**FINAL REPORT: Microbial Dissolution of Silicate Materials,
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9G**

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The objective of this research was to better understand the role of selected thermophilic bacteria in the colonization and dissolution of silicate minerals, with potential applications to the HDR Project.

Preliminary experiments

Preliminary experiments with *Thermus aquaticus* and *T. filiformis*, two thermophilic aerobes with optimal growth temperatures near 70°C were conducted with a 6 week incubation using bytownite and forsterite chips and powders, with periodic monitoring of pH and measurement of dissolution rates of forsterite and bytownite powders by analysis of Ca (bytownite) and Mg,Fe (forsterite) in solution (0.1-0.2g of 60>>80mesh (177-250 micron) fraction was used). Microbial colonization was documented on the chips by SEM examination. There was little or no measured dissolution of the bytownite powders in inoculated or control runs (an upper limit of 0.01%). Significant dissolution of forsterite powders occurred in both inoculated and control runs; on the order of 0.1 to 0.4% of the original powder went into solution, assuming stoichiometric dissolution. Inoculated powder runs became slightly alkaline (pH = 8.0-8.4), while other runs remained near neutral; ammonia was detected in most runs. Significant microbial growth occurred in both normal and "mineral deficient" media (0.1% tryptone and yeast mixture) with the powders present (little growth was evident in their absence). Apparently there was insufficient

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incentive for the microbes to attack the minerals under these conditions, since dissolution occurred under abiotic conditions, providing a supply of mineral nutrients for growth. Significant enhancement of dissolution rates by these microbes, if it occurs at all, apparently will only happen in truly mineral deficient media. The experiments of Susan Welch using soil bacteria confirm this conclusion (personal communication).

Culture conditions for establishing mineral nutrient deficiency was attempted for the two *Thermus* species and the thermophiles *Bacillus stearothermophilus* and *B. thermoglucosidasius* using ultrapure reagents (mineral salts, glucose and water). No growth was observed with this media, even with the presence of a mixture of amino acids. We found that inconsistent results from previous culture experiments resulted from insufficient washing of inoculant with ultrapure water (three repeated washings, i.e., washing, centrifuging, decanting repeated three times, gave consistent results, one or two washings only did not). Further experiments with Castenholz media (mineral salts including trace elements plus dilute yeast and tryptone) gave the following results:

- 1) *T. aquaticus* grew in complete media, but not in media lacking addition of Ca, Mg and Fe salts.
- 2) *T. filiformis* showed growth in both normal and mineral deficient media.
- 3) *Bacillus* sp. showed no growth in these media.

Dissolution experiments were then conducted using powders of bytownite and diopside (a CaMgFe silicate known to have a low dissolution rate under near neutral experimental and natural conditions). *T. aquaticus* and *T. filiformis* were used in both complete and deficient media (both Ca and Ca, Mg, Fe). Media was decanted at weekly intervals in this batch experiment with monitoring of optical density, pH and Ca, Mg and Fe concentrations (flame AAS) and dissolved Si (silico-molybdenum blue method). Modest enhancement in dissolution was observed for diopside and bytownite in *T. filiformis* -inoculated media (1st week, shown by elevated Ca in Ca-deficient media; see Fig. 1,2). Enhanced dissolution for both inoculated and uninoculated media was observed

for some elements in the first two weeks, but erratic fluctuations in measured elemental concentrations were commonly observed, probably the result of microbial "infection" of sterile controls, as well as analytical problems with the silico-molybdenum blue method (see Fig. 3,4). One major problem with this experiment was the non-reproducibility of media/microbial contact with mineral powders, since the tubes were not agitated during the incubation period. A Digisem digital imaging system for SEM (also purchased under DOE subcontract) made possible systematic photodocumentation of chips before and after the experiments, thus revealing potential colonization and etch features, a methodological approach that was originally made using polaroid film but found to be impractical (and hazardous to health, since treatment solutions for negatives were highly irritating to the respiratory tract).

