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Nondestructive Methods for Removal of Bacteria from Silicate Surfaces

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In order to study biosignatures documenting the effects of microbiota on mineral surfaces, microbial matter must be removed without chemically or physically changing the surface. In previous work for analysis by scanning electron microscopy (SEM) we removed soil bacteria from silicate surfaces with lysozyme; however, when lysozyme-treated surfaces were analyzed at nanoscale by atomic force microscopy (AFM), residual matter was observed. Therefore, we tested removal of bacteria by each of five methods, including four different detergents and CO_2 snow cleaning. AFM observations revealed residue on glass surfaces that underwent CO_2 snow cleaning after 13 days of growth of Bacillus sp. *(a soil microbe). In contrast, treatment with each of four detergents followed by acetone completely removed* Bacillus sp. *without altering the topography of the surfaces even after 77 days of growth. However, X-ray photoelectron spectroscopic (XPS) analyses of the upper 25 Å of the glass surfaces revealed chemical alterations by some detergents, but not by the detergent sodium dodecyl sulfate (SDS). Therefore, for strictly topographic analyses such as AFM, any of the four detergents tested can be considered a viable option for bacterial removal, but for analyses of surface chemistry, the pH-neutral, anionic detergent SDS is recommended. Additionally, AFM imaging after detergent treatments revealed etch pits on glass surfaces exposed to bacteria that were similar in size and distribution to small colonies of* Bacillus sp. *These surfaces were also depleted in Fe and Al relative to Si as measured by XPS, yet similar controls incubated in media without bacteria showed no depletion in Fe and Al.*

Keywords AFM, bacteria removal, detergents, etch pits, XPS

Introduction

Microbial activity is a major factor in mineral weathering due to the ubiquity of microorganisms in soils and sediments. Even aquifer sediments hundreds of meters deep contain abundant microbial life, with approximately 10^5 to 10^7 cells/cm^3 (e.g., Barker et al. 1997).

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Furthermore, in nutrient-depleted environments, microorganisms preferentially colonize, and thus may weather, minerals containing limiting nutrients (Rogers et al. 1998).

Understanding the effects of microbiota on mineral alteration requires the ability to recognize evidence of bacterial-promoted dissolution on mineral surfaces. A number of studies have examined such biosignatures on mineral surfaces (e.g., Grantham and Dove 1996; Maurice et al. 1996; Grantham et al. 1997; Maurice and Forsythe 1997; Kalinowski et al. 2000; Liermann et al. 2000b). Most microbe-mineral interaction studies have examined surfaces with microbes still attached, most commonly by using scanning electron microscopy (SEM) to observe cells and to look for pitting in the surfaces adjacent to attachment sites (e.g., Bennett et al. 1996; Barker et al. 1998; Rogers et al. 1998). Researchers have also begun to utilize atomic force microscopy (AFM) to document micro- to atomic-scale physical effects of surface reactions by imaging the topography of samples (e.g., Grantham and Dove 1996; Maurice et al. 1996). The atomic force microscope images a sample by probing a surface with a sharp tip attached to a cantilever and measuring the deflection of the cantilever in response to changes in the topography of the surface. Less commonly, microelectrodes have been used to measure pH in biofilms surrounding cells attached to surfaces (e.g., Barker et al. 1998; Liermann et al. 2000b) and atomic-scale surface analyses such as X-ray photoelectron spectroscopy (XPS) have been employed to document chemical changes at the mineral surface (Balaz et al. 1996; Blight et al. 2000; Kalinowski et al. 2000).

In order to observe weathering effects of long-term growth of microbes on mineral surfaces, microbes must first be removed from the surfaces. This is not difficult after short-term (hours to days) growth and researchers have simply rinsed bacteria off or repeatedly scanned a surface to physically remove cells with AFM tips (Grantham et al. 1997; Maurice et al. 1996). However, for long-term (weeks to months) experiments that better approximate the natural environment, these removal techniques have proven insufficient (Maurice et al. 1996; Liermann et al. 2000b).

In previous work, we removed bacteria from surfaces of hornblende glass and crystal with lysozyme, an enzyme that lyses bacterial cell walls. We assumed lysozyme was adequate for removing aerobic bacteria incubated for less than 3 weeks based upon SEM analyses of the cleaned mineral surfaces (Kalinowski et al. 2000; Liermann et al. 2000b). However, as described here, when these same lysozyme-treated surfaces were analyzed in this study by AFM, we observed residual biomatter. Hence, a more effective method of removal was needed for AFM studies of mineral-microbe interactions.

Some methods of removal can be too abrasive and will remove or damage evidence of bacterial growth. In addition, chemical interactions between the cleaning-agent and the mineral surface may mask or alter chemical signatures of microbial activity. For example, Thorseth et al. (1995) observed enhanced dissolution of basaltic glass when solutions of $KClO_3$ in HNO_3 were used to remove bacteria for SEM imaging. H_2O_2 is also commonly used to oxidize organic matter for removal from mineral surfaces (e.g., Thorseth et al. 1995). However, H_2O_2 can react with the organic matter to form oxalic acid (Barker and Banfield 1996), which can enhance dissolution of the mineral surfaces by lowering pH or complexing cations (Stone 1997). Thus, it is of utmost importance to choose an appropriate cleaning technique for combined textural and chemical analyses.

In using AFM, it is vitally important to determine whether images are representative of the overall surface, a problem particularly applicable to environmental research, in which materials are typically extremely heterogeneous even at the AFM scale (Johnsson 1993). Therefore, instead of using crystalline hornblende, as used in our previous experiments investigating siderophore-producing microbes (Liermann et al. 2000a; Liermann et al. 2000b;

Kalinowski et al. 2000; Brantley et al. 2001), we synthesized and polished an Fe-silicate glass to prepare a chemically homogeneous, smooth surface similar to the mineral hornblende. The use of a smooth, chemically analogous substrate aids in the recognition of features attributable to bacteria and eliminates chemical variability caused by impurities and secondary phases in the mineral structure.

Detergents are an obvious choice for removing bacteria from mineral surfaces because their amphiphilic character reduces the surface tension leading to rupture of cell walls and reduces interfacial tension at interfaces between liquids and solids, enabling release and solubilization of organic substances into solution (Aronstein et al. 1991; Banat et al. 2000). In this study, five cleaning methods, involving CO_2 snow cleaning or one of four different surfactants, were evaluated using AFM, SEM, and XPS on bacteria-exposed hornblende glass surfaces.

