Earth and Planetary Science Letters 269 (2008) 605–613

Contents lists available at ScienceDirect

Earth and Planetary Science Letters

journal homepage: www.elsevier.com/locate/epsl

Microbial perchlorate reduction: A precise laboratory determination of the chlorine isotope fractionation and its possible biochemical basis

Magali Ader [a,b], Swades Chaudhuri [c,d], John D. Coates [d], Max Coleman [b,e,*]

[a] University of Paris 7 & Institut de Physique du Globe de Paris, France
[b] School of Human and Environmental Sciences, The University of Reading, Reading, UK
[c] Department of Chemical Engineering, Texas A&M University, College Station, TX, USA
[d] Department of Plant and Microbial Biology, University of California Berkeley, CA, USA
[e] Jet Propulsion Laboratory, Caltech, CA, USA

ARTICLE INFO

Article history:
Received 16 July 2007
Received in revised form 4 February 2008
Accepted 3 March 2008
Available online 18 March 2008

Editor: H. Elderfield

Keywords:
perchlorate
perchlorate reduction
isotope fractionation mechanism
Azospira suillum
chlorine stable isotopes

ABSTRACT

Perchlorate-reducing bacteria fractionate chlorine stable isotopes giving a powerful approach to monitor the extent of microbial consumption of perchlorate in contaminated sites undergoing remediation or natural perchlorate containing sites.

This study reports the full experimental data and methodology used to re-evaluate the chlorine isotope fractionation of perchlorate reduction in duplicate culture experiments of *Azospira suillum* strain PS at 37 °C ($\Delta^{37}Cl_{Cl^--ClO_4^-}$) previously reported, without a supporting data set by Coleman et al. [Coleman, M.L., Ader, M., Chaudhuri, S., Coates, J.D., 2003. Microbial Isotopic Fractionation of Perchlorate Chlorine. Appl. Environ. Microbiol. 69, 4997–5000] in a reconnaissance study, with the goal of increasing the accuracy and precision of the isotopic fractionation determination. The method fully described here for the first time, allows the determination of a higher precision $\Delta^{37}Cl_{Cl^--ClO_4^-}$ value, either from accumulated chloride content and isotopic composition or from the residual perchlorate content and isotopic composition. The result sets agree perfectly, within error, giving average $\Delta^{37}Cl_{Cl^--ClO_4^-}=-14.94\pm0.15‰$. Complementary use of chloride and perchlorate data allowed the identification and rejection of poor quality data by applying mass and isotopic balance checks. This precise $\Delta^{37}Cl_{Cl^--ClO_4^-}$ value can serve as a reference point for comparison with future in situ or microcosm studies but we also note its similarity to the theoretical equilibrium isotopic fractionation between a hypothetical chlorine species of redox state +6 and perchlorate at 37 °C and suggest that the first electron transfer during perchlorate reduction may occur at isotopic equilibrium between an enzyme-bound chlorine and perchlorate.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

1.1. Perchlorate occurrences in the environment

There was no interest in perchlorate in the environmental community until it was recognized as a major contaminant in some soils, ground and surface-waters (up to 3.7 g L^{-1}) and a potential health threat in the USA (Motzer, 2001; Tikkanen, 2006). Since then, the continued development of new methods for perchlorate quantification lowered its detection limit from 400 to 0.02 μg L^{-1} (Koester et al., 2000; Snyder et al., 2005), allowing the recognition of a more widespread contamination than expected (Urbansky, 2000; Snyder et al., 2005; Tikkanen, 2006) and renewing interest in characterising its sources.

The known natural occurrences of perchlorate were until recently limited to the very rare nitrate deposits found only in the extremely arid Atacama Desert of northern Chile, where perchlorate content is between 0.1% and 1% (Ericksen, 1983). Lately, low levels of perchlorate have been detected also in other potassium-rich evaporite deposits (Orris et al., 2003), in unsaturated zones of the semi-arid Southwest United state (Rao et al., 2007), in rain and snow (Dasgupta et al., 2005) as well as in seawater (Martinelango et al., 2006). Natural perchlorate is thought to be generated in the atmosphere (Bao and Gu, 2004; Böhkle et al., 2005; Dasgupta et al., 2005; Rao et al., 2007) but its accumulation seems to be limited to arid environments.

Man-made perchlorates are mostly used as propellants for rockets, air bags inflators, fireworks and road flares. Because of their high solubility and stability, their careless disposal in the environment has led to contamination of drinking waters now requiring remediation (Urbansky, 1988; Motzer, 2001; Urbansky, 2002; Hatzinger, 2005).

Because of its chemical stability, perchlorate in solution should accumulate in the environment, but recent studies have demonstrated that diverse and ubiquitous bacteria can couple growth to the anaerobic reduction of perchlorate (or chlorate) to chloride (Coates and Achenbach, 2004). These organisms are thought to be responsible for perchlorate consumption in environments where anoxic conditions

* Corresponding author. Jet Propulsion Laboratory, Caltech, CA, USA. Tel.: +1 818 393 6353; fax: +1 818 393 4445.
E-mail address: max.coleman@jpl.nasa.gov (M. Coleman).

0012-821X/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.epsl.2008.03.023

can develop, and present a high potential for in situ bioremediation of perchlorate contaminated sites.

So far, two major issues concerning perchlorate have been (i) monitoring and bioremediation of contaminated sites (Motzer, 2001) and (ii) identification of the sources of contamination (Bao and Gu, 2004). For both of these issues the use of the chlorine stable isotope composition of perchlorate was shown to be very promising (Coleman et al., 2003; Sturchio et al., 2003) especially when coupled to its oxygen isotope composition (Bao and Gu, 2004; Böhkle et al., 2005; Sturchio et al., 2007).

1.2. Stable isotopes: identifying perchlorate sources

As is the case for most of the elements with stable isotopes, there are variations of the abundances of the minor relative to the major isotope of chlorine in its compounds.

These variations can be used either to trace the origin of the element in the compound, and/or to identify the process that controlled its incorporation in this substance.

The chlorine stable isotopic composition in a sample is reported as parts per thousand difference from SMOC (Seawater Mean Ocean Chloride) in the δ-notation, as given by the Eq. (1):

$$\delta^{37}\mathrm{Cl}_{\mathrm{Sample}} = 1000 \times \left[R_{\mathrm{Sample}}/R_{\mathrm{SMOC}} - 1\right] \text{ in ‰} \quad (1)$$

where $R = {}^{37}\mathrm{Cl}/{}^{35}\mathrm{Cl}$.

Manufactured perchlorate salts from a number of manufacturers have chlorine isotope values that vary from about −3‰ to just above 2‰ (Ader et al., 2001; Böhkle et al., 2005). Natural perchlorates from the Atacama Desert present values from −14.5‰ to −9.2‰ (Böhkle et al., 2005). Thus, there are significant isotopic variations between manufactured and natural perchlorate salts. Perchlorate is highly soluble, chemically stable when in solution, and does not seem to significantly exchange its isotopes with chloride during the time scales involved (Bao and Gu, 2004, Sturchio et al., 2007). Hence, provided that perchlorate has not been consumed by bacteria, there is a possibility that the chlorine isotopic composition of perchlorate could be used to trace the source of a contaminant and certainly to identify whether it is natural or manufactured (Böhkle et al., 2005).

1.3. Stable isotope fractionation: identifying processes affecting perchlorate

The partitioning of stable isotopes between two substances 1 and 2 is quantitatively described by a fractionation factor (α_{1-2}) or a fractionation (Δ_{1-2}), respectively defined by the Eqs. (2) and (3).

$$\alpha_{1-2} = R_1/R_2 \quad (2)$$

$$\Delta_{1-2} = 1000 \times (R_1/R_2 - 1) \quad (3)$$

where $R_1/R_2 = (1000+\delta_1)/(1000+\delta_2)$.

During the transfer of chlorine from one molecule to another the relative abundance of the chlorine isotopes can be modified either by kinetic or equilibrium isotopic fractionation (Urey, 1947; Bigeleisen, 1952; Schauble et al., 2003).

