biomass accumulation on the bottom of the reactor. Three bench-scale SBRs were run in parallel at room temperature under anaerobic conditions with cycles consisting of a fill period, a reaction period, a settle period and a decant period. In order to maintain an anaerobic environment the liquid media in the reactor and feed vessels was sparged with nitrogen gas before the mixing was initiated.

### Synthetic wastewater

The composition of the synthetic perchlorate wastewater brines used throughout the studies in the BFRs and SBRs is summarized in Table 1. The initial perchlorate concentration ranged from 610 to 650 mg l<sup>-1</sup> as ClO<sub>4</sub><sup>-</sup>. The NaCl concentration was varied in the wastewater solutions to achieve the desired salinity, and ranged from 0% to 4% (w/v) NaCl. The ionic strength of the wastewater brines calculated using the Debye-Hückel equation was ranged from 0.14 to 0.82 mol l<sup>-1</sup> (Morel and Hering 1993). Potassium acetate (CH<sub>3</sub>COOK) was utilized as the source of carbon and energy for the growth of micro-organism in all wastewaters with an initial acetate : perchlorate ratio of 1.77 g CH<sub>3</sub>COO<sup>-</sup> : 1 g ClO<sub>4</sub><sup>-</sup>

**Table 1** Composition of the synthetic perchlorate wastewaters for the profile study, based on variable NaCl concentrations

Constituent (g l <sup>-1</sup> )	NaCl concentration (% w/v)				
	0%	1.0%	2.0%	3.0%	4.0%
NaClO <sub>4</sub> ·H <sub>2</sub> O			0.88*		
MgSO <sub>4</sub> ·7H <sub>2</sub> O			0.10*		
K <sub>2</sub> HPO <sub>4</sub>			1.55*		
KH <sub>2</sub> PO <sub>4</sub>			0.86*		
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>			0.50*		
CH <sub>3</sub> COOK			1.83*		
Yeast extract			0.05*		
NaCl	0	10	20	30	40
Ionic strength (mol l <sup>-1</sup> )	0.14	0.27	0.39	0.52	0.82
Total dissolved solid	5.4	15.3	24.5	33.8	42.6

\*The same constituent concentration was used for all NaCl concentrations.

(Rikken *et al.* 1996). The wastewater solutions were phosphate-buffered so the pH remained at near-neutral values; the pH range during perchlorate reduction was 6.7–7.8.

Table 2 shows the composition of the synthetic perchlorate wastewaters, as a function of perchlorate not NaCl, used for the batch growth studies. Five different initial concentrations of perchlorate (200, 500, 1100, 1700 and 2400 mg l<sup>-1</sup> ClO<sub>4</sub><sup>-</sup>) were utilized with variable salinity wastewater solutions to determine the growth rates and kinetic parameters. The same initial acetate : perchlorate ratio (1.77 g CH<sub>3</sub>COO<sup>-</sup> : 1 g ClO<sub>4</sub><sup>-</sup>) was utilized during the entire batch growth study.

### Reactor operation procedure

A preliminary study was performed for 2 months using BFRs to simulate the effect of a rapid increase in salinity on freshwater micro-organisms in concentrated perchlorate

**Table 2** Composition of the synthetic perchlorate wastewaters for the batch growth study, based on various perchlorate concentrations

Constituent (g l <sup>-1</sup> )	ClO <sub>4</sub> <sup>-</sup> concentration (mg l <sup>-1</sup> )				
	200	500	1100	1700	2400
NaClO <sub>4</sub> ·H <sub>2</sub> O	0.283	0.706	1.553	2.4	3.388
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1	0.1	0.1	0.2	0.3
K <sub>2</sub> HPO <sub>4</sub>			1.55*		
KH <sub>2</sub> PO <sub>4</sub>			0.86*		
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.5	0.5	0.5	1.0	1.5
CH <sub>3</sub> COOK	0.589	1.471	3.237	5.0	7.063
Yeast extract			0.05*		
NaCl	0, 10, 20, 30, or 40 depending on desired salinity				

\*The same constituent concentration was used for all NaCl concentrations.

ate wastewaters. The freshwater mixed cultures (RAS and ADS) used in all experiments were obtained from the Truckee Meadows Water Reclamation Facility in Reno, NV, USA. Another comparative preliminary study was conducted for 2 months in BFRs to determine the influence of gradual changes in salinity on the freshwater micro-organisms. Previous research has shown that gradual acclimation to increased salinity resulted in better performance of the biological treatment process (i.e. a higher perchlorate reduction rate) at 3% NaCl than in the case where a rapid increase in salinity occurred (Kincannon and Gaudy 1966; Burnett 1974; Park and Marchand 2002). After the preliminary acclimation studies, the RAS and particularly the ADS cultures exhibited increases in their perchlorate-reducing capacity as they became adapted to the concentrated perchlorate and high salinity conditions. At this time, a new operational setup was decided upon and SBRs were used. These systems had a high degree of operational flexibility as the cycle time and reaction sequence could be altered (Harty *et al.* 1993), and the salinity could be extended up to 4% NaCl in a gradual manner. Thus, a comparative profile study was initiated between both the RAS and ADS cultures. During the start-up period of approx. 2.5 months, acclimation to perchlorate-reducing environments was attained by cycling the cultures through seven or eight batch cycles at 0% NaCl. The biomass was regularly wasted from the system, not only to maintain an optimum microbial population, but also to induce the exponential growth of perchlorate-reducing microbes. After the system reached steady-state conditions (based on the stable rates of perchlorate reduction between subsequent cycles) and the cultures acclimated to the high concentrations of perchlorate, perchlorate degradation profiles were monitored to evaluate the system performance. Moreover, after the profile studies, a set of batch growth studies were performed using the BFR system with solutions containing 0.8–0.9 g l<sup>-1</sup> of mixed liquor volatile suspended solids (MLVSS) with varying perchlorate concentrations (200, 500, 1100, 1700 and 2400 mg l<sup>-1</sup> as ClO<sub>4</sub><sup>-</sup>), in order to determine the specific growth rates ( $\mu$ ) of the perchlorate-reducing cultures in a given salinity. Then, the NaCl concentration was increased stepwise to 1%, and then 2%, 3%, and up to 4% NaCl, not only to minimize any salt toxicity shocks to perchlorate-reducing microbes, but also to acclimate to each salinity level without much disruption of the cell functioning.

