Coal Depyritization by the Thermophilic Archaeon

Metallosphaera sedula

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The kinetics of pyrite oxidation by Metallosphaera sedula were investigated with mineral pyrite and two coals with moderate (Pittsburgh no. 8) and high (New Brunswick, Canada) pyritic sulfur content. M. sedula oxidized mineral pyrite at a greater rate than did another thermophile, Acidithiobacillus brierleyi, or a mesophile, Thiobacillus ferrooxidans. Maximum rates of coal depyritization were also greater with M. sedula, although the magnitude of biological stimulation above abiotic rates was notably less than with mineral pyrite. Coal depyritization appears to be limited by the oxidation of pyrite with ferric ions and not by the rate of biotic oxidation of ferrous iron, as evidenced by the maintenance of a high ratio of ferric to ferrous iron in solution by M. sedula. Significant precipitation of hydronium jarosite at elevated temperature occurred only with New Brunswick coal.

Microorganisms that oxidize metal sulfides, especially pyrite (FeS₂), assist in the commercial recovery of metals from sulfide ores (9, 14) and have been studied for the desulfurization of coal in experimental heap leaching (12) and bioreactor (4, 23) systems. Pyritic sulfur is oxidized abiotically in aqueous, oxygenated solutions, resulting in dissolution of the mineral:

\[ 2\text{FeS}_2 + 7\text{O}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{FeSO}_4 + 2\text{H}_2\text{SO}_4 \] (1)

Small amounts of elemental sulfur are formed under certain reaction conditions (19, 20). Ferric ions, produced from either biological or chemical oxidation of ferrous iron, also oxidize pyrite:

\[ \text{FeS}_2 + 14\text{Fe}^{3+} + 8\text{H}_2\text{O} \rightarrow 15\text{Fe}^{3+} + 2\text{SO}_4^{2-} + 16\text{H}^+ \] (2)

In addition to these two routes, oxidation may occur by direct microbial attack where microorganisms have direct access to the surface of pyrite. The relative importance of the direct and indirect (reaction 2) mechanisms to biodegradation is still uncertain. Bacteria such as Thiobacillus ferrooxidans, which can regenerate ferric iron from ferrous iron, have been extensively studied as metal sulfide-oxidizing agents. Strains that can oxidize ores rapidly are sought to improve process economics. Faster rates are especially important in the development of processes for the microbiological removal of pyrite from coal because large quantities of relatively low-value material must be processed.

One way to oxidize the pyrite in coal more rapidly would be to increase the temperature. Thermophilic microorganisms such as Sulfolobus acidocaldarius and Acidithiobacillus brierleyi, formerly Sulfolobus brierleyi (24), were shown to remove pyrite from coal at 70°C (15, 16).

Another thermophilic archaeon, Metallosphaera sedula, was recently isolated and described by Huber et al. (13). According to these authors, this facultative chemolithoautotroph oxidized sulfidic ore "about ten times faster" than A. brierleyi, although few details were provided. In this article, we report on determination of pyrite-leaching kinetics in the presence of M. sedula and the ability of this organism to remove sulfur from coal in comparison with A. brierleyi and T. ferrooxidans. Coal depyritization appears to be dominated by limitations imposed by the reaction involving oxidation of pyrite with ferric ions and not by the rate of biotic oxidation of ferrous iron.

MATERIALS AND METHODS

Coal and pyrite. Mineral pyrite was obtained from the Office of Standard Reference Materials, National Institute of Standards and Technology, Gaithersburg, Md. (standard reference material 8455, pyrite ore bioleaching substrate, 58- to 91-μm particle size). Pittsburgh no. 8 coal was ground to 75 to 150 μm, and New Brunswick coal (11) was ground to less than 75 μm. The samples were sterilized in a vacuum oven for 30 min/day at 95°C on 3 successive days and stored in a vacuum desiccator under a nitrogen atmosphere. Unless otherwise noted, the percentages reported for the forms of sulfur analyzed are provided on a moisture- and ash-free basis.