1.3.1. Thermodynamic equilibrium isotopic fractionation between chloride and perchlorate

Thermodynamic equilibrium isotopic fractionation of a given element between two substances can be calculated theoretically from the reduced partition functions of the element in both substances, which in turn are calculated from quantum mechanics using the measured vibrational energy of the chemical bonds of the atoms of the element (Bigeleisen and Mayer, 1947; Urey, 1947; Richet et al., 1977; Schauble et al., 2003).

Urey (1947) and Schauble et al. (2003) calculated the reduced partition functions for some chlorine-containing substances but not for the chloride anion in solution. Therefore, Urey (1947) did not report the fractionation factor for perchlorate/chloride, although Hoering and Parker (1961) misquoted him and reported it. They apparently mistook the isotopic fractionation factor for perchlorate and the dissociated chlorine atom (reduced partition function of perchlorate) for the isotopic fractionation factor between perchlorate and aqueous chloride. Although Schauble et al. (2003) did not calculate the aqueous chloride reduced partition function; they were able to estimate it using the experimental determination of the equilibrium fractionation between chloride and NaCl given by Eggenkamp et al. (1995) and their theoretical determination of the reduced partition function for NaCl. The reduced partition function for the aqueous chloride anion would be between 2.1 and 3.0 at 22 °C, allowing the calculation of the isotopic fractionation between chloride and perchlorate at 25 °C. Using data from Schauble et al. (2003) or Urey (1947) for perchlorate, chloride is fractionated with respect to perchlorate by −70.0‰ or −75.9‰ respectively.

1.3.2. Isotopic fractionation associated with microbial reduction of perchlorate to chloride

One strain of perchlorate-reducing bacteria (*Azospira suillum* previously named *dechlorosomas suillum*) was shown to fractionate chlorine isotopes during the reduction of perchlorate, reducing ${}^{35}\mathrm{Cl}_{\mathrm{ClO}_4^-}$ faster than ${}^{37}\mathrm{Cl}_{\mathrm{ClO}_4^-}$ (Coleman et al., 2003; Sturchio et al., 2003). Laboratory experiments performed by two independent teams, yielded isotopic fractionation values for chloride relative to perchlorate between −16.6 and −12.9‰ (Sturchio et al., 2003) and −15.8±0.4‰ and −14.8±0.7‰ (Coleman et al., 2003). In an attempt to better characterise this isotope fractionation and especially its strain or temperature dependence, Sturchio et al. (2007) performed laboratory experiments of perchlorate biodegradation by *Dechlorospirillum* sp. FBR2 and *A. suillum* JPLRND at 22 °C and 10 °C. The $\Delta^{37}\mathrm{Cl}_{\mathrm{Cl}^-\mathrm{-ClO}_4^-}$ value ranged between −14.5 and −11.5%. No strain or temperature dependence was identified but $\Delta^{37}\mathrm{Cl}_{\mathrm{Cl}^-\mathrm{-ClO}_4^-}$ values for their duplicate experiment nearly cover the whole range of reported $\Delta^{37}\mathrm{Cl}_{\mathrm{Cl}^-\mathrm{-ClO}_4^-}$ variations, preventing the identification of a smaller influence of temperature or strain if any.

1.4. Purpose of this paper

To date, this isotopic fractionation is by far the largest ever observed for chlorine-bearing species. It places the chlorine stable isotope composition of perchlorate among the most powerful tools for monitoring bioremediation of groundwater pollution or for detecting potential perchlorate-reducing activity in arid environments where perchlorates are likely to accumulate such as the Atacama Desert of Chile or possibly Mars's surface of which it is considered as a possible analogue. The Atacama Desert is so arid that in some places no living organisms can be isolated. This may be one of the reasons why perchlorates are preserved there, but if they have been partly biodegraded, their $\delta^{37}\mathrm{Cl}$ could be used as evidence of metabolic activity.

Beyond these environmental applications more fundamental applications relate to the understanding of interactions between biochemistry of respiratory metabolisms and their isotope effects. The biochemistry of perchlorate reduction has now been sufficiently characterized (Rikken et al., 1996; van Ginkel et al., 1996; Kengen et al., 1999; O'Connor and Coates, 2002; Coates and Achenbach, 2004; Achenbach et al., 2006) to consider (i) identifying internal biochemical controls on the chlorine isotope fractionation and (ii) using this system as a tractable model for understanding other dissimilatory metabolic fractionations.

Improving the precision of the value of this chlorine isotope fractionation would therefore be useful both as a laboratory reference point with which to compare other experimental, environmental or theoretical values, and to investigate the internal biochemical controls that determine the extent of chlorine fractionation.

In a reconnaissance study, Coleman et al. (2003) published relatively precise results of experiments to determine the isotopic fractionation

associated with microbial reduction of perchlorate but without a supporting detailed methodology, comprehensive data set, or discussion of the fundamental processes involved.

Given the fundamental interest in a precise determination of the $\Delta^{37}Cl_{Cl^--ClO_4^-}$, we present here the full experimental data and methodology used to calculate the results reported by Coleman et al. (2003) along with a reevaluation of this result using an improved statistical treatment. On this basis we propose a hypothetical model for the biochemical control of the chlorine isotope fractionation induced by microbial perchlorate reduction.

2. Methods

2.1. Microbial experiments

Duplicate culture experiments (labelled S1 and S3) of *A. suillum* were performed at Southern Illinois University, Carbondale, IL. The experiments were conducted and monitored in a manner similar to that described by Chaudhuri et al. (2002). The culture experiments were performed in a batch fermentor at 37 °C, in a basal medium previously described in which chloride salts were replaced with equivalent sulphate salts (Coleman et al., 2003). Acetate (~15 mM) and perchlorate (~10 mM) were added as the sole electron donor and acceptor, respectively. Assuming 100% dissimilation, the electron demand for the complete reduction of perchlorate is met by the acetate, however, as almost 50% of the acetate was assimilated by the microbial cells to make biomass, the acetate content was insufficient to ensure complete reduction of the perchlorate content. The pH value was maintained at 7 by way of automatic dispensation of either 0.5 M H_2SO_4 or 1.0 M NaOH. The inoculum culture of *A. suillum* had been prepared in an identical chloride-free medium but with nitrate (10 mM) as the electron acceptor. Growth was monitored by microscopic observation and optical density measurements at 600 nm. Preliminary perchlorate and chloride concentrations were measured by ion chromatography as previously described (Chaudhuri et al., 2002).

Experiment C was performed as a preliminary investigation using exactly the same basal medium as described in Chaudhuri et al. (2002). Therefore it started with substantial chloride content and the basal medium contained enough acetate to allow the complete reduction of the perchlorate.

For each of the three culture experiments, samples were collected throughout the growth phase. In order to stop perchlorate reduction in the samples, bacterial cells were removed by filtration through sterile 0.2-μm-pore-size filters. The samples were then introduced into sterile 10-ml serum vials, septum sealed and filled with N_2, and sent to the isotope analysis laboratory in the University of Reading, where they were stored in a refrigerator before their analysis.

2.2. Analytical procedure

The analyses of chloride and perchlorate content and chlorine isotopic composition were performed as described previously (Ader et al., 2001) at the University of Reading, UK. Briefly, chloride is removed from the solution by precipitation as silver chloride and filtration. The perchlorate left in the filtrate is then quantitatively reduced to chloride by alkaline fusion-decomposition at 600 °C in K_2CO_3. The resulting salts are then dissolved in distilled water, and the chloride precipitated as silver chloride. Silver chloride precipitates obtained either from the chloride or from the perchlorate were treated as required for a chlorine isotopic analysis (Eggenkamp, 1994): the silver chloride is reacted with excess iodomethane (CH_3I) and the resultant chloromethane (CH_3Cl) is separated from residual iodomethane by gas chromatography before introduction to the mass spectrometer.

The perchlorate and chloride contents were measured by comparison of the CH_3Cl peak area given by gas chromatography for the sample with that of chloride isotope standards of known size. The relative precision for this method evaluated on the seawater chloride standards for each set of analyses is usually of ±5% (1σ) but can be as high as 10%, depending on the stability of the GC and on the sample size. Although this method may be only comparable or even worse in precision than ion chromatography, it presents two advantages in the context of this study: it is performed on exactly the same sample that is analysed isotopically and the chlorine yields of both perchlorate and chloride are obtained by comparison with the same standard (sea water), which implies that their relative abundances are directly comparable.