### Analyses

Perchlorate, chlorate, chlorite, chloride, and acetate were analysed using a Dionex DX-300 Ion Chromatograph (Dionex Corp., Sunnyvale, CA, USA) with an IonPac<sup>®</sup>

AS16 analytical and guard column for perchlorate, an IonPac<sup>®</sup> AS9-SC analytical and guard column for chlorite and chlorate, an IonPac<sup>®</sup> AS4A-SC analytical and guard column for chloride and acetate, an anion self-regenerating suppressor (ASRS-1), a gradient pump module (GPM-2), and a suppressed conductivity detector (PED conductivity mode). The mobile phase was a 50 mmol l<sup>-1</sup> NaOH eluent used for perchlorate analysis or a 1.8 mmol l<sup>-1</sup> sodium carbonate and 1.7 mmol l<sup>-1</sup> sodium bicarbonate eluent for chlorite, chlorate, chloride and acetate analyses. In addition, all solid analyses [mixed liquor suspended solids (MLSS), MLVSS, and total dissolved solids (TDS)] were determined according to the Standard Methods (APHA 1998).

Profiles of the SBR mixed liquors for the biological reduction of perchlorate and acetate were conducted for three consecutive batch cycles, after each of the salinity systems reached steady-state conditions. In all profiles, an MLSS concentration was maintained at approx. 5 g l<sup>-1</sup> before feeding wastewater brines into the SBRs. After feeding, samples were taken with a regular interval for analysing perchlorate, chlorate, chlorite, chloride and acetate concentrations in the SBR mixed liquors in order to determine the perchlorate reduction rate for each of the salinity systems. Then, specific zero-order reaction coefficients ( $-k$ , mg ClO<sub>4</sub><sup>-</sup> l<sup>-1</sup> h<sup>-1</sup>) for perchlorate were determined using the slope of the perchlorate reduction profiles computed by linear regression (Park and Marchand 2002). Further, values of  $U_{max}$  (maximum specific perchlorate removal rate, mg ClO<sub>4</sub><sup>-</sup> g<sup>-1</sup> MLVSS h<sup>-1</sup>) were calculated for the cultures at different NaCl concentrations by dividing the specific zero-order reaction coefficient for perchlorate by the biomass concentration (g MLVSS l<sup>-1</sup>).

### Modelling

The growth of the RAS and ADS cultures in the batch study for each of the different salinity levels was measured by the change in biomass concentration between the beginning and the end of the reaction period for each of the five different initial concentrations of perchlorate (200, 500, 1100, 1700 and 2400 mg l<sup>-1</sup> as ClO<sub>4</sub><sup>-</sup>). In addition, the amount of acetate utilized with time during perchlorate biodegradation was measured to plot  $\mu$  (h<sup>-1</sup>) vs substrate ( $S$ , in this case, acetate) and determine the relationship between the observed cell yield ( $Y$ , mg biomass mg<sup>-1</sup> acetate) and substrate. Following the batch growth rate experiments, the experimental data obtained were individually examined against various growth kinetic models in order to determine a model which applied to the experimental data. Model parameter estimation required that each of the growth kinetic parameters

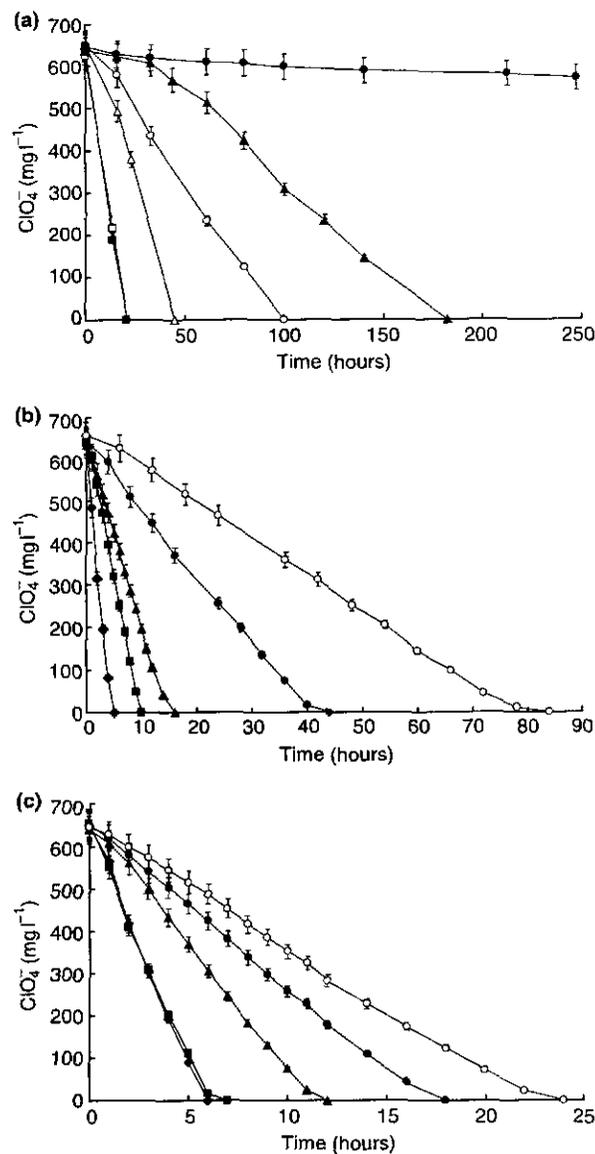
(i.e. the maximum specific growth rate, the half-velocity constant) to be computed to develop the appropriate model. This was achieved by either (i) using a linear curve-fit through linear regression; or (ii) using a nonlinear curve-fit by conducting nonlinear curve fitting best to the experimental data, respectively (Microsoft<sup>®</sup> Excel 2000; Microsoft Corp., Redmond, WA, USA).