Materials and Methods

Substrate and Microbe

A glass with the composition of the mineral hornblende (Table 1), fabricated and characterized as described by Liermann et al. (2000a), was cut into planchets approximately $1 \times 1 \times 1/2$ cm and polished to 0.25 μm. Polished samples were ultrasonicated in acetone for 10 min, air-dried, and stored in a desiccator. Bulk chemistry of the hornblende glass was determined by inductively coupled plasma–atomic emission spectrophotometry (ICP-AES) using the lithium metaborate fusion technique (Suhr and Ingamells 1966).

A *Bacillus sp.* was chosen for its ability to grow vigorously in Fe-deficient growth medium in the presence of hornblende glass. This microbe was isolated from a hornblende-containing soil from Gore Mountain, New York, and was originally identified as an *Arthrobacter sp.* (Kalinowski et al. 2000; Liermann et al. 2000b; Brantley et al. 2001), based on SEM analyses and partial 16S rRNA gene sequencing, which showed a similarity rank of 0.661 to *Arthrobacter oxydans* (Kalinowski et al. 2000). Recently, however, full 16S rRNA gene sequencing (Nucleic Acid Facility, Life Sciences Consortium, The Pennsylvania State University) revealed a sequence similarity of 0.986 (1.0 indicates a perfect match) to *Bacillus mycoides*, a Gram-positive, rod-shaped, nonmotile, endospore-forming bacterium (Sneath 1986). The isolate may be a strain of this species and is referred to here as a *Bacillus sp.*

Growth Conditions

Hornblende glass planchets were placed in sterilized glass test tubes with 10 ml of sterilized, unbuffered, low-Fe medium containing 0.5% (w/v) glucose, 0.5% (w/v) casamino acids, 2 ml l^{-1} of Wolfe's Mineral Elixir (Wolin et al. 1963) prepared without nitriloacetate and without $FeSO_4$ (Liermann et al. 2000a). The glucose and casamino acids were first treated with Chelex-100 (Bio-Rad Laboratories) cation-exchange resin to remove Fe and other trace metals. Fe content of this growth medium is below the lower limit of detection (<0.02 ppm) for ICP-AES (Liermann, unpublished). Samples were inoculated with 0.5 ml of ~1-week cultures of *Bacillus sp.* Parallel control experiments (medium + planchets) were not inoculated. Cultures were incubated at ambient temperature (approximately 25°C) for 13 or 77 days. Upon termination of each experiment, glass planchets were removed from the test tubes, rinsed in deionized water, allowed to air-dry, and cleaned by CO_2 snow cleaning or one of four detergents, chemically fixed and critical point-dried for

TABLE 1 Elemental atomic ratios relative to Si on hornblende glass surfaces[1]

Sample[2]	O/Si	C/Si	C/Si[3]	Na/Si	Al/Si	Fe/Si	Fe/Si[3]	P/Si
Borax	3.17 ± 0.32	0.44 ± 0.04	4.73 ± 0.47	0.11 ± 0.01	0.38 ± 0.038	0.08 ± 0.008	0.16 ± 0.016	0.04 ± 0.004
Sodium pyrophosphate	2.84 ± 0.28	0.40 ± 0.04	3.55 ± 0.36	0.04 ± 0.004	0.34 ± 0.034	0.02 ± 0.002	0.02 ± 0.002	0.03 ± 0.003
Sodium dodecyl sulfate	2.48 ± 0.25	0.30 ± 0.03	6.32 ± 0.63	0.06 ± 0.006	0.31 ± 0.031	0.04 ± 0.004	0.03 ± 0.003	0.02 ± 0.002
Triton X-100	3.37 ± 0.34	1.00 ± 0.10	5.57 ± 0.56	0.05 ± 0.005	0.34 ± 0.034	0.02 ± 0.002	0.04 ± 0.004	0.06 ± 0.006
Control (SDS)[4]	3.91 ± 0.39	0.89 ± 0.09	9.18 ± 0.92	0.10 ± 0.01	0.53 ± 0.053	0.09 ± 0.009	0.08 ± 0.008	0.23 ± 0.023
Blank 1[5]	4.14 ± 0.41	0.40 ± 0.04	—	0.04 ± 0.004	0.56 ± 0.056	0.10 ± 0.010	—	—
Blank 2[5]	4.07 ± 0.41	0.38 ± 0.04	—	0.03 ± 0.003	0.51 ± 0.05	0.08 ± 0.008	—	—
Bulk glass[6]	—	—	0.01 ± 0.001	0.09 ± 0.005	1.00 ± 0.05	0.35 ± 0.018	0.17 ± 0.009	0.24 ± 0.012

[1]Ratios measured by XPS. Error in ratios is assumed to be $\pm 10\%$. Precision of measurement on any one sample is better than this, but based on XPS measurements of glasses in our laboratory (Hamilton et al. 2000), reproducibility of elemental ratios is lowered when measurements are made on multiple samples polished separately.
[2]Samples were incubated with *Bacillus sp*. for 77 days, cleaned with one of 4 detergents and ultraviolet ozone cleaning (UVOC) prior to XPS.
[3]Ratio measured by XPS prior to UVOC treatment.
[4]Control was incubated in medium for 77 days without bacteria, then cleaned with SDS and UVOC.
[5]Blanks were polished planchets of hornblende glass that were not incubated in medium but were cleaned by UVOC.
[6]Bulk glass represents bulk chemistry of the blanks analyzed by ICP-AES using the lithium metaborate fusion technique with maximum relative error reported (Suhr and Ingamells 1966). Differences between XPS-measured ratios and bulk ratios are presumed due to polishing artifacts as seen for other glasses (Hamilton et al. 2000).

SEM imaging of the bacteria, or allowed to air-dry for AFM imaging of the bacteria. The bacteria in the solutions were pelleted by centrifugation, dried overnight at 65°C, and weighed. Small aliquots of the supernatants were filtered with 0.2-μm SFCA syringe filters (Nalgene) and measured for pH.

In these experiments, no attempt was made to replenish glucose. The Fe-depleted medium was used to force the bacteria to obtain Fe from the hornblende glass surfaces. Additionally, ferric iron is at low concentrations in most natural environments and thus, low-Fe medium better approximates environmental conditions.

Cleaning Treatments

The same hornblende glass planchets initially studied by Kalinowski et al. (2000) and Liermann et al. (2000b) were reexamined here. For those studies, the planchets were incubated in unbuffered growth medium with or without *Bacillus sp.* and either allowed to air-dry or cleaned with a 0.1% (w/v) solution of lysozyme, an enzyme that breaks specific polysaccharide bonds in cell walls (see Kalinowski et al. 2000; Liermann et al. 2000b).

