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Use of Silicate Minerals for pH Control during Reductive Dechlorination of Chloroethenes in Batch Cultures of Different Microbial Consortia

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In chloroethene-contaminated sites undergoing *in situ* bioremediation, groundwater acidification is a frequent problem in the source zone, and buffering strategies have to be implemented to maintain the pH in the neutral range. An alternative to conventional soluble buffers is silicate mineral particles as a long-term source of alkalinity. In previous studies, the buffering potentials of these minerals have been evaluated based on abiotic dissolution tests and geochemical modeling. In the present study, the buffering potentials of four silicate minerals (andradite, diopside, fayalite, and forsterite) were tested in batch cultures amended with tetrachloroethene (PCE) and inoculated with different organohalide-respiring consortia. Another objective of this study was to determine the influence of pH on the different steps of PCE dechlorination. The consortia showed significant differences in sensitivities toward acidic pH for the different dechlorination steps. Molecular analysis indicated that *Dehalococcoides* spp. that were present in all consortia were the most pH-sensitive organohalide-respiring guild members compared to *Sulfurospirillum* spp. and *Dehalobacter* spp. In batch cultures with silicate mineral particles as pH-buffering agents, all four minerals tested were able to maintain the pH in the appropriate range for reductive dechlorination of chloroethenes. However, complete dechlorination to ethene was observed only with forsterite, diopside, and fayalite. Dissolution of andradite increased the redox potential and did not allow dechlorination. With forsterite, diopside, and fayalite, dechlorination to ethene was observed but at much lower rates for the last two dechlorination steps than with the positive control. This indicated an inhibition effect of silicate minerals and/or their dissolution products on reductive dechlorination of *cis*-dichloroethene and vinyl chloride. Hence, despite the proven pH-buffering potential of silicate minerals, compatibility with the bacterial community involved in *in situ* bioremediation has to be carefully evaluated prior to their use for pH control at a specific site.

Chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) are among the most common groundwater contaminants in industrialized countries because of their extensive use and their persistence in the environment (1). Among the various decontamination strategies developed in the past decades, *in situ* bioremediation has been recognized as a cost-effective and viable option and has been successfully applied for the remediation of sites contaminated with chlorinated solvents (2, 3). This technique relies on the activity of organohalide-respiring bacteria (OHRB) that reduce chlorinated ethenes stepwise to the innocuous end product ethene, an anaerobic microbial process called organohalide respiration (OHR) (4).

In some situations, *in situ* bioremediation efficiency is reduced by the acidification of groundwater due to substrate fermentation and OHR (3, 5–7). The extent of groundwater acidification is related to the amount of substrate transformed and to the natural buffering capacity of the soil. Due to the higher mass of pollutant present, acidification is more likely to occur in the vicinity of the source zone of chlorinated ethenes (8, 9). The tolerance of OHRB to low pH has been studied for pure cultures (10–18) and some consortia containing OHRB (19, 20). It has been shown that OHRB are inactivated under acidic conditions (pH <5), and therefore, pH buffer amendments are required when the aquifer pH is <5 or when the soil buffering capacity is insufficient (3, 9, 21). In field applications, the most common method used for pH adjustment is injection of a buffering solution such as sodium bicarbonate or sodium carbonate (21). In laboratory studies, the use of KH_2PO_4 buffer encapsulated in a pH-sensitive polymer has successfully been tested for *in situ* pH control (22, 23). However, a field trial has shown that this method had only a short-term effect of a few days (24). Similarly, injection of buffering solution requires constant monitoring and frequent injection, as alkalinity can be rapidly consumed. Moreover, several studies reported that a stable pH was difficult to achieve when this strategy was employed. A study conducted by the Environmental Security Technology Certification Program on pH control with sodium bicarbonate and carbonate solutions showed that in some wells, the pH was >9.0, while locally, the pH dropped below 5.5 (21). In addition, a recent study by Delgado et al. (25) showed that a high bicarbonate concentration can increase hydrogen demand because it stimulates competing H_2-consuming processes such as methanogenesis and homoacetogenesis. More recently, the use of sodium formate as an electron donor has been proposed to minimize acidification (26, 27). Formate dehydrogenation produces sodium bicarbonate, which participates in acidity neutralization

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and does not produce acetic acid (26). However, because formate dehydrogenation is a slow process, pH buffering provided by formate could be insufficient in the case of high dechlorination rates (27).

To overcome the limitation of traditional buffering techniques, the use of ground silicate minerals as a passive pH control system has recently been investigated (28, 29). Silicate minerals are the most common rock-forming minerals (30) and can easily be found worldwide both in natural environments and as by-products of industrial processes. In natural systems, silicate weathering represents the predominant buffering mechanism after carbonate weathering (30). When silicate minerals dissolve, they release cations (K^+, Na^+, Ca^{2+}, Mg^{2+}, Al^{3+}, and Fe^{2+}) and increase pH. Silicate solubility and dissolution rates are pH dependent, both increasing with decreasing pH. Because their dissolution is relatively slow in comparison to that of carbonate minerals, silicate minerals have the potential to act as long-term sources of alkalinity (31). As a result of these characteristics, silicate minerals are potentially good buffering agents to maintain pH in the neutral range. Their dissolution is triggered by acidity production, and the risk of pH overshooting is prevented (31). Wollastonite ($CaSiO_3$) powder, a fast-dissolving calcium silicate, is commonly used in agronomy as a liming agent (32, 33), but the utilization of silicates for remediation of contaminated water bodies and groundwater has rarely been investigated. The addition of wollastonite to an anthropogenically acidified stream was found to be highly effective in increasing the pH and the acid-neutralizing capacity of stream water (34, 35). Other studies have demonstrated the buffering potential of silicate minerals such as nepheline ($Na_3Al_4Si_4O_{16}$) and wollastonite for contaminated water from mining activities (36, 37).

The buffering capacity of ground silicate minerals for pH control during groundwater remediation was previously demonstrated by geochemical modeling (28) and abiotic mineral dissolution experiments (29). According to those studies, four silicate minerals (andradite, diopside, fayalite, and forsterite) have promising groundwater-buffering capacities. In the present study, the use of powders of these silicate minerals as buffering agents in actively dechlorinating batch cultures of consortia containing different OHRB was tested. Special emphasis was put on the effect of pH and the presence of a silicate mineral buffer on each step of PCE dechlorination to ethene.