## Results

### Salinity acclimation effects of freshwater cultures on perchlorate biodegradation

Figure 1(a) illustrates the overall responses of freshwater RAS and ADS during the biological reduction of concentrated perchlorate ( $610\text{--}650\text{ mg l}^{-1}$ ) at NaCl concentrations of 0%, 1.5% and 3.0% with an MLSS concentration of approx.  $5\text{ g l}^{-1}$  in BFRs. Both cultures exhibited similar perchlorate reduction profiles for the 0% NaCl condition with complete perchlorate degradation occurring within 21 h. However, sudden changes in the salinity resulted in significant differences between the two cultures in terms of perchlorate reduction rate. At 1.5% NaCl, complete perchlorate removal by RAS and ADS was accomplished in approx. 45 and 182 h, respectively (Fig. 1a). This result suggests that the RAS culture was inhibited to some degree in the overall perchlorate removal rate, while the ADS culture exhibited a greater inhibition, as indicated by the longer time required to degrade perchlorate. Further, at the 3% NaCl condition, the ADS culture was unable to metabolize perchlorate significantly over 250 h whereas the RAS culture consumed the perchlorate in 100 h with a maximum specific perchlorate removal rate of  $4.4\text{ mg g}^{-1}\text{ h}^{-1}$ . All control experiments operated without biomass addition resulted in no perchlorate degradation or acetate consumption (data not shown).

In contrast to the aforementioned finding, higher perchlorate reduction capacities were achieved by gradual changes in salinity. Figure 1(b,c) depicts the biological reduction of concentrated perchlorate at NaCl concentrations of 0%, 1%, 2%, 3% and 4% with an MLSS concentration of approx.  $5\text{ g l}^{-1}$  in both RAS and ADS sequencing batch reactor systems, respectively. Indeed, the perchlorate removal rates were much higher even at 0% NaCl, likely due to the regular wasting of biomass in the SBR systems which allowed for better acclimation to perchlorate-reducing conditions and enrichment of microbes capable of respiring perchlorate. Overall, reduction of perchlorate at 0% NaCl by RAS and ADS was completed in 5 and 6 h, respectively. In addition, it can be observed from Fig. 1(b,c) that the biodegradation of high concentration of perchlorate was linear; therefore, a simple zero-order kinetic model was used to compare the



**Figure 1** Perchlorate degradation by (a) nonacclimated return activated sludge (RAS, open symbols) and anaerobic digester sludge (ADS, closed symbols) at 0% ( $\square$ ,  $\blacksquare$ ), 1.5% ( $\triangle$ ,  $\blacktriangle$ ), and 3% ( $\square$ ,  $\bullet$ ) NaCl in the batch flask reactor, (b) acclimated RAS, and (c) acclimated ADS at 0% ( $\blacklozenge$ ), 1% ( $\blacksquare$ ), 2% ( $\blacktriangle$ ), 3% ( $\bullet$ ) and 4% ( $\square$ ) NaCl in the sequencing batch reactor with a mixed liquor suspended solid concentration of approx.  $5000\text{ mg l}^{-1}$ . Error bars represent the standard deviation of triplicate reactors.

perchlorate removal rates for each culture as a function of the salinity. Maximum specific perchlorate removal rates ( $U_{\text{max}}$ ) of  $37.8$  and  $34.6\text{ (mg g}^{-1}\text{ h}^{-1})$  were observed for the RAS and ADS cultures at 0% NaCl, respectively (Table 3). In contrast to the results depicted in Fig. 1(a) (where a sharp change in salinity occurred), the gradual

**Table 3** The maximum specific perchlorate reduction rates ( $U_{\max}$ ) for the RAS and ADS cultures during biological reduction of perchlorate in SBRs at pH 6.7–7.8

NaCl concentration (%)	RAS cultures		ADS cultures	
	MLVSS ( $\text{g l}^{-1}$ )	$U_{\max}$ ( $\text{mg g}^{-1} \text{h}^{-1}$ )*	MLVSS ( $\text{g l}^{-1}$ )	$U_{\max}$ ( $\text{mg g}^{-1} \text{h}^{-1}$ )*
0	3.47	$37.8 \pm 0.8$	3.22	$34.6 \pm 1.3$
1	2.96	$22.7 \pm 1.3$	2.86	$34.3 \pm 3.8$
2	2.42	$18.2 \pm 0.3$	2.24	$26.2 \pm 1.5$
3	2.13	$7.3 \pm 0.4$	2.07	$18.7 \pm 0.9$
4	1.89	$4.5 \pm 0.3$	1.82	$16.2 \pm 0.7$

RAS, return activated sludge; ADS, anaerobic digester sludge; SBR, sequencing batch reactor; MLVSS, mixed liquor volatile suspended solid.

\*Average value  $\pm$  standard deviation for triplicate cycle runs.