A planchet, rinsed in deionized water and air-dried after 13 days of incubation, was cleaned using a CO_2 snow cleaning unit (Applied Surface Technologies, New Providence, NJ, USA), which removes particulate matter and organic residues by a combination of aerodynamic drag, momentum transfer, and solvent action, provided by the flow of CO_2 snow particles moving in a high velocity CO_2 gas (Sherman and Adams 1996). The sample was placed in a glove bag and blasted for 3 min with CO_2 snow and gas. This technique has been used previously for cleaning dust, fingerprints, and nose prints from optical instruments, semiconductors, hard disk drive components, scribed silicon wafers, X-ray mirrors, and clean room equipment (Sherman et al. 1991; Sherman et al. 1994; Sherman and Adams 1996).

Four detergents were also used to clean planchets that were not exposed to the medium (cleaned blanks), planchets exposed to the growth medium for 77 days (cleaned controls), and planchets exposed to medium with *Bacillus sp.* for 77 days (cleaned samples): 2% (v/v) sodium dodecyl sulfate (SDS), 1% (v/v) Triton X-100, 5mM sodium pyrophosphate (SPP, $Na_4P_2O_7$ $10H_2O$), and 5 mM sodium tetraborate (borax, $Na_2B_4O_7$ $10H_2O$). These detergents were chosen based on their previous use as bactericides (borax, Al-Hitti et al. 1983) or bacterial lysing agents (SPP, Lee et al. 1994; Triton X-100, Backhus et al. 1997; SDS, Cullen and Hirsch 1998). Planchets were ultrasonicated in detergent solutions for 45 min, then ultrasonicated in distilled and deionized water for 10 min before air-drying. To remove loose particles, condensation, fingerprints, and other contaminants prior to AFM imaging, samples were cleaned by ultrasonication in spectroscopic grade acetone, and blown dry with compressed nitrogen gas, immediately before imaging.

AFM

All planchets (blanks = polished only; cleaned blanks = polished and cleaned; cleaned controls = polished, incubated in sterile medium, and cleaned; cleaned samples = polished, incubated in medium with bacteria, and cleaned) were imaged in air with a Digital Instruments Dimension 3100 Atomic Force Microscope in Tapping-Mode (TM-AFM) using a tapping-mode etched silicon probe tip (TESP–70) at a scan-rate of 0.5–1.0 Hz. Tapping-Mode AFM is commonly used for imaging cells, organic materials, and other sensitive surfaces because the tip only contacts the surface intermittently, minimizing the lateral frictional forces that can damage surfaces during contact-mode imaging

(e.g., Maurice et al. 1996). In TM-AFM, the surface topography is determined by changes in the oscillation amplitude during scanning (Howland and Benatar 1996).

Height images, showing features both above and below the average surface level, were collected for all samples. For some samples, amplitude images, showing only the positive features, were also taken, as well as phase-contrast images, revealing variations in surface adhesive properties (Digital Instruments 1997). To eliminate tilt and S-shaped bow distortions caused by curvature of the piezoelectric stylus, thermal drift, or lateral forces, third-order plane fitting was performed on each image (Ruppe and Duparee 1996; Digital Instruments 1997). The dimensions of the surface features were measured using Digital Instruments Nanoscope IIIa Controller software V.4.31 (1997).

SEM

As a point of comparison to the AFM images, and to document the distribution of the cells on the surfaces, glass planchets with and without bacteria were imaged using scanning electron microscopy (SEM). Air-dried samples of *Bacillus sp*. (see Liermann et al. 2000b) on hornblende glass, as well as abiotic control and lysozyme-cleaned surfaces, were coated with gold and imaged using a Philips XL-20 SEM. Additionally, a sample containing live *Bacillus sp.* was prepared for imaging after 13 days of growth by immersing it in 2.5% glutaraldehyde in 0.1 cacodylate buffer at pH 7.4 for 3 h at about 25°C, then for 17 h at 4°C and washing 3 times for 5 min in 0.1 M cacodylate buffer in order to chemically fix the bacteria. The sample was then placed in 25, 50, 70, 85, and 95% ethanol for 5 min each, then rinsed 3 times for 5 min with 100% ethanol to slowly dehydrate the bacteria. Bone-dry liquid CO_2 was used as the transitional fluid for critical-point drying at 34°C and 1,450 psi with an exchange temperature of 16°C and 900 psi in an Energy Beam Scientific Polaron E3000 Critical Point Dryer. The sample was coated with gold/palladium and imaged using a JEOL JSM 5400 scanning electron microscope operated at 20 kV.

XPS

Elemental analysis of the upper 25 Å of a circular area (diameter 700 μm) of several planchets was performed by XPS using a Kratos Analytical XSAM 800 pci electron spectrometer with a 1253.6 eV Mg standard X-ray source at 350 Watts at a takeoff angle of 65° with respect to the sample plane (see Kalinowski et al. 2000). XPS was performed on two blanks and four planchets incubated with bacteria for 77 days and cleaned with the different detergents as described earlier. One control, incubated for 77 days in medium without bacteria, was also analyzed after cleaning with SDS. In preparation for analysis, samples were initially cleaned with acetone only. Then the samples were re-analyzed after cleaning with acetone followed by ultraviolet ozone cleaning (UVOC) to remove organic contamination (Vig 1992; Zazzera and Evans 1993; Kalinowski et al. 2000). Analyses without UVOC are also reported for comparison.