Chorine isotope compositions were measured on a VG SIRA 12, triple-collector, dual-inlet, isotope ratio mass spectrometer. Results are reported relative to the samples of seawater chloride reference material run with each batch of analyses. Any seawater sample can be considered as representative of Standard Mean Ocean Chlorine (SMOC) (Godon et al., 2004 and references therein). For the complete chloride or perchlorate $\delta^{37}Cl$ analysis processes, reproducibility is ≤0.05‰ (1σ) and chloride blank is lower than 0.1 μmol (i.e. <1% of the sample). Correction for the blank contribution was not needed.

3. Results

As reported in the previous studies (Chaudhuri et al., 2002; Coleman et al., 2003), neither chlorate nor chlorite, the two major potential intermediate products, was detected (detection limit, 0.001 mM) by ion chromatography in the samples. The results of the analyses of chloride and perchlorate content and chlorine isotopic composition are presented in Table 1 for the three culture experiments. Duplicate analyses of some of the samples show a satisfactory reproducibility: the differences between duplicate measurements are lower than 10% of the measured values for the chlorine content and lower than of 0.2‰ for the $\delta^{37}Cl$ except for one chloride $\delta^{37}Cl$ (sample S14). S1 and S3 being duplicate experiments the perchlorate and chloride content and isotopic composition for each sampling time are similar, except for the duplicate samples, S34 and S14, and S35 and S15. S34 and S35 chloride contents are higher than those of their duplicate samples S14 and S15, whereas their perchlorate contents are lower. Comparison with ion chromatography measurements performed immediately after sampling also shows an increase in chloride content and a decrease in perchlorate content in S34 and S35 at the time of the isotopic analyses. This and the white cloudy appearance of both these samples suggest that unexpected bacterial growth and perchlorate reduction have continued during their transport and storage, although the bacteria removal by filtration was believed to be complete.

3.1. Perchlorate content and $\delta^{37}Cl$ evolution

The initial perchlorate contents and isotopic compositions were measured on the un-inoculated control samples, C1, S10 and S30. The initial perchlorate content is 10.41 ± 0.38 mM for the C experiment, and of 9.45 ± 0.30 mM for the S1 and S3 experiments. The initial perchlorate $\delta^{37}Cl$, estimated as the average of those measurements, is +0.65 ± 0.08‰. In all three experiments (Table 1), the perchlorate content decreases as its $\delta^{37}Cl$ increases so that when the perchlorate content reaches about 4 mM (more than 50% of the perchlorate reduced) its $\delta^{37}Cl$ value is close to +13‰.

3.2. Total chloride $\delta^{37}Cl$ evolution

The initial chloride content (chloride blank) was very small. Consequently its concentration and its isotopic composition could be measured for the S1/S3 experiments only by combining samples S10 and S30. Samples S11 and S31 taken after 30 min had not started to react and provide duplicate analyses of the initial chloride content and $\delta^{37}Cl$. The chloride blank content is 0.22 ± 0.02 mM and its $\delta^{37}Cl$ is +0.17 ± 0.05‰. This blank probably results from chloride salts used in the preparation of the vitamin and mineral stock solutions (Bruce

Table 1
Data measured on the samples of the experiments C, S1 and S3: chloride content and isotopic composition (δ^{37}Cl) as well as perchlorate content and isotopic composition

	Experiment C						Experiment S1						Experiment S3			
Sample	Cl^-	δ^{37}Cl	ClO_4^-	δ^{37}Cl	Sample	Time	Cl^-	δ^{37}Cl	ClO_4^-	δ^{37}Cl	Sample	Time	Cl^-	δ^{37}Cl	ClO_4^-	δ^{37}Cl
	mM		mM				mM		mM				mM		mM	
	±5%	0.1‰	±5%	±0.1‰		min	±5%	±0.1‰	±5%	±0.1‰		min	±5%	±0.1‰	±5%	±0.1‰
C1	n.d.	n.d.	10.38	0.73	S10	0	0.22	0.17	9.20	0.80	S30	0	0.22	0.17	9.80	0.65
			10.04	0.56												
			10.80	0.64	S11	30	0.21	0.07	9.20	0.59	S31	30	0.21	0.07	9.60	0.61
			n.d.	0.62												
Average			10.41	0.64	S12	72.5	0.98	−10.70	8.40	1.83	S32	72.5	0.84	−10.29	9.40	1.66
Difference			0.76	0.17												
					S13	77.5	1.55	−11.34	7.80	3.11	S33	77.5	1.49	−11.28	7.75	2.80
C5	1.85	−9.94	8.54	2.91												
	1.90	−9.80	n.d.	3.00	S14	82	2.40	−11.20	7.20	4.96	S34	82	5.50	−9.10	3.40	13.59
Average	1.88	−9.87					2.70	−10.87					5.50	−8.93		
Difference	0.05	0.14			Average		2.55	−11.04			Average		5.50	−9.02		
					Difference		0.30	0.33			Difference		0.00	0.17		
C4	3.17	−10.45	7.5	5.18												
	3.17	−10.26	7.24	5.17	S15	85.5	4.05	−9.76	4.85	9.36	S35	85.5	5.40	−8.85	3.60	12.82
	3.00	−10.35					3.90	−9.75					5.30	−8.90		
Average	3.11	−10.35	7.37	5.18	Average		3.98	−9.76			Average		5.35	−8.88		
Difference	0.17	0.19	0.26	0.01	Difference		0.15	0.01			Difference		0.10	0.05		
C3	7.10	−7.56	4.1	15.19	S16	90	5.50	−8.55	4.00	13.20	S36	90	5.55	−8.64	4.40	13.04
			3.9	15.07			5.45	−8.62					5.55	−8.73		
Average			4.0	15.13	Average		5.48	−8.59			Average		5.55	−8.69		
Difference			0.2	0.12	Difference		0.05	0.07			Difference		0.00	0.09		
C2	11.00	0.62	n.d.	n.d.	S17	99.5	5.80	−8.55	3.90	13.64	S37	99.5	5.65	−8.42	3.60	13.69
	11.50	0.63					5.35	−8.75					5.50	−8.62		
	10.60	0.54			Average		5.58	−8.65			Average		5.58	−8.52		
Average	11.03	0.60			Difference		0.45	0.20			Difference		0.15	0.20		
Difference	0.50	0.09			S18	121.5	6.10	−8.33	n.d.	n.d.	S38	121.5	5.90	−8.25	3.80	14.51
							5.80	−8.41					5.60	−8.35		
						Average	5.95	−8.37			Average		5.75	−8.30		
						Difference	0.30	0.08			Difference		0.30	0.10		

The chloride contents and isotopic compositions reported for S10 and S30 correspond to the chloride content measured on their cumulate. This is also the case for S11 and S31. Where duplicate measurements could be performed, their averages and differences are given.

et al., 1999) or possibly even from the bacteria. Unfortunately, the limited amount of sample C1 available did not allow a chloride blank determination for experiment C, but we expect it to be higher than in the S experiments because no special care was taken to minimise the initial input of chloride.

In all three experiments, as the chloride content starts to increase, its δ^{37}Cl decreases very sharply down to values of −11‰ before increasing more slowly toward the starting isotopic composition of the initial perchlorate. It reaches a value of +0.62 ± 0,05‰ in sample C2 after completion of the perchlorate reduction. This decrease of the chloride δ^{37}Cl at the beginning of the experiment is certainly the result of mixing between the chloride blank and the microbially reduced chloride with negative δ^{37}Cl, and it shows the necessity of correcting the chloride data from the blank contribution before any interpretation of these data in terms of microbial fractionation can be done.

3.3. Blank corrections and produced chloride δ^{37}Cl evolution

The content and isotopic composition of the chloride produced by microbial reduction for each sample of the experiments S1 and S3 were calculated by correcting the chloride content and isotopic composition of the sample for the chloride blank. The latter is determined by analysing the chloride content and isotopic composition of the initial samples S10–S30 sampled prior to inoculation and S11–S31 samples just after the inoculation before any detectable perchlorate reduction had occurred (Table 1). The blank corrected values of chloride content and isotopic composition are reported in Table 2, the average values being given when several determinations were performed. The δ^{37}Cl of the produced chloride increases steadily from values close to −14‰ in samples S12, S32 at the beginning of the experiments, up to values close to −8.7‰ in the samples S18 and S38.