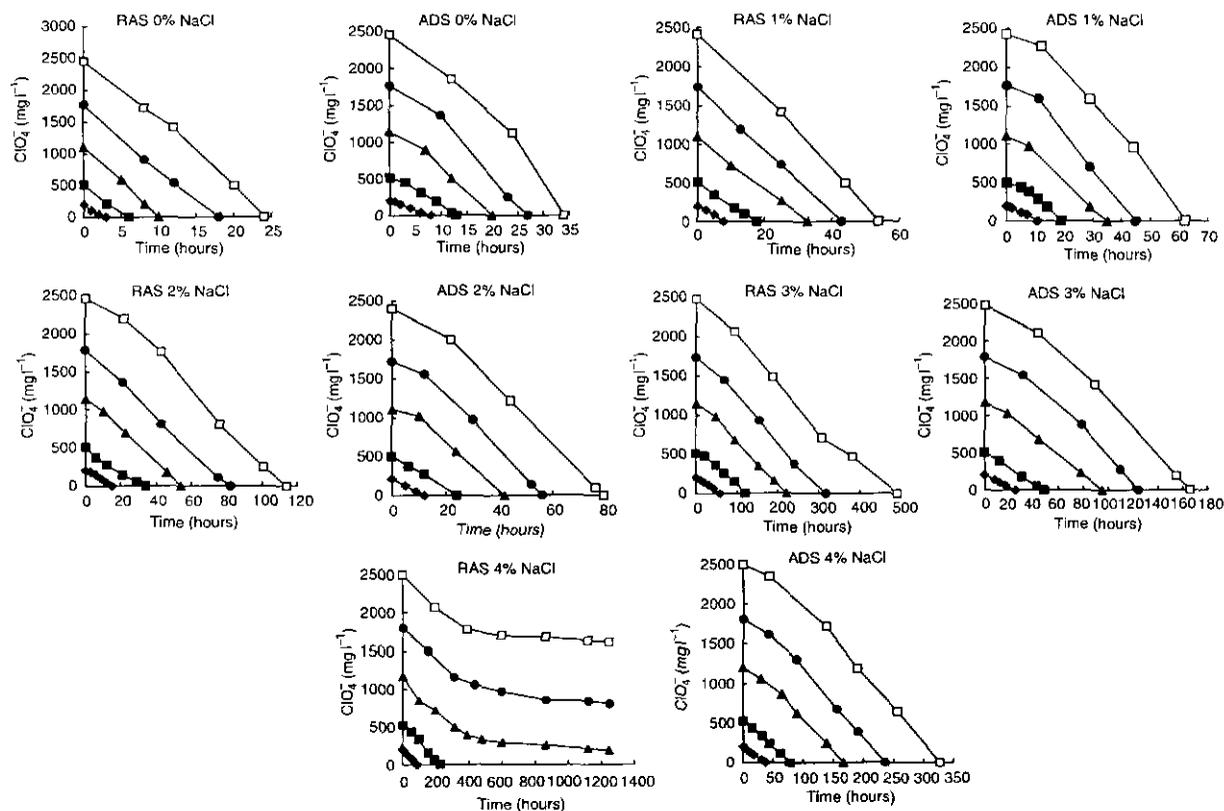
increase to 1% NaCl strongly favoured the ADS culture. As can be seen from Fig. 1(b,c), the ADS culture exhibited a higher overall capacity for perchlorate removal than the RAS cultures, with a  $U_{\max}$  1.5 times higher than the RAS culture at 1% NaCl ( $22.7$  and  $34.3 \text{ mg g}^{-1} \text{h}^{-1}$  for RAS and ADS, respectively). Further increases in salinity above the 1% NaCl level resulted in decreases in  $U_{\max}$  for both RAS and ADS cultures. Comparatively, the ADS culture performed much better than the RAS culture at NaCl concentrations between 1% and 4% with  $U_{\max}$  values between 1.5 times higher (at 1% NaCl) and 3.6 times higher (at 4% NaCl) than the RAS culture. Indeed, the perchlorate degradation rate for RAS at 4% NaCl was only 12% of the rate measured at 0% NaCl whereas the rate for the ADS culture at 4% NaCl was only 47% of the rate measured at 0% NaCl. This finding is significant particularly in the light of the fact that the RAS culture performed better at 0% NaCl (Table 3) and exhibited better growth under nonacclimated conditions (Fig. 1a). It is also interesting to note that increases in salinity resulted in a lower ratio of MLVSS : MLSS for both RAS and ADS cultures (Table 3). While all the SBR experiments were operated at a relatively constant value of MLSS ( $5 \text{ g l}^{-1}$ ), the MLVSS : MLSS ratio for RAS at 0% and 4% NaCl were 0.69 and 0.39, respectively, while the ratio for ADS at 0% and 4% NaCl were 0.64 and 0.36, respectively.

In the light of the results obtained during stepwise increases in NaCl concentration, the RAS cultures showed marked decreases in perchlorate reduction rate under the 5-month SBR operations, while the ADS cultures exhibited gradual decreases in perchlorate reduction rate during the entire experimental period. It is believed that the decrease in perchlorate reduction rate resulted from NaCl toxicity to the perchlorate-reducing microbes. During all experiments, the perchlorate degradation intermediates,

chlorate ( $\text{ClO}_3^-$ ) and chlorite ( $\text{ClO}_2^-$ ) identified by Rikken *et al.* (1996), were not detected at any time during the biological reduction of perchlorate regardless of NaCl concentration. This finding supports the assertion that high salinities do not affect a specific enzyme responsible for a step in the perchlorate reduction pathway, but rather, likely have an influence on the overall growth and physiology of perchlorate-reducing micro-organisms.

#### Growth kinetics of acclimated perchlorate-reducing cultures

A batch growth study was conducted to determine how perchlorate-reducing cultures that were acclimated to a given salinity would grow under varying perchlorate concentrations. Figure 2 shows the biodegradation kinetics of various initial perchlorate concentrations performed for 200, 500, 1100, 1700 and 2400  $\text{mg l}^{-1}$  as  $\text{ClO}_4^-$  by acclimated RAS and ADS cultures. The BFR systems had initial MLSS concentrations of  $1.5$ – $2.0 \text{ g l}^{-1}$  and were operated at NaCl concentrations between 0% and 4% NaCl. It is noteworthy that increasing the NaCl concentration in solution resulted in a decrease in the MLVSS concentration, likely due to an elevated concentration of NaCl within the cells. Therefore, these experiments were started with a gradual increase in the initial MLSS concentration with increasing salinity with a goal of  $0.8$ – $0.9 \text{ g l}^{-1}$  of MLVSS. This served to normalize the MLVSS concentration between experiments with different NaCl levels. Based on the slopes of the perchlorate degradation profiles shown in Fig. 2 and the MLVSS concentrations for each condition, specific perchlorate degradation rates were obtained (Fig. 3). As shown in Figs 2 and 3, at the 0% NaCl conditions, an increase in the perchlorate reduction rate was observed with increases in the perchlorate concentration. This is indicative of perchlorate being a relatively biodegradable compound in freshwater habitats (Attaway and Smith 1993). The RAS culture was able to degrade perchlorate ( $1067 \text{ mg l}^{-1} \text{ ClO}_4^-$ ) with a  $U_{\max}$  as high as  $104 \text{ mg g}^{-1} \text{h}^{-1}$  at the 0% NaCl condition (Fig. 3). In accordance with the previous data, at 0% NaCl concentrations, all of the  $U_{\max}$  values calculated for the RAS culture were 1.5–2.6 times higher than those observed for the ADS culture. However, as depicted in Fig. 3, the RAS culture exhibited a more marked decrease in the perchlorate reduction rate ( $U_{\max}$ ) than the ADS culture as salinity increased to 1% NaCl. As in the SBR experiments previously described, an increase in salinity had a more profound influence on the RAS culture than on the ADS culture. The  $U_{\max}$  value for the RAS culture at 3% NaCl was more than two times lower than the comparable salinity for an ADS system. In addition to the