Results

AFM and SEM Observations

SEM images of the critical point-dried sample (Figure 1) reveal relatively spherical spores of *Bacillus sp.* on the glass surface, enveloped by glycocalyx (Brantley et al. 2001), a polysaccharide biofilm by which the cells attach to the surface (e.g., Brisou 1995; Thorseth et al. 1995). AFM images of CO_2 snow-cleaned planchets after 13 days growth of

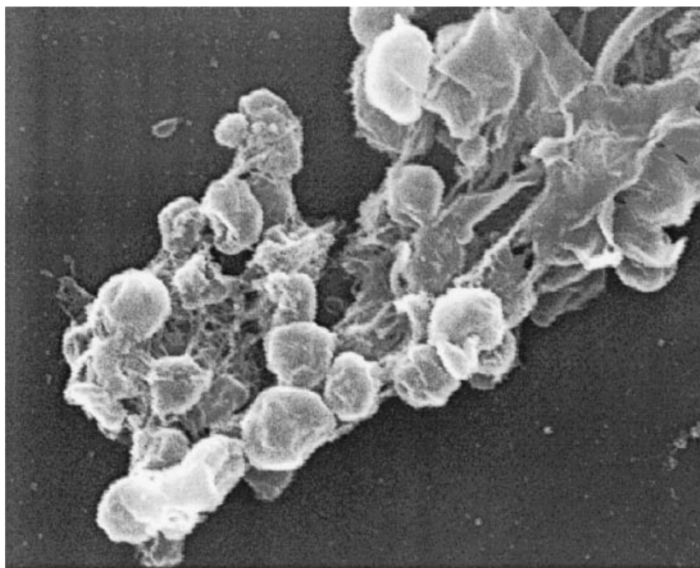

FIGURE 1 SEM image showing endospores (~1 μm diameter) of *Bacillus sp*. surrounded by biofilm on hornblende glass after growth for 13 days. The sample was prepared by critical point drying and sputter coated with gold/palladium.

Bacillus sp. compared to AFM images of air-dried, uncleaned surfaces reveal residual biomatter or precipitates (Figure 2). Similarly, AFM images of the lysozyme-cleaned surfaces reveal damaged, but identifiable, cellular material (images not shown). However, many regions of the CO_2 snow-cleaned surface and of the lysozyme-cleaned surfaces show no evidence of biomaterial, presumably because they were uncolonized in those areas.

Linear depressions, presumed to be etched polishing scratches based on previous work (Mellott et al. 2001), appear similar on all blanks, cleaned blanks, cleaned controls, and cleaned samples, measuring 7–30 nm deep and $<$600 nm wide when imaged by AFM (e.g., Figure 3).

All blanks, cleaned blanks, cleaned controls, and cleaned samples reveal occasional large pits (6–20 μm across, 200–400 nm deep), which are roughly circular or oval and are randomly oriented on the surfaces (Figure 4). However, only the 77-day detergent-cleaned *Bacillus sp*. samples exhibit smaller, shallower, more frequent pits (300–4000 nm across, 30–170 nm deep) that are not observed on the blanks, cleaned blanks, cleaned controls, or cleaned samples after only 13 days growth (Figure 5). These pits are irregular oblong shapes and are generally concentrated along polishing scratches (e.g., Figure 3), sometimes grouped or aligned $<$5 μm apart (Figure 5).

Analysis of Surface Chemistry by XPS

A depletion in the surface elemental ratios measured by XPS as compared to those measured on the blanks is seen on sample surfaces after removal of bacteria using all detergents except borax for Al/Si, and for all detergents for Fe/Si (Table 1, Figure 6). The Al/Si ratios of the post-bacteria detergent-cleaned sample surfaces are approximately equal, as are the Fe/Si ratios of all cleaned sample surfaces except the borax-treated surface.

In contrast to Fe and Al, enrichment in P is seen on all of the post-bacteria detergent-cleaned surfaces relative to the blanks (Table 1, Figure 6). Additionally, as is commonly

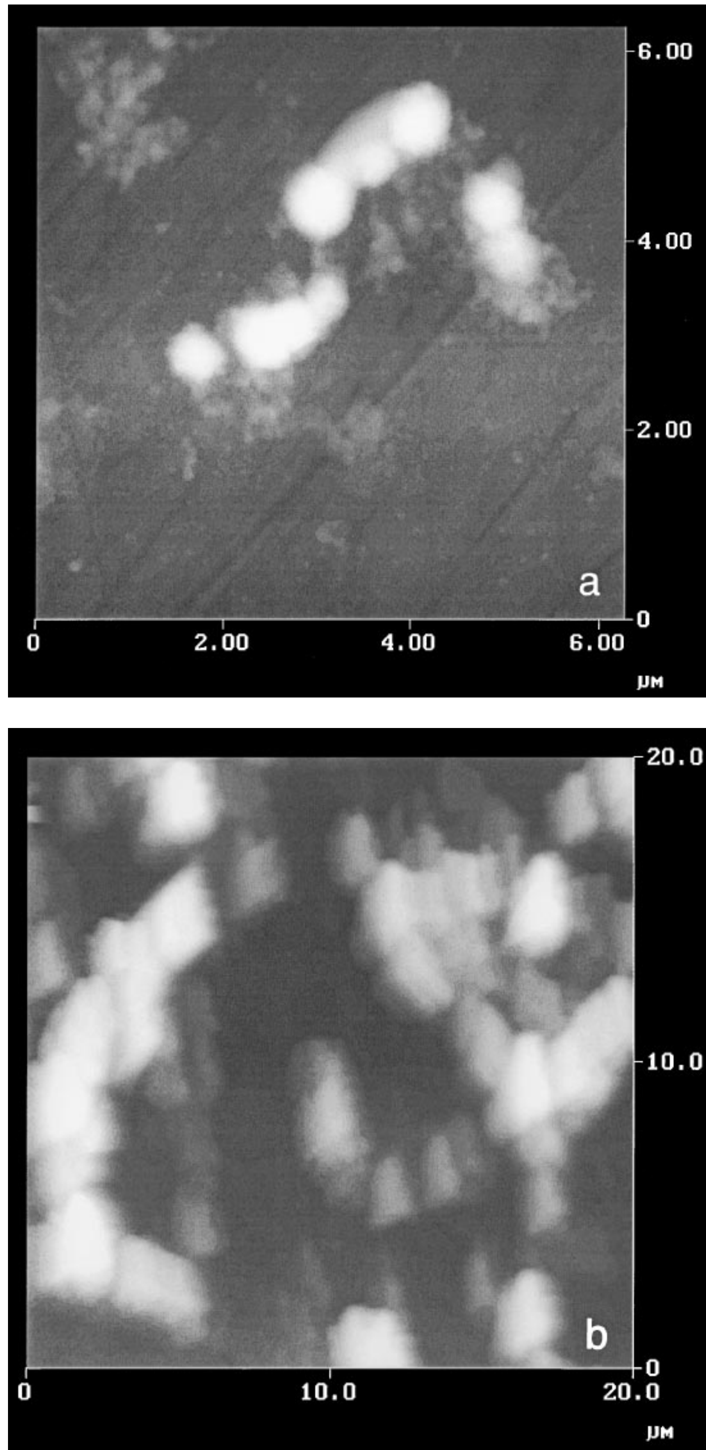

FIGURE 2 AFM height image of (a) a 6 μm × 6 μm area of an uncleaned hornblende glass surface showing air-dried *Bacillus sp*. spores and biofilm. (b) Similar image after CO_2 snow cleaning showing residual material, possibly residual biomatter or precipitates on a 20 μm × 20 μm region of the surface.