MATERIALS AND METHODS

Chemicals. All chemicals were analytical grade and used without purification. Tetrachloroethene (PCE) (99%) and *n*-hexadecane (99%) were obtained from Acros Organics and Merck, respectively. All gases (N_2, CO_2, and H_2) were supplied by Messer Schweiz AG, Switzerland.

Organohalide-respiring consortia. The five organohalide-respiring consortia used in this study, SL2-PCEa, SL2-PCEb, AQ-1, AQ-5, and PM, originated from chlorinated ethene-contaminated aquifers. They were enriched and maintained in the laboratory for several years. Details of the enrichment process were described previously by Szynalski (38) for SL2-PCEa, SL2-PCEb, AQ-1, and AQ-5 and by Yu (39) for the PM culture. Consortia AQ-5, SL2-PCEa, and PM have the ability to dechlorinate PCE to ethene completely. SL2-PCEb is a subculture of SL2-PCEa and dechlorinates PCE only to *cis*-dichloroethene (*cis*-DCE). Consortium AQ-1 dechlorinates *cis*-DCE to ethene but cannot grow on PCE or TCE.

Effect of pH on the OHR rate. The influence of a pH of between 5 and 7.5 on the OHR rates was determined for the five consortia described above. Duplicate batch tests were conducted in 500-ml serum bottles containing 200 ml of anaerobic growth medium. For each consortium, six different pH values were tested, from pH 5 to pH 7.5 with stepwise increments of 0.5 pH units. Tests at pH >7.5 were not possible due to the precipitation of calcium phosphate, which made phosphate unavailable for bacterial growth and activity. The anaerobic growth medium used was similar to the one described previously (17), with the following modifications: $NaH_2PO_4 \cdot 2H_2O$ and $NaHCO_3$ were replaced by zwitterionic buffers, 2-(*N*-morpholino)ethanesulfonic acid (MES) at 100 mM for pH 5 to 6.5 and 3-(*N*-morpholino)propanesulfonic acid (MOPS) at 100 mM for pH 7 to 7.5. The initial pH was adjusted by the addition of an anaerobic NaOH or HCl solution. The bottles were sealed with Viton rubber stoppers, and the gas phase of the bottles was replaced with 100% hydrogen (for SL2-PCEa and SL2-PCEb) or 100% nitrogen (for AQ-1, AQ-5, and PM) by using a gas exchange system. To provide the chloroethenes, a two-liquid-phase system (40) was used with PCE or *cis*-DCE dissolved in hexadecane. This system allows a constant delivery of the chlorinated ethenes from the hexadecane to the aqueous phases and maintains the concentration of the chlorinated ethene constant and below the toxicity limit. The nominal chloroethene concentration in the medium (i.e., the amount of chlorinated ethene present in the hexadecane phase divided by the total volume of the aqueous phase) was 5 mM, except for consortium PM, where it was 1.25 mM. For all consortia, the concentration of PCE dissolved in hexadecane was 100 mM, corresponding to a concentration in the aqueous phase of 0.02 mM. Acetate (final concentration, 2 mM) was added as a carbon source for SL2-PCEa and SL2-PCEb from concentrated stock solutions. Consortia AQ-1 and AQ-5 were amended weekly with an electron donor mixture of ethanol, propionate, and butyrate (0.66 mM each per week), and consortium PM was amended with 1.2 mM lactate per week. The cultures were inoculated with 8 ml of a preculture and incubated at 30°C in the dark without agitation. Measurements of pH and the chloroethene concentrations were performed on a regular basis. Finally, experimental observations were fitted to the equation

$$R_{D,i} = r_{max,i} f(\mathrm{pH}) \quad (1)$$

where $R_{D,i}$ is the degradation rate of chloroethene i, $r_{max,i}$ is the maximum degradation rate, and f(pH) is the pH inhibition function (see equation 3).

Molecular detection of OHRB. Terminal restriction fragment length polymorphism (T-RFLP) analyses were conducted to evaluate the microbial community structure of consortia SL2-PCEa, SL2-PCEb, AQ-1, and AQ-5. The T-RFLP analyses were performed on the culture at the end of the pH experiments described above. DNA extraction and T-RFLP analysis were carried out on cells from a 20-ml culture aliquot, as described previously (41). Amplifications of the 16S rRNA gene were performed by using a T3 thermocycler (Biometra; Biolabo, Châtel-St-Denis, Switzerland), eubacterial forward primer Eub-8F fluorescently labeled with 6-carboxyfluorescein (FAM) at the 5′ position, and reverse primer Eub-518r, with the following thermocycling program: 94°C for 4 min 30 s (1 cycle); 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min 45s (30 cycles); and 72°C for 10 min (1 cycle). For each sample, three T-RFLP analyses with three restriction enzymes (HaeIII, HhaI, and MspI) were conducted. The affiliation of terminal restriction fragments (T-RFs) with known OHRB was determined by the use of a semispecific T-RFLP method using semispecific PCR with nonspecific primer Eub-8F and a specific reverse primer for the genus of interest. Reverse primers were described previously by Smits et al. (42) for *Dehalobacter restrictus*, Adrian et al. (43) for *Dehalococcoides* spp., Lanthier et al. (44) for *Desulfitobacterium* spp., and Daprato et al. (45) for *Sulfurospirillum* spp. Pure cultures and highly enriched consortia of known compositions were used as positive controls. The semispecific T-RFLP analyses were conducted in parallel with three restriction enzymes, HaeIII, HhaI, MspI (Promega), in order to obtain three T-RFs corresponding to one genus (Table 1).