3.4. Calculation of combined statistical uncertainties

The statistical uncertainty of each result depends on the uncertainties of all the individual parameters measured that contribute to that result. We need to calculate mass and isotopic balances, correct the data for the blank and calculate fractionation factors. In order to assess the precision of those calculated numbers and, more importantly, to evaluate the validity of the differences or similarities between them, we need to determine their statistical uncertainties. For this purpose we used Crystal Ball® software, which determines the standard deviation of each of the calculated values from the individual uncertainties determined for the measured values, using a Monte Carlo simulation approach.

4. Treatment and basic interpretation of the results

4.1. Validation of results by mass and isotopic balance

These experiments behave as closed systems with respect to chlorine. The only two identified chlorine species are chloride and perchlorate and there is neither loss nor gain of chlorine, but only its transfer from the perchlorate anion to the chloride anion in solution. The total chlorine content (expressed as the sum of the perchlorate and chloride, without blank correction) and its isotopic composition should therefore be constant through out the experiment. This mass and isotopic balance is shown in the Eq. (4)

$$\delta^{37}\mathrm{Cl}_{\mathrm{tot}} \cdot C_{\mathrm{Cltot}} = \delta^{37}\mathrm{Cl}_{\mathrm{Cl}^-} \cdot C_{\mathrm{Cl}^-} + \delta^{37}\mathrm{Cl}_{\mathrm{Cl04}^-} \cdot C_{\mathrm{Cl04}^-} \quad (4)$$

where C is for concentration and the chloride data are not corrected from the blank. The validity of the chloride and perchlorate content and

Table 2
Treated results for the experiments C, S1 and S3

Samples	Blank corrected Cl^- results		ClO_4^- results		Mass and isotopic balance		$\Delta^{37}Cl_{Cl^-\text{–}ClO_4^-}$ calculated using $f_{ClO_4^-}$			$\Delta^{37}Cl_{Cl^-\text{–}ClO_4^-}$ calculated using f_{Cl^-}			Weighted-mean
	C_{Cl} corrected	δ^{37}Cl corr	$C_{ClO_4^-}$	$\delta^{37}Cl_{ClO4}$	Tot Cl	δ^{37}Cl tot	$f_{ClO_4^-}$	Δ^{37}Cl (ClO_4^-)	Δ^{37}Cl (Cl^-)	f_{Cl}	Δ^{37}Cl (ClO_4^-)	Δ^{37}Cl (Cl^-)	
C1			10.41	0.64	10.74±0.7	0.62±0.07	1.00						
C5			8.54	2.95	10.41±0.5	0.65±0.15	0.82	−11.65±3.5					
C4			7.37	5.18	10.48±0.4	0.57±0.23	0.71	−13.12±1.9					
C3			4.00	15.13	11.10±0.4	0.62±0.37	0.38	−15.03±0.8					
C2					11.03±0.6	0.60±0.05	0.00						
					10.74±0.2	0.61±0.04		−14.61±0.7					
S10	0.01		9.20	0.80	9.42±0.5	0.79±0.05							
S11	−0.01		9.20	0.59	9.41±0.5	0.58±0.05							
S12	0.77±0.05	−13.76±0.36	8.40	1.83	9.38±0.4	0.52±0.09	0.90	±109	−15.15±0.5	0.92	−13.63±1.2	−15.03±0.4	
S13	1.34±0.15	−13.19±0.27	7.80	3.11	9.35±0.4	0.71±0.22	0.84	−13.91±5.5	−15.11±0.5	0.86	−15.79±2.0	−14.95±0.2	
S14	2.34±0.21	−12.07±0.28	7.20	4.96	9.75±0.5	0.77±0.30	0.77	−16.77±3.7	−14.47±0.5	0.75	−14.85±1.6	−14.72±0.3	
S15	3.77±0.40	−10.33±0.09	4.85	9.36	8.83±0.5	0.74±0.54	0.52	−13.30±1.1	−15.39±0.5	0.60	−16.69±2.5	−14.33±0.5	
S16	5.27±0.28	−8.95±0.06	4.00	13.20	9.48±0.5	0.61±0.38	0.43	−14.77±0.9	−14.98±0.4	0.43	−14.93±1.3	−14.90±0.6	
S17	5.36±0.32	−9.00±0.15	3.90	13.64	9.48±0.4	0.53±0.42	0.42	−14.84±0.9	−15.28±0.5	0.42	−15.02±1.4	−15.19±0.7	
S18	5.74±0.30	−8.69±0.07								0.38		−15.55±0.8	for S1
				Weighted-mean for each Δ^{37}Cl calculation				−14.46±0.60	−15.07±0.21		−14.75±0.62	−14.88±0.18	−14.93±0.16
S30	0.01		9.80	0.65	10.02±0.5	0.64±0.05							
S31	−0.01		9.60	0.60	9.81±0.5	0.59±0.05							
S32	0.62±0.09	−13.95±0.67	9.40	1.66	10.23±0.5	0.69±0.11	1.01	±978	−14.52±0.8	0.93	−14.67±2.4	−15.10±0.6	
S33	1.28±0.15	−13.21±0.30	7.75	2.80	9.24±0.5	0.53±0.22	0.83	−11.73±4.6	−15.18±0.5	0.86	−14.51±2.0	−14.92±0.2	
S34	5.29±0.28	−9.39±0.13	3.40	13.59	8.90±0.3	−0.38±0.38	0.37	−12.76±0.7	−17.17±0.6	0.43	−15.29±1.3	−15.63±0.7	
S35	5.14±0.27	−9.26±0.06	3.60	12.82	8.95±0.3	−0.15±0.38	0.39	−12.73±0.7	−16.40±0.5	0.45	−15.04±1.3	−15.11±0.6	
S36	5.34±0.28	−9.05±0.07	4.40	13.04	9.95±0.5	0.92±0.61	0.47	−16.43±2.2	−14.35±0.8	0.43	−14.43±1.2	−15.20±0.6	
S37	5.37±0.28	−8.87±0.15	3.60	13.69	9.18±0.5	0.19±0.60	0.39	−13.64±1.4	−15.76±0.9	0.42	−15.05±1.3	−14.99±0.6	
S38	5.54±0.29	−8.63±0.08	3.80	14.51	9.55±0.4	0.77±0.40	0.41	−15.36±0.9	−14.90±0.4	0.40	−15.20±1.3	−14.98±0.6	for S3
				Weighted-mean for each Δ^{37}Cl calculation				−14.97±0.68	−14.95±0.26		−14.82±0.54	−14.97±0.19	−14.95±0.16
													for S1 and S3
Weighted-mean for S1 and S3					9.52±0.12	0.64±0.02							−14.94±0.15

In the first columns the blank corrected chloride results and the untreated perchlorate results are given. The mass and isotopic balance data calculated using the untreated chloride and perchlorate data are given in the following columns, and finally, the various calculated chlorine isotopic fractionations are reported. The corresponding uncertainties were evaluated using Crystal Ball software.

isotopic analyses reported in this paper therefore can be assessed by chlorine isotopic and mass balance at all stages of the experiments. The results of the calculations together with their standard deviations are reported in Table 2. For most samples, the total chlorine content and isotopic composition, calculated by isotopic and mass balance, are consistent within errors and with the initial (control) samples. This demonstrates that no chloride has been lost or gained during the experiments, nor during the complete analysis process, and that the isotopic compositions measured are accurate within the measurement uncertainty.

The samples S34 and S35, for which bacterial growth and perchlorate reduction continued after sampling, present mass and isotopic imbalance with lower total δ^{37}Cl values and total chlorine content compared to the others samples. These samples therefore will be excluded from the following average calculations and considered separately.

4.2. Rayleigh fractionation model of the results

4.2.1. Basis of the model

We used one of the mathematical expressions of the Rayleigh distillation model:

$$R_i/R_0 = f_i^{(\alpha-1)} \tag{5}$$

where,

R_0 is the stable isotope ratio of the initial reagent
Ri is the stable isotope ratio of the reagent
f_i is the fraction of reagent left, both at stage I
α is the isotopic fractionation factor for the reaction ($\alpha = R_{product}/R_{reagent}$).