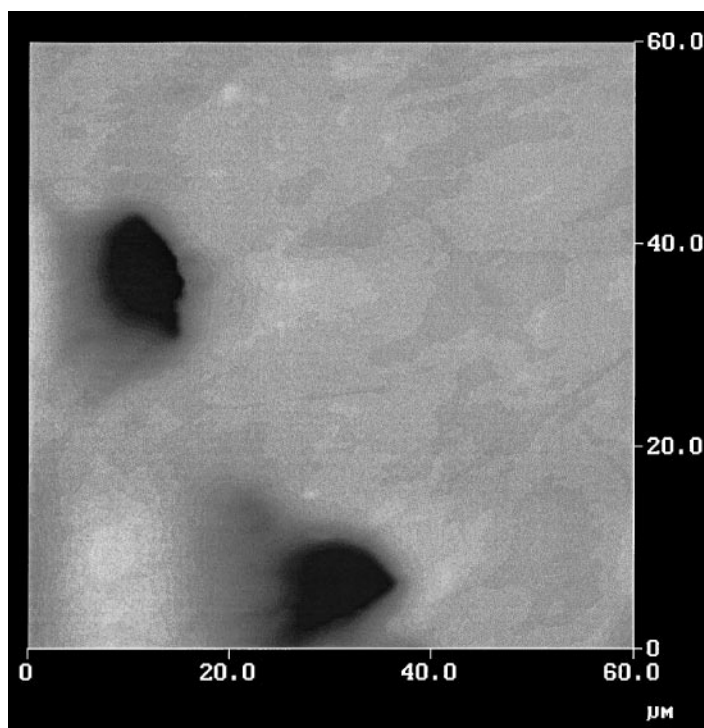

FIGURE 3 AFM height image of a 60 μm × 60 μm area of an untreated (blank) hornblende glass planchet. The large pits, measuring approximately 10 μm–20 μm across and 400 nm deep, are inferred to have been created during preparation of the polished planchets.

observed on silicate surfaces analyzed by XPS after reaction with aqueous solution (Johnsson 1993), all surfaces are coated with a carbon-rich layer of mostly aliphatic hydrocarbons, composing as much as ~60% of the atomic concentration of the upper 25 Å for surfaces not first treated by UVOC (Table 1). Most of the carbon was removed by UVOC, although up to ~18 atomic% remained. After UVOC, the bacteria-exposed, SDS-cleaned surface had the lowest carbon content of all surfaces analyzed. Also, Fe/Si ratios either increased (SDS), decreased (borax, Triton X-100), or remained the same (SPP) after UVOC treatment (Table 1, Figure 6a).

Discussion

Effectiveness of Removal Techniques

When samples, controls, or blanks were imaged without wiping with acetone and blasting with compressed nitrogen gas immediately prior to imaging, the surfaces were extensively obscured by unidentified contaminants. Samples cleaned with lysozyme reveal what appear to be the remains of bacteria attached to the surface. Thus, this method is not effective for complete removal of bacteria from the surfaces. However, the lysozyme-cleaned abiotic controls appear identical to the blanks under both AFM and SEM; therefore, lysozyme and acetone do not alter the topography of the surfaces and may be useful in combination with other, more effective removal techniques.

Although CO_2 snow cleaning has proven effective for removing many organic residues at the XPS and AFM scales (Sherman et al. 1991; Sherman and Adams 1996), we saw a

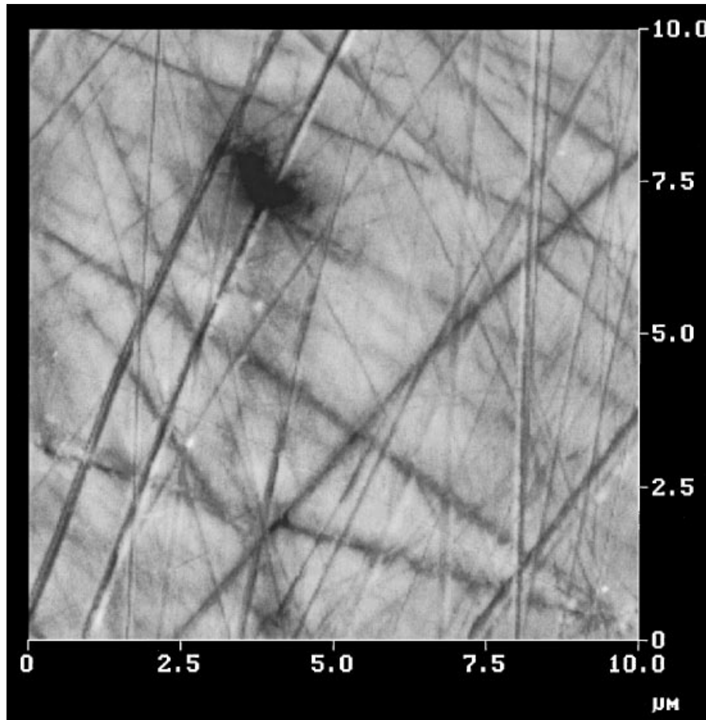

FIGURE 4 AFM height image of a small pit (2 μm × 1.3 μm, 65 nm deep) on a 20 μm × 20 μm area of a hornblende glass planchet after growth of *Bacillus sp*. for 77 days.

significant quantity of cellular material still attached to the hornblende glass surface after treatment. CO_2 snow cleaning removes organics because CO_2 acts as a solvent, dissolving organic substances when dry ice particles liquefy upon impact before resolidifying in the flowing gas. Therefore, although it is possible that longer treatment times could increase effectiveness, it is more likely that the bacterial cells are insufficiently soluble in CO_2 to be removed by this process. Indeed, Hills (1995) found that CO_2 snow cleaning was not effective at removing complex oxygenated organics or organics with functional groups having low solubility in liquid CO_2.

Comparison of detergent-cleaned blanks to untreated blanks under AFM shows that the detergents do not alter the microtopography of the surfaces. However, it is necessary to image numerous controls, blanks, and surface locations to distinguish surface features from dust particles, edge effects, and other artifacts. Comparison of AFM images to SEM images is also helpful for distinguishing surface features from artifacts (Maurice et al. 1996).