Microbial strains and culture conditions. M. sedula was obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen, Germany), and A. brierleyi was obtained from J. A. Brierley (Newmont Metallurgical Services, Salt Lake City, Utah). These organisms were maintained at 70°C in a mineral salts medium consisting of the following (in grams per liter of distilled or deionized water): K₂HPO₄, 0.04; MgSO₄ · 7H₂O, 0.4; and (NH₄)₂SO₄, 0.4. The pH was adjusted to 2.0 with 10 N H₂SO₄. The mineral salts solution was autoclaved at 121°C for 15 min, and an aliquot of a sterile solution of yeast extract (10% [wt/vol]; Difco, Detroit, Mich.) was added to give a final concentration of 0.02% (wt/vol). Pyrite (2.0% [wt/vol]) or a mixture of Pittsburgh no. 8 coal (5% [wt/vol]) and pyrite (0.4% [wt/vol]) was added as an oxidizable substrate. Cells were concentrated in some cases by centrifuging the cell culture supernatant at 12,000 ×

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T. ferrooxidans ATCC 13661 was obtained from the American Type Culture Collection, Rockville, Md., and maintained in 9K iron medium at pH 2.0 (25). The culture was incubated at 28°C on a gyratory shaker at 175 rpm. Cells were acclimated to a coal substrate by growing the inoculum in flasks containing Pittsburgh no. 8 coal (10% [wt/vol]) in 9K basal salts solution diluted 10-fold with 0.01 N H₂SO₄. Cell counts were done with a Petroff-Hausser counting chamber and phase-contrast microscopy. Experiments involving M. sedula or A. brierleyi were conducted at 68 to 70°C, and experiments using T. ferrooxidans were conducted at 28°C. Initial cell concentrations ranged from 3.5 x 10⁶ to 4.5 x 10⁷ cells per ml. Water lost by evaporation was replaced periodically by the addition of sterile deionized water. The pHs of 1.0-ml aliquots of the reaction mixture were determined at intervals during the incubation. Additional 0.5-ml aliquots were centrifuged at room temperature at either 3,000 x g for 10 min or 14,000 x g for 3 min. The supernatant was analyzed for sulfate (barium sulfate turbidimetric method [1]) and iron (o-phenanthroline colorimetric method [2]). At the conclusion of certain experiments, solid coal was collected on filter paper, rinsed three times with deionized water, and dried in a vacuum oven at 95°C for 1 h. The coal was analyzed for sulfur forms by methods of the American Society for Testing and Materials (ASTM) (3). The rate of bioleaching was determined from the slope of the linear portion of plots of the percentage of solubilized iron versus time (28). Mineral precipitates were identified by X-ray diffraction and by use of an X-ray fluorescence detector attached to a scanning electron microscope.

RESULTS

Bioleaching of mineral pyrite. M. sedula grew poorly on pyrite in mineral salts solution. However, when the medium was supplemented with yeast extract (0.02%), M. sedula oxidized mineral pyrite rapidly. After 5.0 days of incubation at a shake rate of 200 rpm, the soluble iron in inoculated flasks was 94% Fe(III), while in an uninoculated control flask it was 81% Fe(II). After 7.7 days, the pH of the solution in inoculated flasks had dropped from 2.0 to 1.3. While 59% of the pyrite in inoculated flasks had been oxidized, on the basis of soluble-iron measurements, only 4% had oxidized abiotically. M. sedula stimulated oxidation at a rate 22.3-fold higher than the abiotic rate (10.05 versus 0.45%·day⁻¹). The rate of pyrite oxidation based on sulfate production was 10.5%·day⁻¹, which is nearly identical to that based on iron. These rates could have been slightly underestimated, since a trace amount of hydronium jarosite, an insoluble iron hydroxysulfate, was detected in the solid residue upon termination of the experiment.

A. brierleyi solubilized iron from mineral pyrite at a rate of only 0.34% of the total Fe·day⁻¹ at a shake rate of 146 rpm, which approximated the abiotic rate. However, at an initial cell concentration of 2.0 x 10⁵ cells per ml and a reduced shake rate of 75 rpm, the rate of iron solubilization in flasks inoculated with A. brierleyi was 67% of the rate in flasks containing M. sedula under these conditions (3.27 versus 4.89%·day⁻¹, respectively), while the abiotic rate was 0.30%·day⁻¹.