Mineral preparation and characterization. Bulk mineral samples of the following four silicate minerals were purchased from F. Krantz Rheinisches Mineralien-Kontor GmbH and Co. KG (Bonn, Germany): andradite ($Ca_3Fe_2Si_3O_{12}$; Erzgebirge, Sachsen, Germany), diopside [$CaMg(SiO_3)_2$; Outukumpu, Finland], fayalite (Fe_2SiO_4; Billiton, Indonesia), and forst-

TABLE 1 Lengths of terminal restriction fragments corresponding to known genera of organohalide-respiring bacteria determined by semispecific T-RFLP analysis with the three restriction enzymes HaeIII, HhaI, and MspI

Genus	Terminal restriction fragment length (bp)		
	HaeIII	HhaI	MspI
Sulfurospirillum	252	90	463
Dehalococcoides[a]	165	194	488[c]
Dehalococcoides[b]	244	194	488[c]
Dehalobacter	212	229	137

[a] Fragment size corresponding to the *Dehalococcoides* strain present in consortia SL2-PCEa and AQ-1.
[b] Fragment size corresponding to the *Dehalococcoides* strain present in consortium AQ-5.
[c] There is no restriction site for the enzyme MspI in the fragment amplified. The fragment length corresponds to the undigested fragment.

erite (Mg_2SiO_4; Aheim, Northfjord, Norway). These minerals were chosen as they were identified as being suitable buffering agents in a previous modeling study (28) and in screening experiments. The minerals were crushed with a hydraulic press and ground with an agate disc mill. The mineral powder was rinsed, sonicated (2 times for 5 min in ethanol and then 5 min in milliQ pore water), and washed for 24 h in milliQ water, as described previously by Barker et al. (46), to remove fine particles. The mineral samples were further dried overnight at 60°C and were sterilized by heating to 150°C for 3 h. Chemical compositions were determined by X-ray fluorescence (XRF) analysis with a Philips PW2400 XRF spectrometer and by laser ablation-inductively coupled plasma mass spectrometry (ICP-MS) with an Elan 6100 DRC quadrupole spectrometer. The specific surface area of the cleaned mineral powder was determined by the multipoint nitrogen adsorption Brunauer-Emmett-Teller (BET) method with a Quantachrome Nova surface area analyzer (Quantachrome GmbH & Co. KG, Germany).

Evaluation of the buffering capacities of silicate minerals in biotic experiments. Biotic experiments to investigate the acid-neutralizing capacities of silicate minerals during OHR of chlorinated compounds were performed with the two consortia SL2-PCEa and SL2-PCEb. Duplicate batch tests were conducted in 120-ml serum bottles containing 50 ml of sterile anaerobic growth medium, modified from a medium described previously by Holliger et al. (17) to reduce the soluble buffering capacity so that the main source of pH buffering was the mineral powder. The following modifications were made: K_2HPO_4 was added at only 0.49 mM, NH_4HCO_3 was added at 0.98 mM, and NaH_2PO_4 and $NaHCO_3$ were completely omitted. The medium was reduced by the addition of Na_2S (1 mM). For the consortium containing *Dehalococcoides* (i.e., SL2-PCEa), additional experiments showed that the ionic strength of the low-buffered medium was too low and resulted in an inhibition of *cis*-DCE degradation by *Dehalococcoides* (see Fig. S1 in the supplemental material). Therefore, for this consortium, the ionic strength of the anaerobic medium was increased by the addition of 32 mM NaCl.

Electron donors, substrates, and PCE were amended as described above, and detailed experimental conditions for the two consortia are listed in Table 2. The nominal PCE concentrations in the medium were equal to 2.5 mM and 1.25 mM for SL2-PCEb and SL2-PCEa, respectively. Acetate (2 mM) was added as a carbon source. The mineral powder was added to the batch cultures under aseptic conditions before gas exchange and the addition of Na_2S. The amount of sterile mineral powder added to each batch culture was based on geochemical simulations described below. The amount of mineral, listed in Table 2, was chosen so as to maintain the pH in the tolerance range for each consortium. Two types of controls without mineral powder were performed, a "positive" control with a standard medium buffered by bicarbonate, as described previously (17), and a "negative" control with the low-buffered medium described above. The batch cultures were incubated in the dark at 30°C on an overhead shaker at 20 rpm. Measurements of pH and chloroethenes were performed on a regular basis. Analytical measurements were performed until complete transformation of chloroethenes to ethene (for SL2-PCEa) or *cis*-DCE (for SL2-PCEb) was achieved or until dechlorination ceased due to inhibition. For consortium SL2-PCEa, measurements of dissolved trace element concentrations (Cd, Cr, Cu, Pb, Zn, Ni, Mn, Co, and As) resulting from mineral dissolution were performed at the end of the experiment.

Analytical methods. PCE, TCE, *cis*-DCE, vinyl chloride (VC), and ethene were analyzed by gas chromatography with a GC Varian Star 3400CX instrument equipped with a GS-GasPro column (30 m by 0.32 mm; J&W Scientific, MSP Friedly & Co., Koeniz, Switzerland) coupled to a flame ionization detector. One hundred microliters of gas samples was collected from the headspace with a Hamilton (Reno, NV) gastight syringe and analyzed on the gas chromatograph with a 1.3-ml min^{-1} flow of nitrogen carrier gas. The initial temperature was 45°C; the column was kept at 45°C for 3 min, and the temperature was then raised to 75°C at a rate of 15°C min^{-1} and then to 200°C at a rate of 25°C min^{-1} and finally kept at 200°C for 5 min. Trace element concentrations were measured by ICP-MS with an Elan DRC II instrument (Perkin-Elmer, USA). The pH was measured with an InLab microelectrode and a SevenEasy pH meter (Mettler Toledo, Switzerland).

Modeling approach. A geochemical model was used to determine the amount of mineral needed to maintain neutral pH during the biodegradation of a given amount of chloroethenes. Numerical simulations were

TABLE 2 Experimental conditions used for batch cultures with consortia containing OHRB and different silicate minerals as pH-buffering agents

Consortium	Electron donor/substrate[a]	Chlorinated ethene	Nominal concn of chlorinated ethene (mM)[b]	Silicate mineral powder		
				Mineral	Mass (g)	Specific surface area (m^2/g)[c]
SL2-PCEb	Hydrogen	PCE	2.5	Andradite	4.0	0.40
				Diopside	4.0	1.55
				Fayalite	4.0	1.14
				Forsterite	0.7	4.41
SL2-PCEa	Hydrogen	PCE	1.25	Andradite	4.3	0.47
				Diopside	3.3	0.96
				Fayalite	3.9	1.11
				Forsterite	1.2	1.54

[a] The headspace of cultures with hydrogen as the electron donor was 100% H_2.
[b] The nominal concentration is a theoretical value and indicates the concentration that would be reached if all chlorinated ethene added as hexadecane stock solution is present in the aqueous phase.
[c] The specific surfaces for the same mineral are different because the mineral powder added did not originate from the same batch of prepared mineral powder.