Influences of Cleaning Treatments on Surface Chemistry

Based on the previously summarized AFM observations, any of the four detergents (borax, SPP, SDS, or Triton X-100) remove biomaterial from the silicate surfaces effectively. However, C/Si ratios on the glass surfaces after incubation with bacteria were elevated before UVOC (Table 1). After UVOC, the C/Si ratios of glasses cleaned with two of the treatments (SDS, SPP) were identical or lower than the blanks, suggesting that SDS- or SPP-cleaning plus UVOC removed C effectively. Neither borax nor Triton X-100 removed all C effectively (Figure 8).

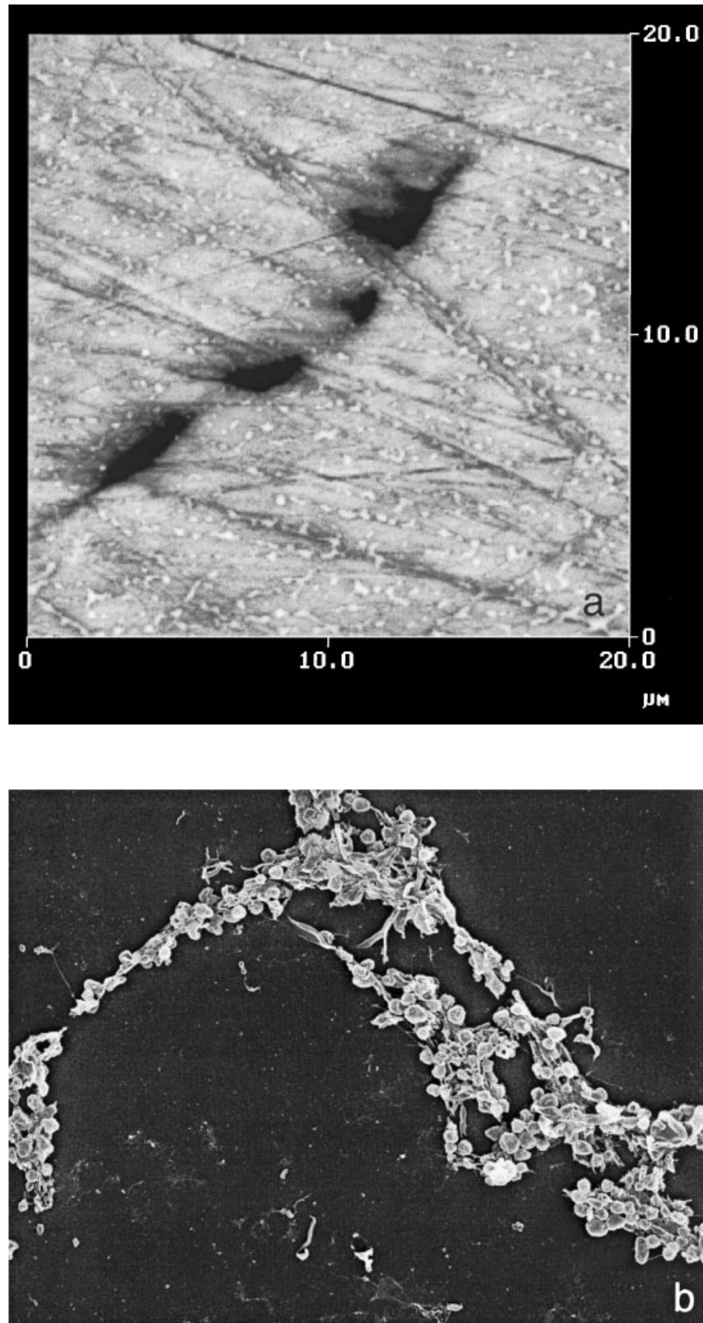

FIGURE 5 (a) AFM height image of a string of pits 3.5 mm long, ≤73 nm deep, on a 20 μm × 20 μm area of a hornblende glass planchet after growth of *Bacillus sp.* for 77 days. (b) Small colonies of *Bacillus sp.* (represented here as mostly endospores ∼1 μm diameter) grown on hornblende glass and imaged under SEM after critical point drying reveal similar size and distribution to the groupings of pits seen in (a).

In contrast, only the surface cleaned with SDS after incubation with bacteria revealed a P/Si ratio within error of the upper limit for P/Si estimated for the blanks. Specifically, no P was measured on blanks, and given the sensitivity of XPS, the highest P/Si ratio that could have been present on blanks is 0.02 (Figure 8). Thus, SDS is the most effective cleaning agent tested.

XPS analyses on glass surfaces after cleaning with all four procedures yielded Al/Si ratios that were identical within error (Table 1, Figure 8a). The loss of Al with respect to Si after incubation with bacteria followed by cleaning with detergents is consistent with loss of Al observed after incubation and cleaning with lysozyme as observed by Kalinowski et al. (2000). This latter Al loss was attributed to leaching by siderophores, a mechanism that presumably also explains our observations.

Three of the cleaning procedures yielded Fe/Si surface ratios that were identical within error and lower than that of the blanks, while the borax-cleaning yielded an Fe/Si ratio identical to the blanks. Low Fe/Si ratios were also observed by Kalinowski et al. (2000) after incubation of glass with bacteria and cleaning with lysozyme. Fe depletion was attributed to leaching by siderophores. The anomalous enrichment of Fe on the borax-treated sample here is therefore attributed to Fe precipitation at the glass surface. Such precipitation might