Bioleaching of pyritic sulfur from Pittsburgh no. 8 coal. The biological environmental of the rate of oxidation of coal-pyrite inclusions was not as significant as that with mineral pyrite. At a pulp density of 10% (wt/vol), M. sedula solubilized iron from Pittsburgh no. 8 coal at three times the abiotic rate (Table 1). After 7 days of incubation, the concentration of soluble iron in inoculated flasks corresponded to 93.4% of the pyrite initially present in the coal. During this interval, the molar concentration of sulfate increased at approximately twice the rate of increase of soluble iron, approximating the expected stoichiometry for pyrite oxidation. There were slight decreases in soluble iron occurring after 7 days, suggesting some iron precipitation (Fig. 1). Soluble sulfate continued to rise to a level corresponding to 100% of the theoretical amount of pyrite in the coal after 11 days. At this time, the bioleached coal was found to be 97% depyritized by ASTM sulfur analysis. The leach solution from the exhaustive leaching experiments contained 5% Fe(II) in inoculated flasks and nearly 100% Fe(II) in uninoculated control flasks by M. sedula ( ) and in an uninoculated control ( ) as determined by solubilized iron concentration.

TABLE 1. Rates of iron solubilization from Pittsburgh no. 8 coal at mesophilic and thermophilic temperatures

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Shake rate (rpm)</th>
<th>Iron solubilization rate (of total Fe·day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. sedula</td>
<td>150</td>
<td>17.36</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>3.67</td>
</tr>
<tr>
<td>A. brierleyi</td>
<td>150</td>
<td>4.71</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>3.60</td>
</tr>
<tr>
<td>T. ferrooxidans</td>
<td>150</td>
<td>8.18</td>
</tr>
<tr>
<td>None</td>
<td>(28–30°C) 150</td>
<td>0.83</td>
</tr>
<tr>
<td>None</td>
<td>(68–70°C) 46</td>
<td>5.74</td>
</tr>
</tbody>
</table>

* 10% (wt/vol) pulp density.

FIG. 1. Removal of pyrite at 70°C from Pittsburgh seam coal in duplicate flasks by M. sedula ( ) and in an uninoculated control ( ).
ultated flasks, demonstrating that a high ferric-to-ferrous iron ratio was maintained in the medium by the organism.

As with mineral pyrite, only a trace amount (<2% [wt/wt]) of hydronium jarosite was detected by X-ray diffraction. These results are supported by sulfur analysis of the coal, which showed little change in the organic sulfur content. Jarosite, which is generally insoluble in dilute mineral acids, is included as a part of the organic sulfur fraction.

For comparative purposes, the rate of iron solubilization from Pittsburgh no. 8 coal by T. ferrooxidans ATCC 13661 was determined and found to be approximately one-half the rate for M. sedula. Although the lag period was reduced from 11 days to less than 4 days by acclimating T. ferrooxidans to a coal substrate, the overall rate of iron solubilization did not increase markedly. Bioleaching rates were 8.6 to 9.9 times the abiotic rates with nonacclimated and acclimated cells, respectively. Analysis of the coal after 21 days showed decreases in pyritic sulfur of 85.2 and 35.6% in inoculated and uninoculated flasks, respectively. Organic sulfur values also decreased by 9.4% in both groups.

We were unsuccessful in demonstrating depyritization of Pittsburgh no. 8 coal with A. brierleyi at a high shake rate. At a reduced shake rate, however, iron solubilization proceeded in inoculated flasks in excess of four times the rate in uninoculated flasks (Table 1), and, as in the case of M. sedula, soluble iron in flasks containing A. brierleyi was predominantly Fe(III) (94.9%). At initial cell concentrations of 2.0 to 2.9 × 10⁶ cells per ml, pyritic sulfur had decreased by 66.7 and 64.8% when the flasks were inoculated with M. sedula and A. brierleyi, respectively, but had decreased by 36.1% in uninoculated controls (Table 2). Hence, at lower shake rates, these organisms showed comparable rates of depyritization of Pittsburgh no. 8 coal, with a slight decrease in organic sulfur noted in the presence of A. brierleyi.