Because we measure δ rather than ratios, we substituted values from Eqs. (1) (2) and (3) in Eq. (5) as shown by Eq. (6). This allows the calculation of the isotopic fractionation between the instant product and the reagent.

$$\Delta_{\text{instant product–reagent}} = \ln\left[\left(10^3 + \delta^{37}\text{Cl}_{\text{reagent}}\right)/\left(10^3 + \delta^{37}\text{Cl}_{\text{initial reagent}}\right)\right] \times 10^3/\ln(f) \tag{6}$$

4.2.2. Modelled results

The results are shown in Fig. 1 where chloride, perchlorate and total chlorine δ^{37}Cl are plotted versus the fraction of perchlorate left ($f_{Cl\text{–}ClO4\text{–}}$) used as an indicator of reaction progress.

Because the experiments are closed systems with respect to chlorine, and because we measured both the chloride and perchlorate content and isotopic composition, we can calculate Δ by two independent paths (and in a total of four different ways). Indeed, $f_{ClO_4^-}$ can be calculated independently using either the perchlorate content ($f_{ClO_4^-} = ClO_4^-/ClO_{4\text{-initial}}^-$) or the chloride content ($f_{Cl^-} = [ClO_{4\text{-initial}}^- - Cl^-]/ClO_{4\text{-initial}}^-$), and for each f, Δ can be calculated using either the measured $\delta^{37}Cl_{ClO_4^-}$ or the $\delta^{37}Cl_{ClO_4^-}$ calculated by mass and isotopic balance using the measured $\delta^{37}Cl_{Cl}$ according to Eq. (7).

$$\delta^{37}\text{Cl}_{\text{ClO}_4^-} = \left[\delta^{37}\text{Cl}_{\text{ClO}_{4\text{–initial}}^-} - \left(1 - f_{\text{ClO}_4^-}\right) \times \delta^{37}\text{Cl}_{\text{Cl}^-}\right]/f_{\text{ClO}_4^-} \tag{7}$$

The initial perchlorate content and isotopic composition can be evaluated by two independent methods. The first one is the average of

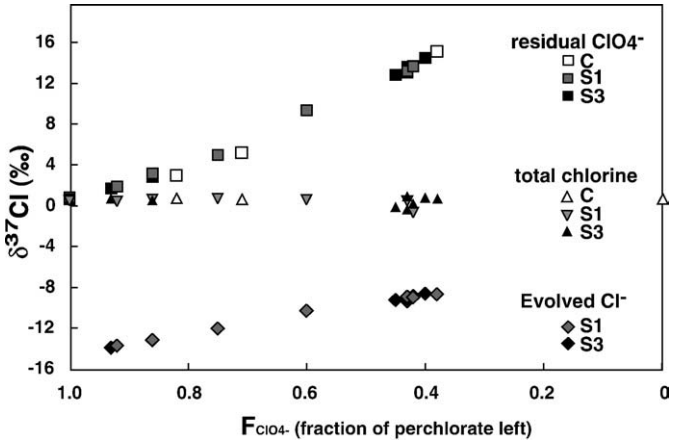

Fig. 1. Evolution of the perchlorate, evolved chloride (blank corrected) and total chlorine isotopic compositions, as a function of the fraction of perchlorate left during the culture experiments C, S1 and S3.

the results obtained for the initial samples S10, S11, S30 and S31: $ClO_{4\text{-initial}}^{-}$ = 9.45 ± 0.32 mM and $\delta^{37}ClO_{4\text{-initial}}^{-}$ = 0.65 ± 0.05‰ (n = 4). The second method uses the average of the blank corrected values of the total chlorine (Cl and ClO_4) content and isotopic composition for every S1 and S3 sample: $ClO_{4\text{-initial}}^{-}$ = 9.30 ± 0.12 mM and $\delta^{37}Cl_{ClO_4^-}$ = 0.65 ± 0.02‰ (n = 15, S34 and S35 excluded). Both methods agree perfectly for the $\delta^{37}Cl_{ClO4.initial}$ and quite well for the $ClO_{4\text{-initial}}^{-}$. The second method is preferred here because the averages are calculated on a higher number of samples, which makes them more precise.

For the samples of experiment C, because the chloride blank could not be determined, only the fractionation derived from perchlorate results could be calculated, and the initial perchlorate content and isotopic composition used had to be those measured for the sample C1.

The results of the fractionation calculations are reported in Table 2, together with their standard deviations (±1σ) obtained by Monte Carlo simulation using Crystal Ball® software. All $\Delta_{\text{instant-}Cl^-\text{–}ClO_4^-}$ values are between −11.73 and −17.17‰. The $\Delta_{\text{instant-}Cl^-\text{–}ClO_4^-}$ calculated using $f_{ClO_4^-}$ and the measured $\delta^{37}Cl_{Cl}$ are homogeneous and present the lowest standard deviations (between ±0.2‰ and ±0.8‰). The Δ values calculated using $f_{ClO_4^-}$ and the measured $\delta^{37}Cl_{ClO_4^-}$ present the largest scatter along with the highest standard deviations (between ±0.7‰ and ±5.5‰); with the exception of S34 and S35 Δ values, this large scatter results from error propagation associated with the calculation (as indicated by the high standard deviations), rather than real differences in the fractionation values. We can therefore conclude that there is no visible change of $\Delta_{\text{instant-}Cl^-\text{–}ClO_4^-}$ with the progress of the perchlorate reduction.

The fact that for S34 and S35, Δ values calculated using f_{ClO4} are so distinct from each others and from most of the remaining values, while those calculated using f_{Cl} are similar to the other samples, suggests that the mass and isotopic imbalance identified in these samples is due to a loss of chlorine initially contained in perchlorate during the analytical procedure. The reason for it remains unknown, but maybe is related to the fact that bacterial growth and perchlorate reduction continued in these samples after their sampling.

4.2.3. Statistical appraisal of final results

To prevent undue emphasis being exerted by possibly erroneous values, we chose to use the weighted-mean (Eq. (8)) to calculate the average fractionation.

$$\Delta_{\text{mean}} = \Sigma w_i x \Delta_i \tag{8}$$

where $w_i = (l/\sigma_i^2)/(\Sigma l/\sigma_i^2)$.

In this calculation each Δ_i is weighted by inverse square of its standard deviation, which ensured that values with the greatest uncertainty will not influence the average value as much as the others.

Weighted-means were calculated for each of the four Δ calculations of each experiment, excluding those calculated for S34 and S35 samples since they present mass and isotopic imbalance. Their standard deviations are determined using Crystal Ball®. They are all in the range of −14.46 to −15.07%. The global $\Delta_{\text{instant-}Cl^-\text{–}ClO_4^-}$ weighted-mean values are −14.93 ± 0.16% for S1, −14.95 ± 0.16% for S3, and −14.94 ± 0.14% (σ) for S1 and S3.

4.3. Comparison with previous results

The fact that the Rayleigh fractionation model describes O_4^- the observed relationships so well, confirms that no significant isotopic exchange between chloride and perchlorate occurred in the samples during the shipping and storage periods preceding their analysis when temperature probably varied between 4 and 30 °C. This lack of exchange confirmed simple laboratory experiments where change in $\delta^{37}Cl$ of Cl^- in the presence of ClO_4^- was less than the analytical uncertainty (Bao and Gu, 2004; Sturchio et al., 2007). The fractionation factor between perchlorate and instant chloride therefore results exclusively from bacterial perchlorate reduction.

The minor difference in value between the $\Delta_{Cl^-\text{–}ClO_4^-}$ value determined for experiments S1 and S3 on *A. suillum* strain PS at 37 °C in this study (−14.94 ± 0.15‰) and by Coleman et al. (2003) (−14.8 ± 0.7‰) is mostly due to the sensitivity of the final result to the initial ClO_4^- content (particularly for the results from experiment C). The improved precision in this study is due to the use of comprehensive statistical treatment of the data and error propagation.

The $\Delta_{Cl^-\text{–}ClO_4^-}$ value of −14.94 ± 0.15‰ determined in this study for experiments S1 and S3 on *A. suillum* strain PS at 37 °C is within the range of −16.6 to −11.5‰ determined for experiments on *Dechlorospirillum* sp. FBR2 and *A. suillum* JPLRND at room temperature, 22 °C and 10 °C (Sturchio et al., 2003, 2007). The uncertainties reported here are nearly of an order of magnitude smaller than those reported by Sturchio et al. (2003) and (2007).