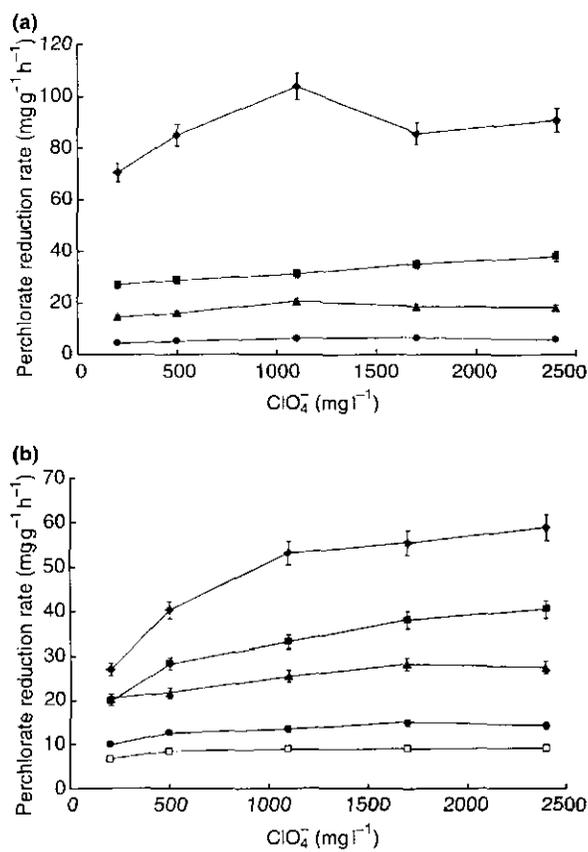
**Figure 2** Perchlorate profiles of the return activated sludge (RAS) and anaerobic digester sludge (ADS) cultures during biological reduction of various perchlorate concentration [ $\text{mg l}^{-1}$ : 200 ( $\blacklozenge$ ), 500 ( $\blacksquare$ ), 1100 ( $\blacktriangle$ ), 1700 ( $\bullet$ ) and 2400 ( $\square$ ) as  $\text{ClO}_4^-$ ] under 0%, 1%, 2%, 3% and 4% NaCl conditions, respectively, with a mixed liquor volatile suspended solid concentration of approx. 800–900  $\text{mg l}^{-1}$  in the batch growth experiment.

salinity effects, it was observed that increased initial perchlorate concentrations typically resulted in higher perchlorate reduction rates, particularly at the lower values of salinity. As the salinity increased, however, there was less variability in the perchlorate reduction rates with changing initial perchlorate concentrations suggesting that salinity had a more profound influence than perchlorate concentration on the observed perchlorate removal rates.

Finally, it can be observed in Fig. 2 that there was complete inhibition of perchlorate reduction for the RAS culture in the presence of 4% NaCl when the initial perchlorate concentration exceeded 500  $\text{mg l}^{-1}$ . The three RAS culture systems with initial perchlorate concentrations of 1100, 1700 and 2400  $\text{mg l}^{-1}$  were only able to reduce approx. 900  $\text{mg l}^{-1}$  as  $\text{ClO}_4^-$  before the perchlorate reduction rate approached zero. As mentioned earlier, there were no measured perchlorate degradation intermediate compounds (i.e.  $\text{ClO}_3^-$  or  $\text{ClO}_2^-$ ) during the experiment and sufficient acetate remained in solution (i.e. these profiles were not electron donor limited). These

results suggest that the combination of high salinity (4% NaCl) and high concentrations of perchlorate (or acetate) exerted some undetermined toxic influence or inhibited the perchlorate reduction rate of the microbes in the RAS culture.

Figure 4 depicts the average observed cell yields ( $Y_{av}$ , mg biomass per mg acetate) for the RAS and ADS cultures for all initial perchlorate concentrations (200, 500, 1100, 1700 and 2400  $\text{mg l}^{-1}$  as  $\text{ClO}_4^-$ ) as a function of the culture salinity. The figure reveals that there were distinct contrasts in the trends in  $Y_{av}$  between the two cultures. The ADS cultures showed a fairly constant decrease in the observed yield due to the increase in salinity. On the other hand, the RAS culture exhibited an increase in yield between 0% and 1% NaCl with a large decrease in yield for 2% and 3% NaCl (Fig. 4). In general, the yield for the RAS culture was higher than the ADS culture for 0% and 1% NaCl concentrations whereas the ADS had a higher growth yield at the 2% and 3% NaCl levels (only the 1% and 3% NaCl values were significantly different at the 95% confidence interval).