Bioleaching of pyritic sulfur from New Brunswick coal. New Brunswick coal contains an unusually large amount of pyritic sulfur and had an appreciable abiotic leach rate at 70°C. At a shake rate of 175 rpm and a pulp density of 10%, M. sedula in nonbaffled flasks solubilized iron from New Brunswick coal at a rate (5.60% of the total Fe - day⁻¹) that was not significantly higher than the abiotic rate (5.22% of the total Fe - day⁻¹). Soluble iron concentrations approached 200 mM after 18 days in medium in both uninoculated and inoculated flasks and remained largely as Fe(II). The pHs dropped to 1.3 and 1.1, respectively.

Abiotic leach rates of iron increased in uninoculated baffled flasks, suggesting insufficient oxygen mass transfer to solution in nonbaffled flasks. However, M. sedula did not desulfurize New Brunswick coal at a 10% pulp density at 175 rpm, even at a greater initial cell concentration and when baffled flasks were used to increase aeration. The rates of iron solubilization for uninoculated and inoculated flasks were 7.45 and 7.28%·day⁻¹, respectively. M. sedula did, however, readily depyritize New Brunswick coal in unshaken flasks at 10% (wt/vol) solids (3.93% of the total Fe·day⁻¹, versus 0.29% of the total Fe·day⁻¹ in an uninoculated flask). Soluble iron in flasks containing M. sedula was 85% Fe(III), versus nearly 100% Fe(II) in uninoculated flasks. Again, the pH had dropped to 1.2 in inoculated flasks after 18 days but remained at 1.9, near the initial value, in uninoculated flasks.

M. sedula also depyritized New Brunswick coal in shaken and baffled flasks at a reduced pulp density (1.4% [wt/vol]). In this case, M. sedula leached iron from New Brunswick coal at 2.4 times the abiotic rate (Table 3). As in the unshaken flasks at the higher pulp density, the soluble iron in these flasks was present largely as Fe(III) (99%), while 93% of the soluble iron in uninoculated flasks remained unoxidized.

Direct analysis of the residual coal showed that the pyritic sulfur content decreased by 67.7 and 98.9% in uninoculated and inoculated flasks, respectively, after 5.6 days (Table 4). However, some redistribution of sulfur did occur. The amount of sulfur in the organic fraction increased markedly in coal from both inoculated and uninoculated flasks. Also, a decrease in the concentration of soluble iron in inoculated flasks was noted after 2.8 days. Precipitation of jarosite was confirmed by X-ray diffraction analysis of residual coal from flasks in a parallel experiment. Scanning electron microscopy and X-ray fluorescence analysis indicated that the precipitate was hydronium jarosite. The form of the precipitate on coal from uninoculated flasks could not be identified.

A comparison of rates of depyritization of New Brunswick coal by the thermophiles at a reduced shake rate and pulp

### TABLE 2. Comparison of residual sulfur forms in Pittsburgh no. 8 coal after depyritization by M. sedula and A. brierleyi at a low shake rate

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Pyritic</th>
<th>Sulfatic</th>
<th>Organic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5.19</td>
<td>2.16</td>
<td>0.12</td>
<td>2.91</td>
</tr>
<tr>
<td>Uninoculated*</td>
<td>4.30</td>
<td>1.38</td>
<td>&lt;0.02</td>
<td>2.90</td>
</tr>
<tr>
<td>M. sedula*</td>
<td>3.72</td>
<td>0.72</td>
<td>0.10</td>
<td>2.91</td>
</tr>
<tr>
<td>A. brierleyi*</td>
<td>3.52</td>
<td>0.76</td>
<td>0.11</td>
<td>2.64</td>
</tr>
</tbody>
</table>

* Data are percentages on a moisture- and ash-free basis.