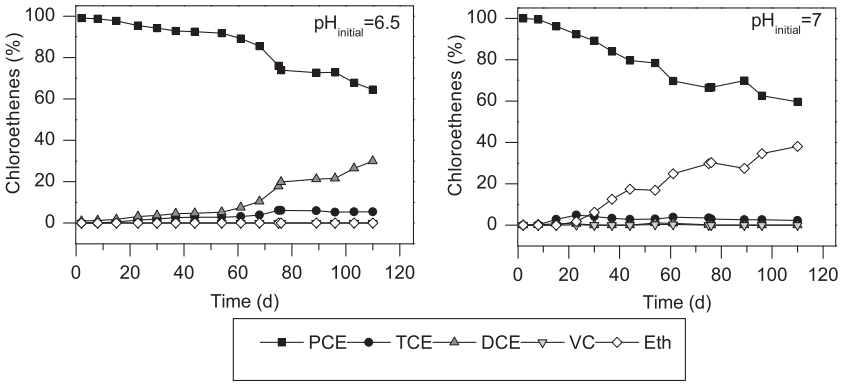

FIG 1 Dechlorination of PCE by cultures of consortium AQ-5 at initial pHs of 6.5 and 7.0. The data points represent the average value of duplicates. The maximum variation between the average value and each replicate is equal to 10%.

performed with the geochemical software PHREEQC-2 (47) and with the MINTEQA2 database (48), using a modified version of the geochemical model described previously by Lacroix et al. (28). The amount of mineral needed was chosen such that the cations released by mineral dissolution counterbalanced acidity production by bacterial activity. The primary goal of the model was to determine the amount of mineral needed to counterbalance a given rate of acidity production. According to the approach of Robinson et al. (9), the acidity rate for each step of the dechlorination pathway was fixed and modeled as a first-order kinetic rate. The kinetic constant was defined according to preliminary experiments where the consortia were grown in the same medium with the same amount of chloroethenes. The acidity production rate was expressed as

$$R_i = k_i C_i f(\mathrm{pH}) \quad (2)$$

where R_i is the degradation rate of chloroethene i, C_i (mol liter^{-1}) is the aqueous concentration of chloroethene I, and k_i (s^{-1}) is a first-order kinetic rate constant. Note that this rate expression is valid only under the given conditions of this experiment (see values in Table S1 in the supplemental material). The pH inhibition function, f(pH), was expressed as

$$f(\mathrm{pH}) = \exp\left[-\frac{\left|\mathrm{pH}_{opt} - \mathrm{pH}\right|^n}{\sigma^2}\right] \quad (3)$$

where pH_{opt} is the optimal pH and n and σ are empirical parameters that were estimated by fitting equation 1 to experimental observations. Partitioning of chloroethenes between the hexadecane, gas, and water phases was expressed as

$$M = V_w C_w + V_g C_g + V_h C_h = C_w\left(V_w + V_g H_{cc} + V_h K_{h\text{-}w}\right) \quad (4)$$

where M (mol) is the total mass of chloroethenes in the system; C_w, C_g, and C_h (mol liter^{-1}) are the concentrations of the chloroethenes in the aqueous, gas, and hexadecane phases, respectively; V_w, V_g, and V_h are the volumes of the three phases; H_{cc} is Henry's constant for partitioning between the aqueous and gas phases; and $K_{h\text{-}w}$ is the water-hexadecane partition coefficient. The parameters used in this equation are listed in Table S2 in the supplemental material.

The mineral dissolution rate (31, 49) was expressed as

$$R_{Diss} = \left[k_{H^+}\left(10^{-\mathrm{PH}}\right)^{n_{H^+}} \exp\left(-T_{inf}E_{H^+}\right) + k_w \exp\left(-T_{inf}E_w\right)\right]\frac{A_0}{V}\left(\frac{m}{m_0}\right)^{\frac{2}{3}}\left(1-\Omega\right) \quad (5)$$

with $T_{inf} = \frac{1}{R}\left(\frac{1}{T} - \frac{1}{298}\right)$, where R_{Diss} (mol m^{-2} s^{-1}) is the mineral dissolution rate; k_{H^+} and k_W (mol m^{-2} s^{-1}) are the rate constants for the acidic and neutral ranges, respectively; n_{H^+} is the reaction order of proton-promoted dissolution; E_{H^+} and E_W (J mol^{-1}) are the activation energies for the neutral and basic ranges, respectively; R (J K^{-1} mol^{-1}) is the universal gas constant; T (K) is the absolute temperature; A_0 (m^2) is the initial surface area; V (liters) is the solution volume; Ω is the mineral saturation index; and m and m_0 are the actual and initial masses of mineral, respectively.

The following hypotheses were made: (i) the reactive surface area is equal to the measured BET surface area, and (ii) no passivation of mineral surfaces occurred. The values of all the parameters related to mineral dissolution are listed in Table S3 in the supplemental material.

RESULTS

Influence of pH on OHR rates. Five organohalide-respiring consortia were used to test the influence of pH on OHR rates. The incubation periods of these tests were 23 days for consortium SL2-PCEb, 91 days for consortium PM, and 110 days for the other three consortia. A small pH drift (between 0.3 and 1 pH unit) was always observed due to the insufficient buffering capacity of the zwitterionic buffer. To overcome the impact of pH drift on data analysis, OHR rates were calculated between two time steps during which pH variations were negligible. The dechlorination patterns under standard conditions (i.e., at pH 7) were different for the five consortia tested: SL2-PCEb transformed PCE to *cis*-DCE without a transient accumulation of TCE, SL2-PCEa dechlorinated PCE to ethene with a transient accumulation of *cis*-DCE and VC, PM dechlorinated PCE to ethene with a transient accumulation of VC, and AQ-1 transformed *cis*-DCE to ethene with transient VC accumulation. The dechlorination pattern of AQ-5 was particularly interesting because PCE was transformed to ethene without the accumulation of intermediate products (Fig. 1). The pH sensitivities of OHR rates exhibited significant differences between consortia and between each step of the OHR pathway (Fig. 2). The parameters of the pH inhibition function (equation 3) for each consortium are listed in Table 3. The degradation of the lesser chlorinated compounds was more sensitive to acidic pH. During the experiment conducted with SL2-PCEa, *cis*-DCE was formed down to pH 4.8, VC was formed down to pH 5.3, and ethene was formed down to pH 5.9. The tolerance to acidic pH conditions was also variable among the five consortia. SL2-PCEb and SL2-PCEa were the most tolerant, while AQ-5 was extremely sensitive to acidic conditions. For this consortium, the lowest pH at which dechlorination was observed was 6.15, and a small change in the initial pH had a strong impact on the dechlorination pattern. At pH 7, AQ-5 transformed PCE directly to ethene without accumulation of intermediate products, while at pH 6.5, accumulation of