Two week batch experiment

In light of the experimental experience described above, a two week batch experiment was undertaken, utilizing inoculants of *T. filiformis*, which showed suggestive dissolution enhancement effects with diopside and bytownite powders. Powders of both minerals were used in Ca-deficient media and controls, all at pH near neutral, a region of minimum expected dissolution in an abiotic experiment (pH remained between 6 and 7 at the end of the experiment). Experiments were conducted in round bottomed polystyrene tubes set in a waterbath with shaker at 70°C. Media was removed at the end of the first week, again a week later at the close of the experiment. Obvious filamentous growth was seen in all inoculated tubes, with no growth apparent in sterile controls. Microbial levels were measured by optical density. SEM-documented chips were used in normal media. Each chip was scanned, with high magnification documentation of a selected area (both before and after). As in previous experiments, media removed from the powder experiments was digested in ultrapure acid in teflon bottles and evaporated to dryness, made up to the desired volume with ultrapure water. These samples were analyzed for Na, Mg, Ca, Al, Si, K, Fe by ICP-MS at the Microscopic and Chemical Analysis Research Center, The Ohio State University, Columbus. Results are shown in Table 1, 2, 3.

To sum up these results:

1) No systematic change in Na, Mg, Ca media concentrations relative to the original media was observed over the course of the powder experiments.

2) An apparent drop in media Si and Fe concentrations was observed in the powder experiments (possible uptake of these elements by microbial growth is suspected, with biofilms adhering to powders, thereby escaping analysis of the vortexed and decanted media).

3) An increase in the Al concentration was observed in the bytownite powder experiment, in both inoculated and uninoculated samples, with no significant difference between the two. The media was apparently too high in the concentrations of nutrient elements to have the mineral powder serve as "bait" for microbial attack. The computed dissolution rate was higher in the first week, with results (10^{-14} moles Al $\text{cm}^{-2} \text{sec}^{-1}$) within an order of magnitude of published dissolution rates for long duration experiments on bytownite at near neutral conditions (Blum and Stillings, 1995) (see Table 3)

4) Detailed photodocumentation of bytownite and diopside chips showed no evidence of microbial colonization or etching (see Appendix for bytownite chip, before and after incubation in inoculated media). Even though this aspect of the experiment was negative, it demonstrated the potential of this methodology to track microbial colonization and dissolution effects.

Conclusions

The demonstration of enhanced dissolution from microbial effects is critically dependent on providing a mineral bait within a media deficient in the critical nutrient found in the mineral (e.g., Fe). Reproducible experimental conditions in batch experiments require agitation to expose mineral powders, as well as nearly similar initial conditions for both inoculated cultures and controls. It is difficult, but not impossible to insure reproducible conditions with microbes favoring filamentous growth habits.

Future plans

Batch and Chemostat experiments

As part of our current research associated with Brookhaven National Laboratory, we will focus on identifying thermophilic and mesophilic bacteria and fungi that solubilize Fe from Fe-bearing

silicates. Future batch experiments will focus first on *Pseudomonas* and thermophilic fungi (*Rhizomucor*, *Thermomyces* and *Rhizopus*) with identified siderophores. Once short term batch experiments identify the conditions for microbial enhancement of dissolution, chemostat experiment will be undertaken. A teflon chemostat has been designed to allow long term dissolution to occur through mineral powders in a continuously replenished deficient media (deficient in necessary trace element such as Fe, which is present in silicate powder). Selected thermophiles will be used, with priority on those with siderophores, and demonstrated enhancement of dissolution from batch experiments. Stage one will be to optimize steady state growth with complete media, little or no growth with deficient media. Stage two will add mineral powders, with continual monitoring of microbial levels, pH and selected trace element concentrations over time to determine the extent of microbial enhancement of mineral dissolution. Surface areas of the mineral powders will be determined on the original powder and after the experiment using the BET method (this subcontract has provided a Quantasorb surface analyzer).