### TABLE 3. Rates of iron solubilization from New Brunswick coal in baffled flasks at mesophilic and thermophilic temperatures

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Shake rate (rpm)</th>
<th>Iron solubilization rate (wt/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. sedula</td>
<td>46</td>
<td>5.67</td>
</tr>
<tr>
<td>A. brierleyi</td>
<td>146</td>
<td>26.10</td>
</tr>
<tr>
<td>T. ferrooxidans</td>
<td>146</td>
<td>6.07</td>
</tr>
<tr>
<td>None (28–30°C)</td>
<td>146</td>
<td>13.28</td>
</tr>
<tr>
<td>None (68–70°C)</td>
<td>146</td>
<td>0.80</td>
</tr>
</tbody>
</table>

### TABLE 4. Comparison of residual sulfur forms in New Brunswick coal after depyritization by T. ferrooxidans and M. sedula

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Pyritic</th>
<th>Sulfatic</th>
<th>Organic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>19.89</td>
<td>18.28</td>
<td>0.40</td>
<td>1.21</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>11.80</td>
<td>9.43</td>
<td>&lt;0.01</td>
<td>2.27</td>
</tr>
<tr>
<td>T. ferrooxidans</td>
<td>4.11</td>
<td>1.58</td>
<td>&lt;0.01</td>
<td>2.53</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>9.88</td>
<td>5.90</td>
<td>0.15</td>
<td>3.83</td>
</tr>
<tr>
<td>M. sedula</td>
<td>4.93</td>
<td>0.20</td>
<td>1.78</td>
<td>2.95</td>
</tr>
</tbody>
</table>

* Data are percentages on a moisture- and ash-free basis.

** Data are percentages after 9.8 days.

Data are percentages after 5.6 days.
density was also made. The rates of iron solubilization by \textit{M. sedula} and \textit{A. brierleyi} exceeded the abiotic rate by factors of 7.4 and 7.9, respectively (Table 3). Both thermophiles depyritized New Brunswick coal to approximately the same extent (79.7 and 76.7\% for \textit{M. sedula} and \textit{A. brierleyi}, respectively). Abiotic oxidation reduced the pyrite content by 60.8\% in uninoculated flasks (Table 5). Increases in the amounts of sulfur in the organic fractions, suggestive of pyrite deposition, were least problematic in flasks inoculated with \textit{A. brierleyi}. Although organic sulfur values were consistent in replicate flasks within experiments, there was substantial variation between experiments.

\textit{T. ferrooxidans} readily oxidized pyrite in New Brunswick coal (Tables 3 and 4). After 9.8 days, 48.4 and 91.4\% of the pyrite had been oxidized at 146 rpm in uninoculated and inoculated flasks, respectively. At a pulp density of 1.4\% (wt/vol), the biotic rate exceeded the abiotic rate by a factor of 16.6. The overall rate was approximately one-half the rate demonstrated by \textit{M. sedula}.

**DISCUSSION**

Ferric ions are generally thought to be the dominant oxidants of pyrite (reaction 2) at low pH (6, 21, 26), especially since pyritic sulfur is oxidized by ferric ions at the same rate in aerobic and anaerobic solutions (26). In view of the rapid oxidation of pyrite by ferric ions at 70°C (7), Bos and Kuenen (8) suggested that the rate of microbial oxidation of ferrous iron might limit the overall dissolution of pyrite at an elevated temperature (70°C). However, we have shown that a high ratio of Fe(III) to Fe(II) can be achieved with \textit{M. sedula} at a high shake rate on mineral pyrite or coal containing pyritic sulfur or by both \textit{M. sedula} and \textit{A. brierleyi} at a lower shake rate. Pyrite oxidation in the coals tested, therefore, was limited not by microbial oxidation of Fe(II) to Fe(III) but instead by the rate of reaction of ferric ions with accessible pyrite surfaces in coal. Because of this limitation, selection of organisms solely on the basis of increased rates of iron oxidation would not necessarily result in more rapid desulfurization of coal at mesophilic and thermophilic temperatures.

Abiotically, mineral pyrite reacted comparatively slowly at 70°C. However, pyrite in Pittsburgh no. 8 and New Brunswick coals demonstrated significant abiotic rates of oxidation at an elevated temperature. This agrees with previous observations (5) that coal pyrite is more reactive than mineral pyrite. While the net biological rate (biotic minus abiotic) of iron solubilization from mineral pyrite by \textit{M. sedula} was over four times greater than the rate previously reported for \textit{T. ferrooxidans} (22), the net rate of biological enhancement for either coal pyrite tested was only slightly higher for the thermophile.