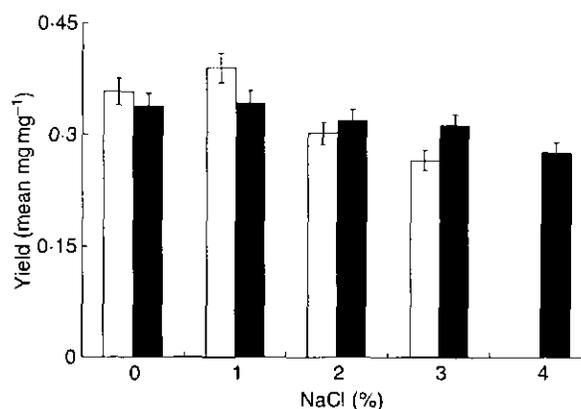


**Figure 3** Maximum specific perchlorate reduction rate ( $U_{max}$ ) for (a) return activated sludge (RAS) and (b) anaerobic digester sludge (ADS) cultures during biological reduction of perchlorate (200, 500, 1100, 1700 and 2400  $\text{mg l}^{-1}$  as  $\text{ClO}_4^-$ ) at 0% (◆), 1% (■), 2% (▲), 3% (●) and 4% (□) NaCl. Error bars display 95% confidence interval.

Specific growth rates ( $\mu$ ,  $\text{h}^{-1}$ ) were calculated for the acclimated RAS and ADS cultures as a function of the initial acetate concentration. The specific growth rate was computed by the following expression:

$$\mu = \frac{\ln(X_t/X_0)}{\Delta t}, \quad (1)$$

where,  $X$  represents the biomass concentration ( $\text{mg l}^{-1}$ ) at the beginning ( $X_0$ ) and at the end ( $X_t$ ) of the measured time period ( $\Delta t$ ) for each of the initial substrate concentrations tested. As it is customary to use the energy source (in this case, acetate) as the independent variable in a growth rate expression, the initial acetate concentration was used instead of the initial perchlorate (recall, the acetate : perchlorate value was  $1.77 \text{ g g}^{-1}$ ). The growth rates were calculated at salinity levels between 0% and 4% NaCl (0–3% for RAS) corresponding to the different initial perchlorate concentrations (200, 500, 1100, 1700 and

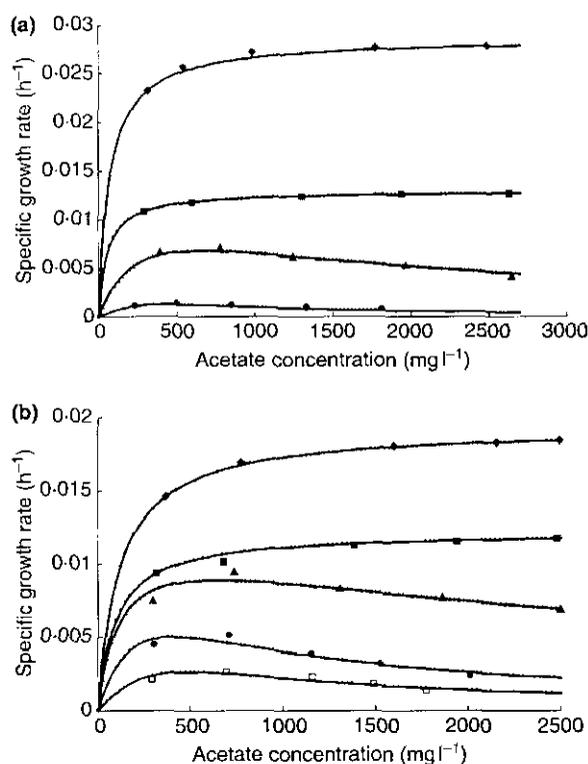


**Figure 4** Yield ( $\text{mg biomass per mg acetate}$ ) between return activated sludge (RAS, □) and anaerobic digester sludge (ADS, ■) cultures, calculated in averaging the value, for all initial perchlorate (200, 500, 1100, 1700 and 2400  $\text{mg l}^{-1}$  as  $\text{ClO}_4^-$ ) degradations in each of the salinity systems. Error bars display the 95% confidence interval.

up to 2400  $\text{mg l}^{-1}$   $\text{ClO}_4^-$ ). For all salinity conditions and initial acetate concentrations studied, the  $r^2$  values for the linear regressions of  $\ln(X)$  vs time were  $>0.91$ , with 80% of the regression data having an  $r^2$  exceeding 0.96 (data not shown). Figure 5 illustrates the variation in  $\mu$  with respect to the initial acetate concentration for the RAS (Fig. 5a) and ADS (Fig. 5b) cultures. The data collected were also fit with a Monod-based model (represented by the curves) which is described in detail in the subsequent section. The RAS culture exhibited higher specific growth rates than the ADS culture at the 0% NaCl condition, albeit both cultures possessed relatively low specific growth rates compared with other substrate-electron acceptor systems for wastewater treatment systems (Grady *et al.* 1999). However, the growth of the RAS cultures was inhibited more significantly than that of the ADS cultures as the NaCl concentration increased to 1% NaCl and above. For NaCl concentrations  $>1\%$ , both the RAS and ADS cultures exhibited a decrease in specific growth rate with increasing initial concentrations of acetate (and perchlorate). Thus, two distinct phenomena were observed: (i) the decrease in the maximum specific growth rate with increasing salinity, and (ii) the decrease in specific growth rate at higher initial acetate (or perchlorate) concentrations for NaCl concentrations of 2% or higher.

### Modelling

Many growth models for biological treatment systems are based on Monod kinetics (Grady *et al.* 1999; Rittmann and McCarty 2001) which mathematically describes the influence of substrate concentrations on microbial growth



**Figure 5** Specific growth rates for (a) return activated sludge (RAS) and (b) anaerobic digester sludge (ADS) cultures as a function of acetate concentration during biological perchlorate reduction (200, 500, 1100, 1700 and 2400 mg l<sup>-1</sup> as ClO<sub>4</sub><sup>-</sup>) under elevated salinity conditions [0% (◆), 1% (■), 2% (▲), 3% (●) and 4% (□) NaCl]. Solid lines are based on the model described in the text and shown in eqn (3).

rate. The Monod equation has the general form shown below where  $\mu$  is the specific growth rate (h<sup>-1</sup>),  $\mu_m$  is the maximum specific growth rate (h<sup>-1</sup>),  $K_s$  is the half-saturation constant (mg l<sup>-1</sup>), and  $S$  is the substrate concentration (mg l<sup>-1</sup>).

$$\mu = \mu_m \left( \frac{S}{K_s + S} \right), \quad (2)$$

Since salinity has a marked influence on the growth rate of perchlorate-reducing microbes, a goal of the current study is to develop a kinetic model that can be used to describe the functional relationship between different salinity levels and the specific growth rate, on the basis of the substrate concentration.