5. Discussion: isotope insights into the biochemistry of microbial perchlorate reduction

5.1. Comparison with thermodynamic equilibrium fractionation calculations

Before discussing the significance of this isotope fractionation in terms of biochemical control, it is important to ensure that it is not entirely controlled by a thermodynamic isotopic equilibrium between chloride and perchlorate.

Urey (1947) and Schauble et al. (2003) gave theoretical determinations of chlorine isotope equilibrium fractionation for most of the inorganic chlorine-containing chemical species. Because the two papers report isotopic fractionation results differently, we have recalculated them here as Δ using Eq. (3), for a temperature of 37 °C (the temperature of our experiments) by linear interpolation between the values at 25 °C and those at 100 °C. The values are reported in Table 3 as a matrix of Δ values, fractionations between the chemical species in the first row and the first column. From a general point of view this table illustrates that the equilibrium isotopic fractionation depends on the difference of oxidation states of chlorine in the chemical species considered; the larger the differences, the larger the chlorine isotopic equilibrium fractionation. For chlorine isotopes, this link between oxidation state and equilibrium fractionation factor was reported in detail by Schauble et al. (2003) and is not dependent on whether Cl is bonded to C or not.

Recalculated values for the theoretical equilibrium $\Delta_{Cl^-\text{–}ClO_4^-}$ at 37 °C (around −70‰) are significantly greater than for the $\Delta_{Cl^-\text{–}ClO_4^-}$ measured for the microbial reduction of perchlorate to chloride (around −15‰), which implies that this microbially mediated reaction is not occurring at isotopic equilibrium between perchlorate and produced chloride. The fact that the fractionation expressed by

Table 3
Matrix showing the equilibrium chlorine isotope fractionations between species with various Cl oxidation numbers, calculated at 37 °C by linear interpolation, using Urey (1947) and Schauble et al. (2003) data

Species		ClO_4^-	ClO_3^-	OClO	ClO	HOCl	Cl_2	HCl	Cl^-	
	Oxidation number	7	5	4	2	1	0	−1	−1	
ClO_4^-	7	0.0	−32.1	−46.4			−67.2	−69.5	−71.3	Urey
	7	0.0		−42.3	−59.6	−60.6	−61.6	−63.8	−65.8	Schauble
$Cl0_3^-$	5		0.0	−14.7			−36.2	−38.6	−40.5	Urey
ClO	4			0.0			−21.8	−24.2	−26.1	Urey
	4			0.0	−18.1	−19.1	−20.2	−22.5	−24.5	Schauble
Cl0	2				0.0	−1.0	−2.1	−4.5	−6.5	Schauble
HOC1	1					0.0	−1.1	−3.4	−5.5	Schauble
$C1_2$	0						0.0	−2.4	−4.5	Urey
	0						00.0	−2.3	−4.4	Schauble
HC1	−1							0.0	−2.0	Urey
	−1							0.0	−2.1	Schauble
$C1^-$	−1								0.0	Urey
	−1								0.0	Schauble

the microbial metabolic activity is smaller than the equilibrium fractionation, appears to be an almost ubiquitous feature of these systems. It was in particular observed for microbial sulphate reduction, which expresses a much smaller fractionation between sulphate and sulphide (typically <35‰ but highly variable; see for example Canfield, 2001) than the equilibrium fractionation (>60‰, Tudge and Thode, 1950; Thode et al., 1971; Ohmoto and Lasaga, 1982; Farquhar et al., 2003), as well as for microbial iron reduction (Anbar, 2004; Schauble et al., 2001).

5.2. The plausibility of kinetic isotope fractionation?

Such discrepancies between the equilibrium isotopic fractionation and the expressed biological isotopic fractionation are usually ascribed to kinetic isotope fractionation. This is the case in particular for microbial sulphate reduction, for which the classical model proposes that the expressed isotope fractionation results from a combination of steps, each operating with a kinetic isotope effect (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968; Rees, 1973; Canfield et al., 2006).

To account for the fractionation induced by microbial iron reduction, Kavner et al. (2005) proposed a kinetic isotope fractionation associated with electron transfer. To support this hypothesis, they have extended Marcus's theory for the kinetics of electron transfer to show that there should be a kinetic isotope fractionation associated with it and have substantiated this theory experimentally by measuring a voltage-dependent isotope fractionation associated with iron electroplating. The magnitude of the electrochemically-induced fractionation being similar to that of Fe reduction by certain bacteria, they suggest that electrochemical processes may be responsible for biogeochemical Fe isotope fractionation. This hypothesis might apply to other respirative isotope fractionation and possibly to perchlorate reduction.

5.3. A rate limiting step occurring at thermodynamic isotope equilibrium

It is now considered likely that some of the internal steps may operate at thermodynamic isotopic equilibrium (Brunner and Bernasconi, 2005; Johnston et al., 2007). This hypothesis has also been put forward to explain the isotope fractionation associated with microbial iron reduction (Anbar, 2004; Schauble et al., 2001), and is worth investigating in the case of perchlorate reduction.

As summarised below, microbial perchlorate reduction offers the advantage of behaving relatively simply compared to sulphate and dissimilatory iron reduction processes in terms of processes that might contribute to the expressed isotopic fractionation. The identification of a thermodynamic isotope fractionation control on the expressed biological fractionation, if any, may thus be more straightforward.

The biochemistry of perchlorate reduction can be summarised as follows. Perchlorate, after its incorporation into the cell, is reduced via at least a two-step pathway, $ClO_4^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$ (Coates et al., 1999; Coates and Achenbach, 2004; Rikken et al., 1996), involving two different enzymes. The perchlorate reductase, located in the periplasm and probably anchored to the cytoplasm membrane, is responsible for the first step, which may be a combination of two steps ($ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^-$) since, although we did not detect any, chlorate was identified as an intermediate species (Sturchio et al., 2003, 2007), and perchlorate reductase also reduces chlorate to chlorite (Bender et al., 2005; Kengen et al., 1999). Chlorite dismutase, located in the outer cell membrane, is responsible for the disproportionation of chlorite to chloride and molecular oxygen, and seems to directly transfer the produced chloride out of the cell. This enzyme is highly conservative among the perchlorate reducers (O'Connor and Coates, 2002), specific to chlorite and, most importantly when considering isotope fractionation effects, it reduces the chlorite quantitatively (Bender et al., 2002; Coates et al., 1999; van Ginkel et al., 1996). Both chlorite disproportionation and chloride transfer out of the cell are therefore very likely not to be rate limiting, thus not to fractionate chlorine isotopes. Similarly, the absence of chlorate in our experiments (<0.001 mM) suggests that even if it is an intermediate, its reduction to chlorite is not rate limiting and will not fractionate chlorine isotopes. This leaves only two processes liable to induce isotopic fractionation: perchlorate transfer into and out of the cell and perchlorate reduction to its first intermediate species by perchlorate reductase. Assuming that the ClO_4^- transfer into and out of the cell does not fractionate chlorine isotopes, the rate limiting step responsible for the overall expressed isotope fractionation would be perchlorate to chlorate reduction, in which case it would not operate at thermodynamic isotopic equilibrium, the theoretical equilibrium fractionation between chlorate and perchlorate at 37 °C being ~−30‰ and not −15‰ (Table 3).