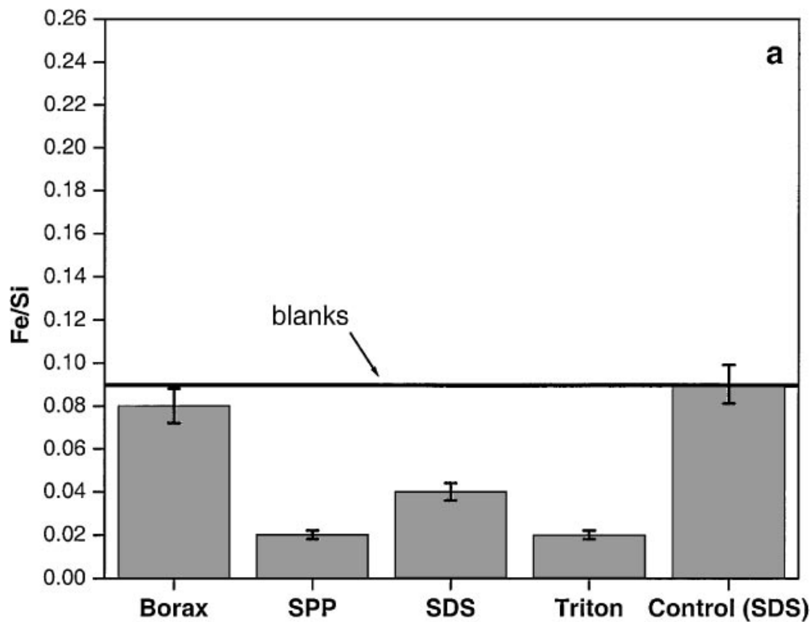

FIGURE 6 XPS-measured elemental ratios in the upper 25 Å of hornblende glass planchets cleaned with detergents after 77 days incubation with or without bacteria. Error bars indicate ±10% reproducibility of elemental ratios based on XPS measurements of glasses in our laboratory (Hamilton et al. 2000). Elemental ratios of the blanks (polished only) are indicated by solid lines. (a) The Fe/Si ratios of the detergent-cleaned surfaces exposed to bacteria compared to a cleaned hornblende glass control (glass exposed to medium only, cleaned with SDS) suggest that the Fe loss observed on the surfaces after removal of bacteria by SPP, SDS, or Triton X-100 (Triton) can be attributed to the bacteria rather than to the medium or the detergents. Formation of an Fe-precipitate may account for the relative enrichment of Fe/Si on the sodium tetraborate (borax)-cleaned surface. *(Continued)*

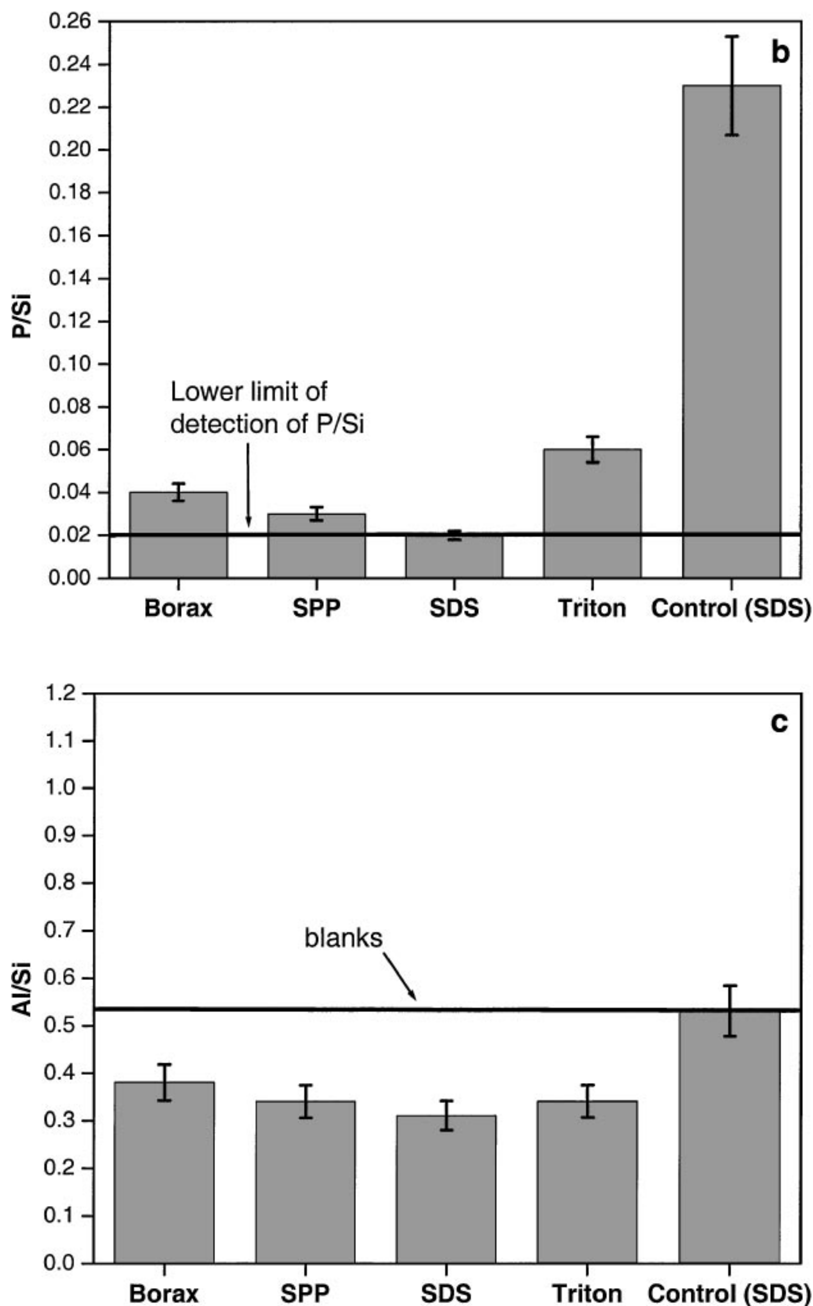

FIGURE 6 (Continued) (b) The P/Si ratios of the detergent-cleaned surfaces reveal enrichment in P relative to the blanks (P content of the blanks was below the XPS detection limit). Formation of phosphate surface complexes may account for this enrichment. The control has the highest P/Si ratio because no bacteria were present to consume the P. (c) The Al/Si ratios of the detergent-cleaned surfaces after removal of bacteria are lowered relative to the Al/Si ratios of a hornblende glass control (medium only, cleaned with SDS) and of the blanks (polished only) suggesting that the bacteria caused the Al loss. *(Continued)*