An attempt has been made to include the two inhibition effects discussed in the previous section (i.e. the influence of salinity on lowering the maximum growth rate and the combination of high salinity and high substrate concentrations on inhibition) without making significant changes to the Monod models. To address the

observed decrease in growth rate with increased salinity (Fig. 5), we propose the inclusion of a salinity inhibition parameter which would effectively decrease the maximum specific growth rate ( $\mu_m$ ) at high NaCl concentrations. This inhibition function may be expressed by modifying  $\mu_m$  to  $(\mu_m - I_s)$  in the Monod growth rate equation (where  $I_s$  represents the salinity inhibition constant, h<sup>-1</sup>). To address the observed decrease in growth rate at higher substrate concentrations in high salinity wastewaters (>1% NaCl), it is proposed to include an inhibition term similar to the term included in the Andrews equation (Andrews 1968; Grady *et al.* 1999). The Andrews equation is traditionally used to model an inhibitory growth substrate; however, in the present case, it is believed that incorporating the Andrews modification will mathematically describe the decrease in growth rate at high substrate concentrations shown by the data points in Fig. 5 for 2% NaCl and higher. Incorporating these two modifications to the Monod expression results in the following equation:

$$\mu = \frac{(\mu_m - I_s)S}{K_s + S + (S^2/K_i)}, \quad (3)$$

where  $I_s$  represents the salinity inhibition constant (h<sup>-1</sup>),  $K_i$  is the substrate inhibition constant (mg l<sup>-1</sup>), and the other terms are as previously described. It should be noted that the values of  $\mu_m$ ,  $I_s$ ,  $K_s$ , and  $K_i$  were determined by regression of the experimental data to the proposed model. For the proposed model to adequately describe experimental conditions over a range of salinity conditions, the  $I_s$  term needs to be equal to zero for the no salinity condition and a constant for NaCl  $\geq$  1%. This can be mathematically handled by considering the following relationship:

$$I_s = \frac{I_s^*(\% \text{NaCl})}{0.01 + \% \text{NaCl}}, \quad (4)$$

where  $I_s^*$  is constant depending on the culture. Thus, at 0% salinity, the  $I_s$  term is equal to zero and is a constant at higher salinity levels.

Using eqn (3), the best model fit curves are shown in Fig. 5 with the specific growth rate data for each of the RAS and ADS salinity systems. The model parameters were obtained by two sequential processes. First, kinetic parameter values for the 0% and 1% NaCl systems were estimated using a linear curve-fit for each of the specific growth rate data sets and are shown in Table 4. The  $r^2$  values of the linear specific growth rate curves ranged between 0.99 and 1, which is indicative of good agreement between the model and experimental data. The  $K_i$  term in the model reflects the decrease in growth rate and perchlorate reduction at high substrate concentra-

**Table 4** Summary of individual growth kinetic coefficients ( $\mu_m, I_S, K_S$  and  $K_i$ ) for the RAS (return activated sludge) and ADS (anaerobic digester sludge) cultures as a function of the NaCl concentration in the system

Culture	NaCl concentration (%)	Growth kinetic parameters			
		$\mu_m$ ( $h^{-1}$ )	$I_S$ ( $h^{-1}$ )	$K_S$ ( $mg\ l^{-1}$ )	$K_i$ ( $mg\ l^{-1}$ )
RAS†	0	0.0286	0	72.8	$K_i \gg S$
	1	0.0286	0.0157	57.6	$K_i \gg S$
	2	0.0286	0.0157	300	1500
	3	0.0286	0.0157	3500	50
ADS†	0	0.0193	0	119.3	$K_i \gg S$
	1	0.0193	0.0071	97.7	$K_i \gg S$
	2	0.0193	0.0071	120	3500
	3	0.0193	0.0071	295	575
	4	0.0193	0.0071	900	275

† $I_S = 0.0157\ h^{-1}$  for RAS and  $0.0071\ h^{-1}$  for ADS.

tions, particularly when the NaCl level was at 2% or higher. For both cultures, analysis of data for the 0% and 1% conditions resulted in values of  $K_i \gg S$  ( $K_i$  was more than 100 times higher than the highest  $S^2$  tested for the RAS-0% NaCl condition); hence, the inhibition term was effectively negligible for 0% and 1% NaCl. The decline of the maximum specific growth rate at 1% NaCl compared with the 0% NaCl condition was used in the model to set the value of  $I_S$  for the RAS and ADS cultures. The salinity inhibition constant for RAS was  $0.0157\ h^{-1}$ , whereas  $I_S$  was  $0.0071\ h^{-1}$  for ADS; the larger magnitude of  $I_S$  for RAS was consistent with the higher decrease in maximum specific growth rate with increasing salinity compared with ADS. Regarding the determination of the other growth parameters, it is noteworthy that we assigned the specific growth rate curve at 1% NaCl as a baseline and the parameter value of both  $\mu_m$  and  $I_S$  as a standard value in order to produce specific growth rate curves for salinities in excess of 1% NaCl. Then, the values of the kinetic model parameters ( $K_S$  and  $K_i$ ) for 2%, 3% and 4% NaCl systems were estimated for each of the curves using a nonlinear curve-fit for the experimental data (Table 4). As shown in Table 4, the values of  $K_S$  considerably increased with increasing salinity above the 1% NaCl level, while the values of  $K_i$  were markedly decreased with increasing salinity. This is further indication of how the model captures the inhibition of microbial growth by high salinity: (i) high values of  $K_S$  signify a low affinity for the substrate (or lower microbial ability to readily grow under specific conditions) and (ii) low values of  $K_i$  describe the high degree of inhibition at low substrate concentrations. Based on this, it is interesting to note for the RAS system at 3% NaCl that the extremely high value of  $K_S$  and relatively low value of  $K_i$  reported in Table 4

validate the finding of inhibition and culture failure at the 4% NaCl level, which was depicted in Fig. 2.