Ferrous iron oxidation by the thermophiles was inhibited in shaken flasks containing a high pulp density of New Brunswick coal. \textit{A. brierleyi} was also inhibited at high shake rates, regardless of the source of pyrite. Lawrence and Marchant (17) previously reported that the oxidation kinetics of a thermophilic \textit{Sulfobolus} sp. were not superior to those of \textit{T. ferrooxidans} at higher pulp densities. It has been suggested that these organisms are more susceptible to physical damage than are cells with a rigid cell wall (8). The thermophiles may differ in their sensitivity to particular culture conditions, however, since \textit{M. sedula} actively oxidized Fe(II) at high shake rates with mineral and coal pyrites. Studies have shown that inhibition in shake flask experiments can be overcome by static incubation (10, 27) or by a reduction in the pulp density (10). We also found this to be true, indicating that inhibition was not due to a soluble toxin, as with mineral pyrite (10) or during arsenopyrite leaching as described by Lindstrom and Gunnerjusson (18), since biological leaching by \textit{M. sedula} proceeded readily in static flasks at the higher pulp density.

The lack of biological enhancement in the oxidation of highly reactive pyrite in New Brunswick coal at high shake rates was also consistent with the finding of Baldi et al. (5) that transient inhibition of growth of \textit{T. ferrooxidans} on pyrites correlated with the chemical reactivity of the pyrites. Hence, a second factor may have been insufficient oxygen availability to the cells at 70°C, since we noted that the abiotic rate continued to increase as the shaking rate increased or when baffled flasks were used in rate determinations. Suspended cells at an oxygenated interface in the static flasks may have been responsible for enhancement of the oxidation rate.

Although it has been stated that jarosite precipitation is problematic in desulfurization of coal at elevated temperature (23), we found this phenomenon to be coal dependent. Minimal precipitation of hydronium jarosite occurred with Pittsburgh no. 8 coal when leached by mesophilic \textit{T. ferrooxidans} or thermophilic \textit{M. sedula}. However, this problem was significant in the case of New Brunswick coal at high and low temperatures, indicating that the rates we have determined for pyrite oxidation in this coal, based on soluble iron concentrations, are minimum values.

A knowledge of the rate of microbiological pyrite oxidation is critical to development of this process for desulfurization of coal. Though microorganisms can remove finely disseminated pyrite from coal, which is difficult to achieve in most physical coal-cleaning processes, microbiological rates of depyritization have been generally viewed as too slow to merit further development. Our results show that \textit{M. sedula} in shake flasks is clearly superior to \textit{A. brierleyi} and \textit{T. ferrooxidans} in the oxidation of mineral pyrite and somewhat faster in pyrite removal from different coals.

**ACKNOWLEDGMENTS**

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We acknowledge the technical support of Elizabeth Frommell in the preparation of X-ray diffraction spectrographs and Sidney Polack for their interpretation as well as the technical assistance of Rhonda Spiess in the pyrite oxidation studies.

**TABLE 5.** Comparison of residual sulfur forms in New Brunswick coal after depyritization by \textit{M. sedula} and \textit{A. brierleyi} at a low shake rate

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Pyritic</th>
<th>Sulfatic</th>
<th>Organic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated(b)</td>
<td>10.31</td>
<td>7.16</td>
<td>0.50</td>
<td>2.64</td>
</tr>
<tr>
<td>\textit{M. sedula}(c)</td>
<td>7.98</td>
<td>3.71</td>
<td>0.45</td>
<td>3.82</td>
</tr>
<tr>
<td>\textit{A. brierleyi}(d)</td>
<td>6.98</td>
<td>4.26</td>
<td>0.82</td>
<td>1.90</td>
</tr>
</tbody>
</table>

\(a\) Data are percentages on a moisture- and ash-free basis. 
\(b\) Data are means of six uninoculated samples from two separate experiments. 
\(c\) Data are means of three samples after 5.9 days. 
\(d\) Data are means of three samples after 5.8 days.
REFERENCES