However, this first step involves a two-electron transfer as it reduces the perchlorate (oxidation state, +7) to chlorate (oxidation state, +5). In the electron transfer chain this step might comprise two steps of one electron transfer each, the first of which produces an intermediate product of oxidation state of +6. Because no inorganic chlorine compound is known with such an oxidation state, if a chlorine-containing molecule of oxidation state +6 exists it has to be as an intermediate or a radical in the reaction mechanism, for example through transient binding to a protein. Based on the observed relationship between the equilibrium isotope fractionation and the difference in oxidation state between two chlorine molecules (Schauble et al., 2003), the thermodynamic equilibrium isotopic fractionation between chlorine of oxidation state +6 and perchlorate can be predicted. In Fig. 2, the equilibrium isotopic fractionations involving perchlorate are reported as a function of the Cl oxidation

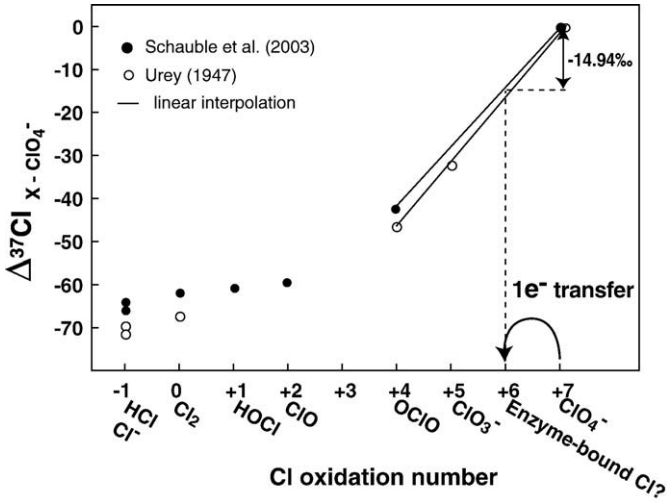

Fig. 2. Equilibrium chlorine isotope fractionation between a chlorine species X and perchlorate ($\Delta^{37}Cl_{x-ClO_4^-}$), as a function of the Cl oxidation number of X, calculated at 37 °C using Urey (1947) and Schauble et al. (2003) data. The species considered are indicated along the *X*-axis. A linear interpolation is drawn to model the data. The microbial fractionation ($\Delta^{37}Cl_{Cl^- - ClO_4^-}$) determined in this study is very close to the modelled isotope fractionation between perchlorate and a molecule with a chlorine oxidation number of 6, suggesting the likelihood of an isotopic equilibrium between perchlorate and an unknown chlorine species of oxidation state +6, which could be enzyme-bound and result from 1 electron transfer.

number of other molecules. The fractionation expected for a molecule of chlorine oxidation state +6 and perchlorate is around −15‰, which is very close to the biological fractionation measured in this study, suggesting that this one electron transfer may proceed at isotopic equilibrium and might be the rate limiting step that controls the overall isotope effect of the microbial perchlorate reduction process. This hypothesis is consistent with the observations that (i) there are no differences in the expressed microbial fractionations by different strains (Sturchio et al., 2007) and (ii) the biological fractionation factor associated with perchlorate reduction remains constant throughout the experiments, implying that it is not affected by the variations in bacterial activity or perchlorate availability during the experiments (this study; Sturchio et al., 2007). A definite test for this hypothesis would be that the isotopic fractionation varies with temperature as predicted by thermodynamic equilibrium isotopic fractionation, i.e. between −14.94‰ at 37 °C and −17‰ at 10 °C. Such a variation is not supported yet by the results of Sturchio et al. (2007), but the large uncertainties in their data may prevent the identification of a temperature dependence if any.

6. Conclusions

In this paper we have for the first time reported the full experimental data set and the comprehensive methodology used to calculate a precise chlorine isotope fractionation ($\Delta_{Cl^- - ClO_4^-}$) induced by the perchlorate reducer *A. suillum* at 37 °C previously reported extremely briefly by Coleman et al. (2003). By doing so, we were able to demonstrate unequivocally that the Rayleigh Distillation process is a good model to describe the relationship between perchlorate contents and isotopic values as well as the relationship between chloride contents and isotopic values. Using a more sophisticated statistical treatment of these data we were able to improve the accuracy and precision of the calculated $\Delta_{Cl-ClO4-}$ value compared to the values initially published by Coleman et al. (2003). Calculated using both the chloride and perchlorate independent sets of data, $\Delta_{Cl-ClO4-}$ value is of −14.94± 0.15‰ and is constant throughout the reaction, indicating that it is not sensitive to the perchlorate concentration in the basal medium, nor to the rate of perchlorate reduction. The advantages of the method rely on the complementary use of chloride and perchlorate content and chlorine isotopic composition. It enables (i) the identification and exclusion from the statistical treatment of samples that do not satisfy mass and isotopic balance criteria and (ii) the calculation of $\Delta_{Cl-ClO4-}$ by two independent sets of data.

Compared to the values published by Sturchio et al. (2003) and (2007) for similar experiments, the precision is improved by a factor of 10, which should be sufficient to allow future detailed investigation of the strain, temperature and other parameter dependency of the $\Delta_{Cl-ClO4-}$, and to make meaningful comparison of the results obtained with theoretical values of thermodynamic fractionation.

Given our present knowledge of perchlorate reduction biochemistry, and the coincidence between the $\Delta_{Cl-ClO4-}$ and the extrapolated thermodynamic equilibrium isotope fractionation between a chlorine molecule of oxidation state +6 (enzyme-bound intermediate?) and perchlorate, we suggest as a working hypothesis that the biochemical step controlling the microbial isotope effect is the first electron transfer of perchlorate reductase, and that it might occur at thermodynamic equilibrium.

In any case, whether this isotopic fractionation results from a kinetic or equilibrium process as envisaged here, it constitute an indisputable biosignature since even in the hypothesis of a thermodynamic equilibrium, the suspected +6 chlorine molecule requires a bond to an enzyme and thus cannot be expressed in an abiotic system.

Acknowledgements

This study was supported by the U.S. Department of Defense SERDP program. This paper is IPGP contribution No. 2352. Part of the work was carried out by the Jet Propulsion Laboratory (JPL), California Institute of Technology, under contract with the National Aeronautics and Space Administration (NASA) with support from JPL's Research and Technology Development Program via a grant to MC.

References

Achenbach, L.A., Bender, K.S., Sun, Y., Coates, J.D., 2006. The biochemistry and genetics of perchlorate reduction. In: Gu, B., Coates, J.D. (Eds.), Perchlorate: Environmental Occurrence, Interactions, and Treatment. Springer, pp. 297–310.
Ader, M., Coleman, M.L., Doyle, S., Stroud, M., Wakelin, D., 2001. Methods for the stable isotopic analysis of chlorine in chlorate and perchlorate compounds. Anal. Chem. 73, 4946–4950.
Anbar, A.D., 2004. Iron stable isotopes: beyond biosignatures. Earth Planet. Sci. Lett. 217, 223–236.
Bao, H., Gu, B., 2004. Natural perchlorate has a unique oxygen isotope signature. Environ. Sci. Technol. 38, 5073–5077.
Bender, K.S., O'Connor, S.M., Chakraborty, R., Coates, J.D., Achenbach, L.A., 2002. Sequencing and transcriptional analysis of the chlorite dismutase gene of *Dechloromonas agitata* and its use as a metabolic probe. Appl. Environ. Microbiol. 68, 4820–4826.
Bender, K.S., Shang, C., Chakraborty, R., Belchik, S.M., Coates, J.D., Achenbach, L.A., 2005. Identification, characterization, and classification of genes encoding perchlorate reductase. J. Bacteriol. 187, 5090–5096.
Bigeleisen, J., 1952. The effects of isotopic substitution on the rates of chemical reactions. J. Phys. Chem. 56, 823–828.
Bigeleisen, J., Mayer, M.G., 1947. Calculation of equilibrium constants for isotopic exchange reactions. J. Chem. Phys. 15, 261–267.
Böhkle, K.B., Sturchio, N.C., Gu, B., Horita, J., Brown, G.M., Jackson, W.A., Batista, J., Hatzinger, P.B., 2005. Perchlorate isotope forensics. Anal. Chem. 77, 7838–7842.
Bruce, R.A., Achenbach, L.A., Coates, J.D., 1999. Reduction of (per)chlorate by a novel organism isolated from a paper mill waste. Environ. Microbiol. 1, 319–331.
Brunner, B., Bernasconi, S.M., 2005. A revised isotope fractionation model for dissimilatory sulfate reduction in sulfate reducing bacteria. Geochim. Cosmochim. Acta 69, 4759–4771.
Canfield, D.E., 2001. Isotope fractionation by natural populations of sulphate-reducing bacteria. Geochim. Cosmochim. Acta 65, 1117–1124.
Canfield, D.E., Olesen, C.A., Cox, R.P., 2006. Temperature control of isotope fractionation by a sulfate-reducing bacterium. Geochim. Cosmochim. Acta 70, 548–561.
Chaudhuri, S.K., O'Connor, S.M., Gustavson, R.L., Achenbach, L.A., Coates, J.D., 2002. Environmental factors that control microbial perchlorate reduction. Appl. Environ. Microbiol. 68, 4425–4430.
Coates, J.D., Michaelidou, U., Bruce, R.A., O'Connor, S.M., Crespi, J.N., Achenbach, L.A., 1999. The ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl. Environ. Microbiol. 65, 5234–5241.
Coates, J.D., Achenbach, L.A., 2004. Microbial perchlorate reduction: rocket-fuelled metabolism. Nat. Rev. Microbiol. 2, 569–580.
Coleman, M.L., Ader, M., Chaudhuri, S., Coates, J.D., 2003. Microbial isotopic fractionation of perchlorate chlorine. Appl. Environ. Microbiol. 69, 4997–5000.