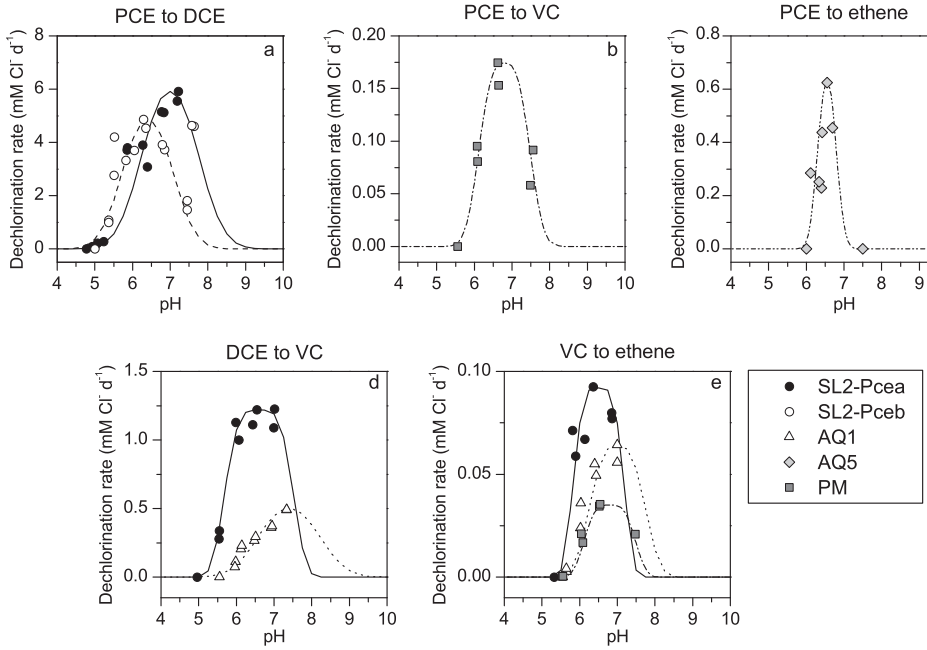

FIG 2 Effect of pH on the organohalide respiration rate for each step of the PCE dechlorination pathway and for the five consortia tested. (a) SL2-PCEa and SL2-PCEb; (b) PM; (c) AQ-5; (d) SL2-PCEa and AQ-1; (e) SL2-PCEa, AQ-1, and PM. The dechlorination rates of all cultures are plotted. The lines represent the fit of the experimental data to equation 3. The organohalide respiration rates (in mM chloride produced per day) were calculated based on the results of the dechlorination products measured by gas chromatography.

cis-DCE was observed, and there was no formation of ethene (Fig. 1).

Predominant OHRB at different pHs. All consortia except SL2-PCEb contained *Dehalococcoides*. The results of T-RFLP analysis indicated that the *Dehalococcoides* strains present in the different consortia were not the same. When T-RFLP analysis was performed with the restriction enzyme HaeIII, the *Dehalococcoides* population present in consortia SL2-PCEa and AQ-1 produced a T-RF with a length of 165 bp, while *Dehalococcoides* present in AQ-5 produced a T-RF of 244 bp. Consortia SL2-PCEa and SL2-PCEb both contained a population identified as *Sulfurospirillum* spp. (50), and consortium AQ-5 contained a population that was affiliated with *Dehalobacter* spp. (38).

T-RFLP analysis performed at the end of the pH sensitivity experiments showed that consortia SL2-PCEa and AQ-5, containing more than one OHRB, had different predominant OHRB at the end of the experiment, which correlated with the dechlorination end product observed (Table 4). At pH 5 and 5.5 with *cis*-DCE as the dechlorination product, SL2-PCEa was dominated by *Sulfurospirillum* spp. (78% ± 7% at pH 5), while at pH 6 to 7.5 with ethene as the dechlorination end product, *Dehalococcoides* spp. were the most abundant OHRB (77.5% ±

TABLE 3 Fitted parameters of the pH inhibition function[a] and goodness of fit for the five consortia SL2-PCEa, SL2-PCEb, AQ-1, AQ-5, and PM[b]

Consortium	Dechlorination step	Mean value ± SD			
		pH_{opt}	σ	*n*	r^2
SL2-PCEa	PCE to *cis*-DCE	6.99 ± 0.05	1.10 ± 0.06	2.09 ± 0.32	0.99
	cis-DCE to VC	6.60 ± 0.02	0.97 ± 0.05	5.28 ± 0.87	0.99
	VC to ethene	6.50 ± 0.05	0.67 ± 0.14	2.75 ± 0.42	0.92
SL2-PCEb	PCE to *cis*-DCE	6.44 ± 0.07	0.92 ± 0.09	1.94 ± 0.60	0.94
AQ-1	*cis*-DCE to VC	7.43 ± 0.12	1.22 ± 0.15	1.86 ± 0.59	0.93
	VC to ethene	6.99 ± 0.10	1.01 ± 0.16	3.42 ± 1.60	0.95
AQ-5	PCE to ethene	6.56 ± 0.04	0.21 ± 0.02	2.15 ± 0.08	0.99
PM	PCE to VC	6.78 ± 0.11	0.76 ± 0.15	2.60 ± 1.24	0.88
	VC to ethene	6.78 ± 0.02	0.70 ± 0.04	4.17 ± 0.33	0.99

[a] See equation 3.
[b] The model was fitted to the data by using a nonlinear least-squares method and the trust region algorithm implemented in MATLAB. For each parameter, the 95% confidence interval obtained with MATLAB is given.