## Discussion

Sharp changes in salinity to values as high as 3% NaCl resulted in little or no biodegradation of perchlorate by freshwater ADS cultures. Conversely, RAS cultures were able to degrade perchlorate at markedly higher rates under nonacclimated conditions. It was assumed that abrupt changes to high salinity might be detrimental to perchlorate-reducing microbes with respect to lowering the microbial activity and cell growth. However, through the stepwise acclimation of both the RAS and ADS cultures to higher salinity levels, a higher degree of tolerance to salinity was observed and acclimation to salinity levels as high as 4% NaCl was possible. It is believed that the key factor involved in overcoming inhibition at high salinities relates to maintaining perchlorate-reducing populations at high cell densities, which would act as a buffer against changes to elevated salinity. Another important aspect is believed to be related to coaggregation of biomass. Once the salinity was increased stepwise to 1% NaCl, aggregated biomass was observed within the reactors. Aggregated biomass with different species appears to play an essential role in either obtaining sufficient resources (in this case, perchlorate, acetate, and other nutrients) efficiently for maintaining the high populations or building a community acclimated to elevated salinity (Shapiro 1991). Even if cell lysis due to the environmental stress occurs near the outer surface of the aggregated biomass, the cells inside the aggregated biomass might be capable of sustained growth, in effect utilizing the cells near the outer surface as a barrier against high salinity (Atlas and Bartha 1993). The inclusion of the  $I_S$  term in eqn (3) would account for this phenomena, effectively reducing the maximum growth rate due to the inactivation of a subpopulation in the mixed culture. It was found that the ADS mixed cultures that possessed high population densities of perchlorate-reducing microbes with biomass aggregation could maintain fairly consistent values of  $U_{max}$ ,  $Y_{av}$ , and  $\mu$  during successive transfers to higher salinities. In contrast to the ADS culture, the RAS culture was characterized by somewhat different properties. The RAS culture exhibited higher dynamic capacities in terms of  $U_{max}$ ,  $Y_{av}$  and  $\mu$  than the ADS cultures under salinity-free conditions, probably as a result of the greater species diversity in RAS than ADS (Grady *et al.* 1999) and higher potential to contain fast growing microbes capable of reducing perchlorate. However, it appears that the perchlorate-reducing microbes in RAS cultures had a lower tolerance for high concentrations of NaCl than those in ADS cultures. Consequently, the RAS culture

exhibited lower kinetic growth rates than the ADS culture when the salinity was higher than 1% NaCl. Overall, these results suggest that there were significant physiological differences between RAS and ADS cultures, which might have been caused by microbial composition, cellular changes, cell lysis, and cell acclimation and/or tolerance to high salinity (Thiemann and Imhoff 1991). This is rather surprising in the light of the fact that the microbial communities associated with anaerobic digesters tend to be more specialized and sensitive to environmental stress than activated sludge micro-organisms (Grady *et al.* 1999).

The growth kinetic model presented here was developed based on experimental growth rate data in an attempt to include an inhibition function relating the influence of salinity and substrate concentration on the specific growth rate of the two mixed cultures studied. Compared with the other studies addressing perchlorate degraders, the mixed populations studied in this investigation exhibited relatively low growth rates, despite containing high microbial densities (800–900 mg l<sup>-1</sup> as MLVSS). The maximum specific growth rates observed in the current study were approx. 10-fold lower than those measured for a single perchlorate-reducing population with low population densities (e.g. 0.14 h<sup>-1</sup> for perchlorate-reducing strain KJ and 0.21 h<sup>-1</sup> for strain PDX), probably because of the absence of competition (Veldkamp *et al.* 1984; Logan *et al.* 2001b). However, comparing values of  $U_{max}$  and  $\mu$  at 0% and 1% NaCl, it appears that the acclimated mixed populations described herein may allow for more efficient growth and utilization of highly concentrated perchlorate solutions than a single population because each of the single populations (strain KJ and PDX) grew optimally in a freshwater environment with up to 500 mg l<sup>-1</sup> as ClO<sub>4</sub><sup>-</sup>, but the growth of other single populations (strains WD, PS and strain CKB) were inhibited at more than 995 mg l<sup>-1</sup> as ClO<sub>4</sub><sup>-</sup> (Bruce *et al.* 1999; Michaelidou *et al.* 2000; Logan *et al.* 2001b). This observation lends credence to the suggestion that other microbial interactions (i.e. cooperation, commensalism, mutualism and/or competition plus commensalism) may be important factors involved in the growth of perchlorate-reducing microbes at high perchlorate concentrations. Indeed, complex population dynamics cannot be predicted and understood from pure culture studies alone (Fredrickson 1977; Shapiro 1991), particularly for a compound such as perchlorate where a competitor electron acceptor (O<sub>2</sub>) is formed during the perchlorate degradation process.

It is still not clear how the variables associated with perchlorate reduction such as the perchlorate or acetate concentration and the salinity are interrelated with respect to the inhibition of mixed culture microbial growth. The

data support the claim that at salinities >1%, high initial concentrations of perchlorate or acetate resulted in decreased growth rates for both RAS and ADS. However, it appears that the impact of high concentrations of perchlorate and acetate on inhibition was limited because nearly constant values of  $U_{max}$  were maintained over variable substrate concentrations for salinities >1% NaCl in both cultures (Fig. 3). That is, high substrate concentrations were readily degradable in the absence of high NaCl concentrations and the most significant growth-limiting factor was the high salinity.

It is anticipated that the salinity inhibition model developed in this study will be useful in many practical applications and can result in improved biological reactor design and helping to gain a better understanding of high salinity inhibition. The research findings can also be useful in assessing appropriate salinity loading levels for the design and management of biological processes for treating hypersaline (>3.5% w/v TDS) perchlorate wastewaters.

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