Dasgupta, P.K., Martinelango, K., Jackson, W.A., Anderson, T.A., Tian, K., Tocks, R.W., Rajagopalan, S., 2005. The origin of naturally occurring perchlorate: the role of atmospheric processes. Environ. Sci. Technol. 39, 1569–1575.
Eggenkamp, H.G.M., 1994. δ^{37}Cl, the geochemistry of chlorine isotopes. Ph. D. Thesis, Utrecht University.
Eggenkamp, H.G.M., Kreulen, R., Van Groos, A.F.K., 1995. Chlorine stable isotope fractionation in evaporates. Geochim. Cosmochim. Acta 59, 5169–5175.
Ericksen, G.E., 1983. The Chilean nitrate deposits. Am. Sci. 71, 366–374.
Farquhar, J., Johnston, D.T., Wing, B.A., Habicht, K.S., Canfield, D.E., Aireau, S., Thiemens, M.H., 2003. Multiple sulphur isotopic interpretations of biosynthetic pathways: implications for biological signatures in the sulphur isotope record. Geobiology 1, 27–36.
Godon, A., Jendrzejewski, N., Eggenkamp, H.G.M., Banks, D.A., Ader, M., Coleman, M.L., Pineau, F., 2004. A cross-calibration of chlorine isotopic measurements and suitability of seawater as the international reference material. Chem. Geol. 207, 1–12.
Harrison, A.G., Thode, H.G., 1958. The kinetic effect in the chemical reduction of sulphate. Trans. Faraday Soc. 54, 84–92.
Hatzinger, P.B., 2005. Perchlorate biodegradation for water treatment. Environ. Sci. Technol. A-Pages 39, 239A–247A.
Hoering, T.C., Parker, P.L., 1961. The geochemistry of stable isotopes of chlorine. Geochim. Cosmochim. Acta 23, 186–199.
Johnston, D.T., Farquhar, J., Canfield, D.E., 2007. Sulfur isotope insights into microbial sulfate reduction: when microbes meet models. Geochim. Cosmochim. Acta, 71, 3929–3947.
Kavner, A., Bonet, F., Shahar, A., Simon, J., Young, E., 2005. The isotopic effects of electron transfer: An explanation for Fe isotope fractionation in nature. Geochim. Cosmochim. Acta, 69, 2971–2979.
Kaplan, I.R., Rittenberg, S.C., 1964. Micro-biological fractionation of sulphur isotopes. J. Gen. Microbiol. 34, 195.
Kemp, A.L.W., Thode, H.G., 1968. The mechanism of the bacterial reduction of sulphate and of sulphide from isotope fractionation studies. Geochim. Cosmochim. Acta 32, 71–91.
Kengen, S.W., Rikken, G.B., Hagen, W.R., Van Ginkel, C.G., Stams, A.J.M., 1999. Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain GR-1. J. Bacteriol. 181, 6706–6711.
Koester, C.J., Beller, H.R., Halden, R.U., 2000. Analysis of perchlorate in groundwater by electrospray ionization mass spectrometry/mass spectrometry. Environ. Sci. Technol. 34, 1862–1864.
Martinelango, P.K., Tian, K., Dasgupta, K., 2006. Perchlorate in seawater bioconcentration of iodide and perchlorate by various seaweed species. Anal. Chim. Acta 567, 100–107.
Motzer, W.E., 2001. Perchlorate: problems detection and solutions. Environ. Sci. Technol. 2, 301–311.
O'Connor, S.M., Coates, J.D., 2002. A universal immuno-probe for (per)chlorate-reducing bacteria. Appl. Environ. Microbiol. 68, 3108–3113.
Ohmoto, H., Lasaga, A.C., 1982. Kinetics of reactions between aqueous sulfates and sulfides in hydrothermal systems. Geochim. Cosmochim. Acta 46, 1727–1745.
Orris, G.J., Harvey, G.J., Tsui, D.T., Eldrige, J.E., 2003. Preliminary analysis for perchlorate in selected natural materials and their derivative products. USGS Open-File Report, pp. 03–314.
Rao, B., Anderson, T.A., Orris, G.J., Rainwater, K.A., Rajagopalan, S., Sandvig, R.M., Scanlon, B.R., Stonestrom, D.A., Walvoord, M.A., Jackson, W.A., 2007. Widespread natural perchlorate in unsaturated zones of the Southwest United States. Environ. Sci. Technol. 41, 4522–4528.
Rees, C.E., 1973. A steady-state model for sulphur isotope fractionation in bacterial reduction process. Geochim. Cosmochim. Acta 37, 1141–1162.
Richet, P., Bottinga, Y., Javoy, M., 1977. A review of hydrogen, carbon, nitrogen, oxygen, sulphur, and chlorine stable isotope fractionation among gaseous molecules. Ann. Rev. Earth Planet. Sci. 5, 65–110.
Rikken, G.B., Kroon, A.G.M., van Ginkel, C.G., 1996. Transformation of (per)chlorate into chloride by a new isolated bacterium: reduction and dismutation. Appl. Microbiol. Biotechnol. 45, 420–426.
Schauble, E.A., Rossman, G.R., Taylor Jr., H.P., 2001. Theoretical estimates of equilibrium Fe-isotope fractionations from vibrational spectroscopy. Geochim. Cosmochim. Acta 65, 2487–2497.
Schauble, E.A., Rossman, G.R., Taylor Jr., H.P., 2003. Theoretical estimates of equilibrium chlorine-isotope fractionations. Geochim. Cosmochim. Acta 67, 3267–3281.
Snyder, S.A., Vanderford, B.J., Rexing, D.J., 2005. Trace analysis of bromate, chlorate, iodate, and perchlorate in natural and bottled waters. Environ. Sci. Technol. 39, 4586–4593.
Sturchio, N.C., Hatzinger, P.B., Arkins, M.D., Suh, C., Heraty, L.J., 2003. Chlorine isotope fractionation during microbial reduction of perchlorate. Environ. Sci. Technol. 37, 3859–3863.
Sturchio, N.C., Böhlke, J.K., Beloso, A.B., Streger, S.H., Heraty, L.J., Hatzinger, P.B., 2007. Oxygen and chlorine isotopic fractionation during perchlorate biodegradation: laboratory results and implications for forensics and natural attenuation studies. Environ. Sci. Technol. 41, 2796–2802.
Thode, H.G., Cragg, C.B., Hulston, J.R., Rees, C.E., 1971. Sulphur isotope exchange between sulphur dioxide and hydrogen sulphide. Geochim. Cosmochim. Acta 35, 35–45.
Tikkanen, M.W., 2006. Development of a drinking water regulation for perchlorate in California. Analytica Chimica Acta 567, 20–25.
Tudge, A.P., Thode, H.G., 1950. Thermodynamic properties of isotopic compounds of sulphur. Can. J. Res. 28, 567.
Urbansky, E.T., 1988. Perchlorate chemistry: implications for analysis and remediation. Bioremed. J. 2, 81–95.
Urbansky, E.T., 2000. Quantitation of perchlorate ion: practices and advances applied to the analysis of common matrices. Crit. Rev. Anal. Chem. 30, 311–343.
Urbansky, E.T., 2002. Perchlorate as an environmental contaminant. Environ. Sci. Pollut. Res. 9, 187–192.
Urey, H.C., 1947. The thermodynamic properties of isotopic substances. J. Chem. Soc. 562–581.
van Ginkel, C., Rikken, G., Kroon, A., Kengen, S., 1996. Purification and characterization of chlorite dismutase: a novel oxygen-generating enzyme. Arch. Microbiol. 166, 321–326.