TABLE 4 Community composition at the end of pH experiments as determined by T-RFLP analyses in consortia SL2-PCEa and AQ-5[a]

	Mean composition (%) ± SD					
	SL2-PCEa			AQ-5		
pH	*Dehalococcoides*	*Sulfurospirillum*	Others	*Dehalococcoides*	*Dehalobacter*	Others
5	2.8 ± 0.6	78.3 ± 6.7	18.9 ± 6.2			
5.5	1.9 ± 0.4	68.1 ± 3.0	30 ± 3.3			
6	81.8 ± 1.8	14.7 ± 1.9	3.5 ± 1.0			
6.5	76.9 ± 4.5	21 ± 2.8	2.1 ± 2.1	2.9 ± 1.3	74.1 ± 1.1	23.0 ± 1.9
7	79.4 ± 2.2	18.1 ± 1.1	2.5 ± 1.2	75.6 ± 4.6	23.8 ± 4.2	0.9 ± 1.3
7.5	77.5 ± 1.1	21.6 ± 1.7	0.9 ± 0.8			

[a] Each T-RFLP analysis was performed in triplicate with three different restriction enzymes. In consortia SL2-PCEb and AQ-1, no significant changes of the community composition were observed, since only one OHRB was present.

1% at pH 7.5). In consortium AQ-5, *Dehalobacter* spp. were predominant at pH 6.5 (74% ± 1%), while *Dehalococcoides* spp. were predominant at pH 7 (76% ± 4.5%) at the end of the experiment.

Acid-neutralizing capacity of silicate minerals during growth of OHRB. Figure 3 shows the evolution of pH in the batch cultures containing minerals and OHRB. In the positive controls, the pH remained rather constant, in the range of 7.2 to 7.5. On the contrary, in the negative control, OHR activities resulted in a pH decrease to the inhibition value for OHRB.

In batch cultures containing silicate mineral powders, the pH remained in a range that was above the acidic limit, below which the OHR activity of the consortia was fully inhibited. In cultures of SL2-PCEb, the pH was maintained at between 5.5 and 7.5 by andradite, fayalite, forsterite, and diopside, while in the cultures of SL2-PCEa, the same minerals maintained the pH in the range of 6.6 to 8.3.

OHR activity with silicate minerals as pH-buffering agents. Figure 4 presents the dechlorination pattern over time for the positive and negative controls and for the batches containing a silicate mineral with SL2-PCEa as the inoculum. An increase of the lag phase was observed from 2 days for the positive control to 5 days in the negative control, 9 days with fayalite and forsterite, and 16 days with diopside. The same observation of an increased lag phase in the presence of minerals was also made for cultures of consortium SL2-PCEb. In the negative controls, a stop of the dechlorination process was observed due to acidification of the medium. For instance, dechlorination stopped after 4 days in the negative control of SL2-PCEb with 41% TCE and 60% *cis*-DCE as the dechlorination end products. Table 5 summarizes the compositions of dechlorination end products at the end of the experiments.

Dechlorination of PCE to *cis*-DCE was complete in the presence of fayalite, diopside, and forsterite with the two consortia SL2-PCEa and SL2-PCEb. However, in the presence of andradite, the transformation of PCE to *cis*-DCE was either partially (for SL2-PCEb) or fully (for SL2-PCEa) inhibited (Fig. 4). Dechlorination of *cis*-DCE to VC and ethene was evaluated with SL2-PCEa. These two dechlorination steps occurred in the presence of forsterite, fayalite, and diopside but not with andradite (Fig. 4). However, transformation of *cis*-DCE and VC occurred at much lower rates than with the positive control, although the pH remained neutral. Transformation of VC to ethene took 16 days for the positive control, while it took about 148 days for the batch with forsterite. With diopside and fayalite, VC dechlorination was completed to 81% and 34%, respectively, after 185 days of incubation.

Influence of silicate mineral dissolution on redox potential. An indication of a changing redox state of the medium was given by resazurin, which is a redox indicator that is colorless at a redox potential of ≤100 mV and pink under more-oxidizing conditions (51). In batch cultures amended with andradite, the medium became pink

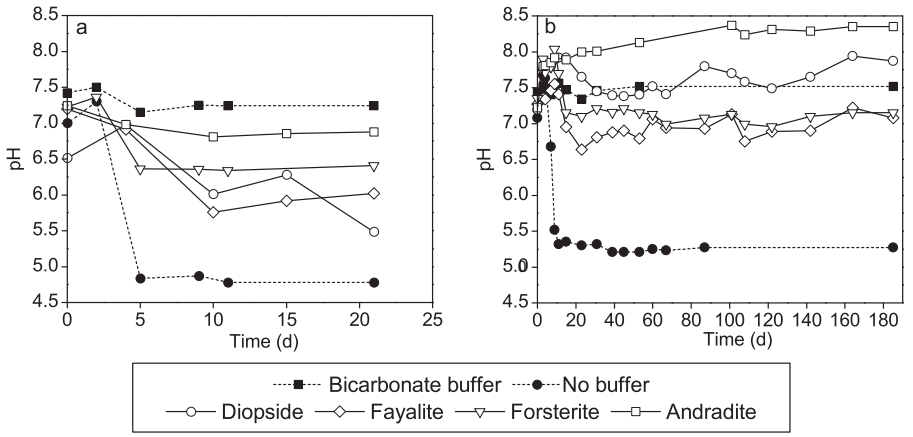

FIG 3 Evolution of pH in cultures of consortia SL2-PCEb (a) and SL2-PCEa (b) with different ground silicate minerals as buffering agents. The values plotted are the average values of duplicates. The maximum variation between the average value and one replicate is equal to 0.25 pH units.