Thermostability of Heat Shock Proteins (HSP)

A fundamental question is the biophysical/biochemical constraint on the upper temperature limit of microbial groups, which may have been critical in the history of microbial evolution on Earth (Schwartzman et al., 1993). Heat Shock Proteins are likely candidates to investigate because of their multifold roles in cells (molecular chaperones, involvement in mitochondrial membrane synthesis etc.). Dr. Theodore Bremner, a molecular biologist in our department, has joined our group to direct experiments with HSP 70 and 90, to determine the upper temperature limit of their activity in selected thermophilic fungi (upper temperature limit of about 60°C).

References

- Blum, A.E. and L.L. Stillings, 1995, Feldspar dissolution kinetics. Ch.7, in *Chemical Weathering Rates of Silicate Minerals. Reviews in Mineralogy Vol. 31* (eds. A.F. White and S.L. Brantley) pp. 291-351, Mineralogical Society of America, Washington, D.C.
- Schwartzman, McMenamin, M., and T. Volk, 1993, Did surface temperatures constrain microbial evolution?, *BioScience* 43, 390-393.

TABLE 1.

Microscopic and chemical Analysis Research Center

The Ohio State University
026 Mendenhall Laboratory
125 South Oval Mall
Columbus, OH 43210

Date of analysis: March 21-23, 1999
Instrument operator: Eric Grunwald
Analysis performed using a
Perkin-Elmer Sciex ELAN 5000
ICP Mass Spectrometer

Results of Water Sample Analysis

Samples (concentrations in ppb)

Precision +/- 5% relative standard deviation

Sample ID	Na	Mg	Al	Si	K	Ca	Fe
1	61	12	2	52	39	204	<5
2	62	12	2	41	38	211	<5
3	111	181	145	588	104	589	44
4	74	12	2	40	39	203	<5
5	80	12	2	62	44	214	<5
6	175	191	162	690	372	594	59
7	189	456	233	177	1554	1292	<5
8	78	11	3	40	40	189	<5
9	164	350	299	254	497	1373	53
10	158	561	247	294	678	1615	42
11	89	92	78	403	204	407	12
12	88	12	3	19	41	211	<5
13	84717	2997	556	127	11790	1394	134
14	100676	3497	422	144	14976	724	176
15	79689	2657	243	163	11309	433	141
16	108519	3755	362	488	16547	580	199
17	114179	4031	216	602	17229	382	220
18	107550	3848	358	545	16775	577	227
19	121152	4222	237	702	18665	405	245
20	115193	4833	57	1755	17836	930	454
21	105457	4087	37	608	16569	509	283
22	106841	4528	54	578	17249	777	297
23	116090	4482	37	790	18237	544	338
24	118149	4625	63	460	18833	658	334
25	112127	4273	35	503	18235	476	296
26	81101	2768	56	515	11647	589	259
27	99457	3789	36	556	16606	489	317
28	37962	1171	25	498	5635	299	273
29	48217	1476	32	384	7152	305	245
30	320	41	33	487	366	639	262
31	214	39	26	406	316	306	266
32	57937	1780	34	404	8541	576	276
33	53772	1629	30	369	7958	437	267
34	412	51	1755	371	348	1025	262
35	518	62	2198	461	372	1860	260
36	330	91	38	395	381	526	304
37	230	149	48	765	353	990	282
38	350	120	29	596	404	341	<5
39	364	66	31	288	423	304	<5

TABLE 2. ANALYTICAL RESULTS 2 WEEK BATCH EXPERIMENT (T. filiformis)