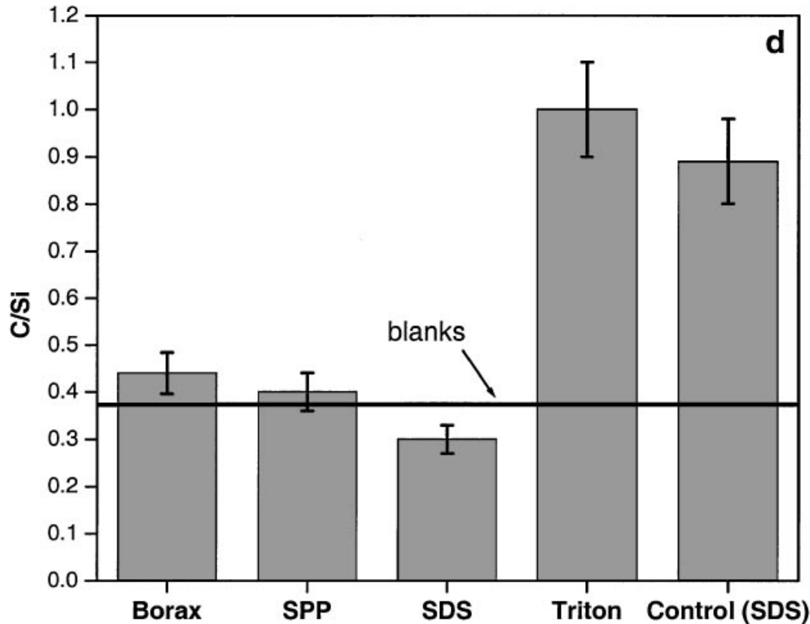

FIGURE 6 (Continued) (d) The C/Si ratios of the detergent-cleaned surfaces after UVOC reveals an enrichment in C/Si (mostly aliphatic hydrocarbons) relative to the blanks on all surfaces except that cleaned with SDS-cleaned after growth of bacteria.

be expected at the high pH of the borax treatment (measured pH of borax solution = 9.0). The low Fe/Si ratios on glasses cleaned with the other reagents are attributed to Fe leaching by siderophores, in agreement with Kalinowski et al. (2000). However, the low Fe/Si ratios measured after treatment with SPP and Triton X-100 may also be due to further effects of cleaning. Specifically, chelation of trivalent metal cations by pyrophosphate has been noted (e.g., Higashi and Shinagawa 1981; Stevenson 1982; Yuan et al. 1993; Hall et al. 1996) and Triton X-100 releases a biochemical reductant (Backhus et al. 1997). Omueti and Lavkulich (1988) also reported that SPP should not be used in mineralogical studies because it can cause mineral hydration.

Therefore, insufficient removal of the C and P deposited during incubation, as well as chemical alteration by borax, SPP, and Triton X-100, argues against the use of these three detergents in chemical studies of microbe-mineral interactions. The remaining detergent, SDS, may therefore be the best cleaning reagent. To further clarify the effects of this detergent, another sample (a control) was incubated in medium without bacteria for 77 days and cleaned with SDS. This control allows analysis of the effect of incubation in medium (without bacteria) and cleaning.

If Fe- and Al-depletion of the glass surface after incubation with bacteria occurs because of siderophore-leaching, then the Fe/Si and Al/Si ratios of the SDS-cleaned control should be identical to the blanks. Correspondence within error between the SDS-cleaned control and the blanks is documented in Figure 6. However, the P/Si and C/Si ratios on the control surface are significantly higher than that of the blanks (Figure 6). Enrichment in P and C is consistent with formation of phosphate complexes and aliphatic carbon coatings on the surfaces due to exposure to medium. The lower P and C contents on surfaces in the biotic experiments relative to the abiotic controls may document uptake of these nutrients by bacteria.

These results indicate that SDS is an effective removal agent for *Bacillus sp*., and because SDS is commonly used as a lysing agent in microbiological studies (e.g., Hilger and Myrold 1991) SDS may be useful for removing many other soil microbes from surfaces. However, some bacteria degrade surfactants such as SDS (Marshall et al. 2000). Biodegradation of SDS was documented for concentrations of 0.069 mM (Marshall et al. 2000), while this study used SDS at 69 mM concentration. Thus, it is likely that SDS is helpful to bacteria at low concentrations but lethal at high concentrations as is the case for a number of substances (Madigan et al. 2000).

The results presented here are consistent with the conclusions of Kalinowski et al. (2000), except that we found that lysozyme cannot remove all of the biomaterial from the hornblende glass surfaces.

Etch Pits

The occasional large pits seen on AFM images of all hornblende glass surfaces are thought to be inherent defects in the glass, possibly air bubbles exposed during preparation of the planchets. This is supported by the fact that these pits have no apparent correlation with polishing scratches and are seen on the untreated blanks as well as the surfaces exposed to the growth medium, bacteria, or cleaning agents.

The tendency of the smaller pits, seen only on the surfaces exposed to bacteria, to congregate along polishing scratches is consistent with findings that abiotic etching (e.g., Honess 1929; Lasaga and Blum 1986; Johnsson 1993; MacInnis and Brantley 1993; Maurice et al. 1995), microbial colonization (e.g., Brisou 1995; Barker and Banfield 1996; Grantham et al. 1997), and microbial pitting (Thorseth et al. 1995; Bennett et al. 1996) of mineral surfaces are more pronounced along high-energy sites such as grain boundaries, dislocations, cleavages, and defects. However, Maurice et al. (2001) recently used AFM and XPS to examine mica surfaces before and after burial for 39 days and observed small etch pits, bacteria, organic films, and decreased Fe/Si ratios after the mica chips were exhumed. Additionally, several studies have documented etch pits in the vicinity of attached microbes using SEM (e.g., Bennett et al. 1996; Barker et al. 1998; Rogers et al. 1998). Thus, the pits may be a consequence of bacterial colonization, although abiotic dissolution caused by a bacterial secretion cannot be ruled out. In a follow-up study, microbial etch pits will be compared to those caused by abiotic processes under the same conditions.

Conclusions

This study has shown that use of lysozyme or CO_2 snow cleaning alone is not effective for removing all biomatter after growth of bacteria from surfaces for AFM-scale analyses. Four detergents were found to completely remove bacteria from Fe-silicate glass without damaging the surfaces as observed by AFM, but upon review of XPS results, suggestions of chemical alterations by some of the detergents were discovered.

On the basis of the results presented here, the following recommendations can be made. First, sodium tetraborate (borax) should not be used for chemical studies of microbial interactions with Fe-silicates because of the possibility of precipitating Fe-oxides that can mask chemical biosignatures. Additionally, sodium dodecyl sulfate (SDS) appears to be useful for removing Fe-phosphates formed by exposure of Fe-containing surfaces to P-containing growth media. SDS is also the most effective detergent tested for removing environmental carbon contamination from silicate surfaces. SDS is therefore recommended for removing bacteria from silicate surfaces for analysis of physical and chemical biosignatures. However,

UVOC is an essential sample preparation step for XPS analyses because the carbon layer it removes can distort the elemental signatures detected by XPS.

Finally, these experiments also documented two possible biosignatures on hornblende glass: lowered Al/Si and Fe/Si ratios as well as the presence of small ($\sim 900 \times 100$ nm) etch pits on the surfaces incubated with *Bacillus sp*.

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