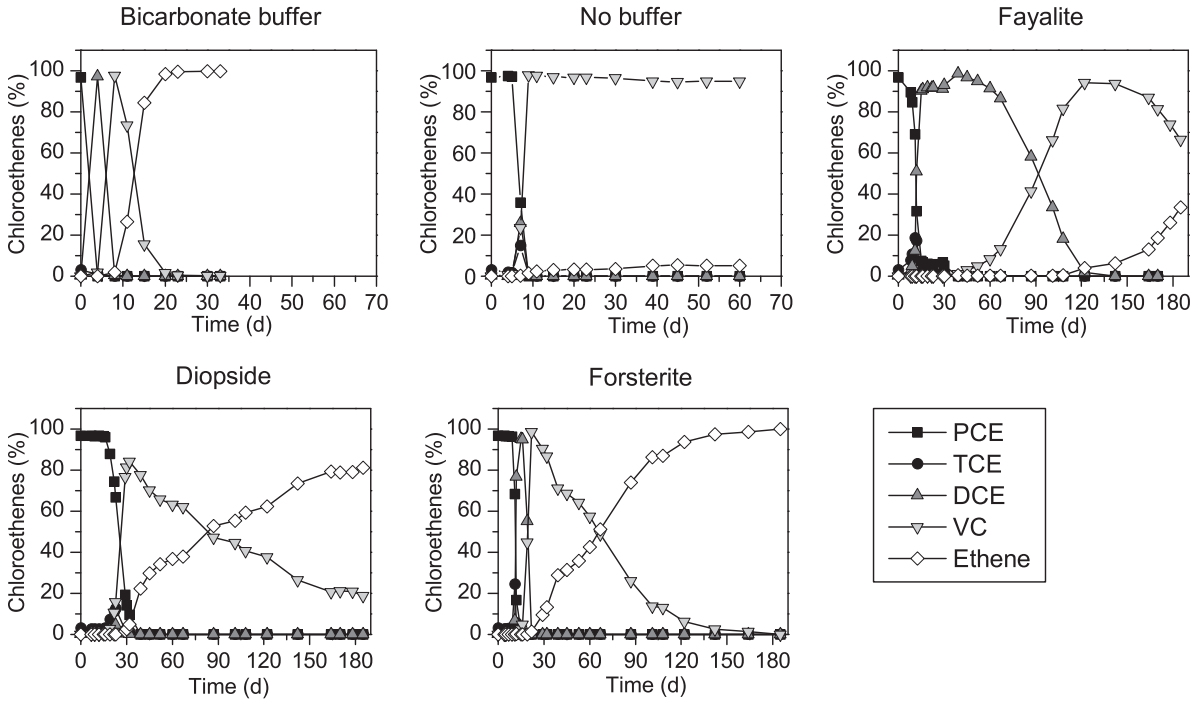

FIG 4 Dechlorination pattern in batch cultures of consortium SL2-PCEa with the three minerals diopside, fayalite, and forsterite as pH-buffering agents. In the cultures with andradite, no PCE dechlorination was observed, and therefore, the results are not presented. Data for just one culture are reported since duplicates behaved similarly yet not identically, with some differences in the lengths of the lag phase.

after 1 day for consortium SL2-PCEa and after 5 days for consortium SL2-PCEb. No change in medium coloration was observed for the other minerals. It has been reported that at a redox potential of >100 mV, growth of OHRB is not possible (52).

Release of heavy metals by silicate minerals. The silicate minerals used in this study contain traces of heavy metals that could possibly have an inhibitory effect on dechlorination by OHRB. The concentrations of dissolved heavy metals in solution at the end of the experiments and known to be toxic for bacteria are presented in Table S4 in the supplemental material. Of the nine heavy metals tested (Zn, Cu, Mn, Cd, Co, Cr, Ni, Pb, and As), only two (Mn and As) were present in higher concentrations in batches with silicate minerals than in the controls. For instance, manganese was present at a concentration of up to 1 mg liter^{-1} in the batch with fayalite, and arsenic was present at a concentration of 75 μg liter^{-1} in the batch with andradite.

DISCUSSION

Influence of pH on OHR activity. This study showed noticeable differences in pH sensitivities between the different steps of the PCE dechlorination pathway. The last steps from *cis*-DCE to ethene were more sensitive to acidic pH than was the transformation of PCE to *cis*-DCE. This pattern can be explained by the different sensitivities of the different OHRB involved in PCE dechlorination. Consortium AQ-5 is composed of mainly a *Dehalobacter* population that dechlorinated PCE to *cis*-DCE and a *Dehalococcoides* population able to form ethene. At pH 7.0, where ethene was formed, *Dehalococcoides* species was the predominant

TABLE 5 Proportion of dechlorination products at the end of the incubation period of cultures inoculated with consortia SL2-PCEb, SL2-PCEa, and AQ-1 and amended with different ground silicate minerals[a]

Consortium	End product	Proportion (%) of dechlorination product after amendment with:					
		Positive control	Negative control	Andradite	Diopside	Fayalite	Forsterite
SL2-PCEb	PCE	0	0	2	0	0	0
	TCE	0	93	49	3	1	0
	cis-DCE	100	7	49	97	99	100
SL2-PCEa	PCE	0	0	98	0	0	0
	TCE	0	0	2	0	0	0
	cis-DCE	0	0	0	0	0	0
	VC	0	95	0	19	66	0
	Ethene	100	5	0	81	34	100

[a] Proportions of parent compounds and dechlorination products were determined at the end of the experiment, on day 40 for SL2-PCEb. For SL2-PCEa, data were determined after 33 days of incubation for the positive control, after 87 days for the negative control, and after 185 days for the cultures containing silicate mineral powders.

population at the end of the experiment, while at pH 6.5, *Dehalobacter* species was predominant and *cis*-DCE was the dechlorination end product, indicating that the former population was extremely pH sensitive, unlike the latter. For consortium SL2-PCEa, composed of *Sulfurospirillum* spp. and *Dehalococcoides* spp., the latter were also almost not detected in cultures with acidic pH, where PCE dechlorination stalled at *cis*-DCE. These results indicate that bacteria belonging to the genus *Dehalococcoides* are quite sensitive to acidic pH. In addition, the transformation from VC to ethene seemed to be more sensitive to pH than was the dechlorination from *cis*-DCE to VC, both performed by *Dehalococcoides*. These results are in agreement with practitioner knowledge and with a previous study by Rowlands (53) which showed that the range of complete degradation from PCE to ethene was observed at between pH 6 and 8.3, whereas partial degradation of PCE to *cis*-DCE and VC occurred in a broader pH range of 5 to 9 in bacterial consortia. In addition, Löffler et al. (54) showed previously that pure cultures of *Dehalococcoides* are active only between pH 6.5 and 8.0. The results of this study can have important implications for the application of OHR in groundwater bioremediation and showed that if the pH is not controlled properly and is between 5 and 6, it could lead to the accumulation of toxic intermediates such as *cis*-DCE and VC.