Los Alamos Table 2

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Sample (ICP #)	MEDIA CONCENTRATIONS (ppm)						Fe
	Na	Mg	Al	Si	K	Ca	
By 1 (13)	266.000	9.410	1.750	0.399	37.000	4.380	0.420
By 2 (14)	280.000	9.720	1.170	0.400	41.600	2.010	0.490
By 2-II (15)	321.000	10.700	0.980	0.657	45.600	1.740	0.570
By 3 (16)	302.000	10.400	1.000	1.360	46.000	1.610	0.550
By 3-II (17)	317.000	11.200	0.600	1.670	47.900	1.060	0.610
By 4 (18)	309.000	11.000	1.030	1.560	48.100	1.660	0.650
By 4-II (19)	326.000	11.400	0.640	1.890	50.200	1.090	0.660
Di 1 (20)	320.000	13.400	0.160	4.880	49.600	2.590	1.260
Di 1-II (21)	320.000	12.400	0.110	1.840	50.200	1.540	0.860
Di 2 (22)	307.000	13.000	0.150	1.660	49.500	2.230	0.850
Di 2-II (23)	323.000	12.500	0.100	2.200	50.700	1.510	0.940
Di 3 (24)	328.000	12.900	0.180	1.280	52.400	1.830	0.930
Di 3-II (25)	312.000	11.900	0.097	1.400	50.700	1.320	0.820
Di 4 (26)	338.000	11.500	0.230	2.150	48.600	2.460	1.080
Di 4-II (27)	307.000	11.700	0.110	1.720	51.200	1.510	0.980
M1 (28)	316.000	9.750	0.210	4.150	46.900	2.490	2.270
M2 (29)	402.000	12.300	0.270	3.200	59.600	2.920	2.040
A (32)	483.000	14.800	0.280	3.370	71.100	4.800	2.300
B (33)	448.000	13.600	0.250	3.070	66.300	3.640	2.220

Notes: By: bytownite; Di: diopside; Both are powders (60-80 mesh, washed, ultrasonically cleaned)
 Inoculated samples: By 1, 2, 2-II, Di 1, Di 1-II, Di 2, Di 2-II
 Uninoculated samples: By 3, 3-II, 4, 4-II, Di 3, 3-II, 4, 4-II
 M1, M2: Ca deficient media
 A, B: inoculated Ca deficient media
 Data: 2-3 significant places

TABLE 3 Bytownite Dissolution Results (incremental Al levels in media)

	moles ($\times 10^{-6}$)	weight powder (g)	Surface area (cm^2)	Dissolution rate (10^{-14} moles Al $\text{cm}^{-2} \text{ sec}^{-1}$)
<u>Inoculated samples</u>				
1st week				
By 1	0.56	0.1563	15.6	5.9
By 2	0.34	0.1509	15.1	3.7
2nd week				
By 2-II	0.27	0.1509	15.1	1.5
<u>Uninoculated samples</u>				
1st week				
By 3	0.28	0.0738	7.4	6.2
By 4	0.29	0.0653	6.5	7.4
2nd week				
By 3-II	0.13	0.0738	7.4	1.5
By 4-II	0.14	0.0653	6.5	1.8

**Figure 1. Diopside powder dissolution
(Ca deficient media, *T. filiformis*)**

Figure 2. Bytownite powder dissolution
(Ca deficient media, *T. filiformis*)

**Figure 3. Diopside powder dissolution
(Ca, Mg, Fe deficient media, *T. aquaticus*)**

**Figure 4. Diopside powder dissolution
(Ca, Mg, Fe deficient media, *T. filiformis*)**

APPENDIX

PHOTOMICROGRAPH (SEM) DOCUMENTATION

BYTOWNITE CHIP INOCULATED WITH

Thermus filiformis (2 WEEK EXPERIMENT)

B7 (7) 8d

Log# 0 10.0 RU Notes: B7(7)8af 3/17/1996 04:25 a.m.
Mag# 749X 48.954um 1.0:112

After

B7 (7) 9 ab

Log# 0 10.0 RU Notes: B7(7)9af 3/17/1996 04:31 a.m.
Mag# 749X 48.954um 1.0:128

After

Log# 0 10.0 RU Notes: DB7(7)8 10/22/1995 03:40 a.m.
Mag# 749X 48.954um 1.0:112

Before

Log# 0 10.0 RU Notes: DB7(7)9 10/22/1995 03:50 a.m.
Mag# 749X 48.954um 1.0:128

Before

B7(7) 6af

After

B7(7) 7af

After

Before

Before

Before

Before

L7C7)4A6

After

L7C7)5A6

After

B-761206 → 3

After

B-761206 → 3

After

Before

Before

Area blown up (10 shots)

Before

by [signature]

After

17(1)af 1312 72

Δ32

B7aft

"After"

DB7(1) No Δ

"Before"

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