The five consortia tested in this study presented different tolerances to acidic pH. SL2-PCEa was the most tolerant consortium, with transformation of VC to ethene down to pH 5.9, while AQ-5 presented a very narrow tolerance range (production of ethene down to pH 6.4). These two consortia both contained members of the genus *Dehalococcoides* but probably of different strains, as indicated by the results of T-RFLP analysis. Identification of *Dehalococcoides* populations that are tolerant to mildly acidic pH, such as the one present in SL2-PCEa, might be of interest for bioaugmentation applications. In addition, it is of importance for application to know the pH sensitivity of the OHRB populations present at a specific site in order to design the appropriate bioremediation approach and to ensure that the success of the remediation approach is not hampered due to pH inhibition. The mere detection of the presence of *Dehalococcoides* spp. does not provide sufficient information, and laboratory tests could help in obtaining the necessary information. For instance, the influence of pH on each step of the dechlorination pathway with OHRB from the site could be tested by following the approach presented in this study.

Suitability of silicate minerals as pH-buffering agents during OHR of chloroethenes. The results of cultures amended with ground silicate minerals confirmed the potential of the latter as acid-neutralizing agents. Previous studies using numerical simulations (28) and abiotic dissolution experiments (29) already indicated that these minerals could be used as pH buffers. The four minerals tested (andradite, fayalite, forsterite, and diopside) were predicted to maintain the pH in a range suitable for dechlorination and could theoretically sustain the transformation of PCE to ethene. The results obtained here showed that three of the four minerals indeed enabled PCE dechlorination to ethene, whereas with the negative controls, dechlorination stalled at VC. However, these results also showed that other mechanisms associated with silicate dissolution can negatively influence chloroethene dechlorination rates.

Among the four minerals tested, andradite was the only one which inhibited the transformation of PCE. Additional experiments demonstrated that the extent of PCE dechlorination inhibition was proportional to the amount of andradite dissolved (results not shown). This observation could be due to the presence of an oxidizing component in the mineral. Indeed, andradite is the only mineral tested that contained a high concentration of Fe(III) (55). Ferric iron is a recognized oxidizing agent, and the addition of Fe(III) is known to increase the redox potential of anaerobic solutions (56, 57). The increase of redox potential was confirmed by the color change of the redox indicator resazurin. OHRB are strict anaerobic bacteria (58) and cannot dechlorinate PCE when the redox potential is >100 mV, which was the case with andradite. Our results suggest that the presence of redox-active compounds inside the minerals, such as Fe(III), has to be considered carefully prior to the selection of a buffering agent. For iron-containing minerals, the oxidation state of iron should be evaluated, and Fe(III)-containing minerals should not be used if the remediation strategy requires a low redox potential to proceed.

The experiments with consortium SL2-PCEa indicated that the transformation of *cis*-DCE to ethene seems to be more sensitive to mineral dissolution than is the transformation of PCE to *cis*-DCE. Since *Dehalococcoides* spp. seemed to be responsible for *cis*-DCE and VC dechlorination, the results indicated that OHRB of this genus are quite sensitive to the effects that silicate mineral dissolution might have on biological activity. Some trace metals released during mineral dissolution, such as manganese or arsenic, might have been responsible for the lower *cis*-DCE dechlorination rates observed. To the best of our knowledge, there are no studies to date investigating the toxicity of these metals to *cis*-DCE-dechlorinating bacteria. Metal toxicity studies have been conducted with other bacteria involved in the biodegradation of halogenated compounds, but they are limited to a restricted number of organic compounds (trichloroaniline, 2-chlorophenol, 3-chlorobenzoate, hexachlorobenzene, and pentachlorophenol) (59). Those studies reported that the lowest concentration at which inhibition by manganese was observed was equal to 28.2 mg liter^{-1} (i.e., 0.51 mM), which is much higher than the concentration measured in this study (i.e., around 1 mg liter^{-1}, or 0.018 mM). Additional studies should be conducted to investigate the exact reason for the inhibition observed in the presence of silicate mineral.

The lower rates of *cis*-DCE and VC dechlorination were perhaps a consequence of the experimental approach chosen. Indeed, in a batch system, nutrient depletion and the accumulation of toxic or redox-active compounds increase with time. In contrast, under field conditions, these effects are less likely to occur due to the renewal of the pore water through groundwater flow. Further studies should be conducted in continuous-flow systems to evaluate the feasibility of overcoming this issue and to assess the long-term buffering efficiency of the minerals.

An important issue for field applications of the pH control approach developed in this study is the delivery of the mineral to the subsurface. This was beyond the scope of this study but has been addressed by Piegat and Newman (60), who studied experimentally the transport of an insoluble solid buffer made of calcium carbonate in column experiments. Those researchers showed that a colloidal suspension of $CaCO_3$ particles stabilized with selected additives to produce a negative charge was able to travel sufficiently in the subsurface with significant alkalinity retained and with a decrease of permeability of <10%.

In conclusion, this study showed that each step of the PCE dechlorination pathway presents a different sensitivity to acidic

pH, with an increasing sensitivity for the degradation of lesser chlorinated ethenes. In addition, it is very likely that different populations of *Dehalococcoides* can have different sensitivities to acidic pH, which has important implications for *in situ* bioremediation and demonstrates the need to carefully evaluate the pH sensitivity of the native OHRB present at a site. This study also evidenced the need to develop appropriate pH control strategies to avoid the accumulation of toxic intermediates. The results obtained with silicate mineral powders as pH-buffering agents showed that these minerals are able to neutralize the acidity produced by OHRB without leading to pH overshooting and allowed ethene formation from PCE. However, interactions between minerals and OHRB activity need to be carefully evaluated, as silicate mineral decreased the transformation rates of *cis*-DCE to ethene.

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