Abstract: A large number of microorganisms are responsible for the oxidation of Mn$^{2+}$ to insoluble Mn$^{3+}$ oxides ($\text{MnO}_x$) in natural aquatic systems. This paper reports the structure of the biogenic MnO$_x$, including a quantitative analysis of cation vacancies, formed by the freshwater bacterium *Leptothrix discophora* SP6 (SP6-MnO$_x$). The structure and the morphology of SP6-MnO$_x$ were characterized by transmission electron microscopy (TEM), X-ray absorption spectroscopy (XAS), including full multiple-scattering analysis, and powder X-ray diffraction (XRD). The biogenic precipitate consists of nanoparticles that are approximately 10 nm by 100 nm in dimension with a fibrillar morphology that resembles twisted sheets. The results demonstrate that this biogenic MnO$_x$ is composed of sheets of edge-sharing of Mn$^{4+}$O$_6$ octahedra that form layers. The detailed analysis of the EXAFS spectra indicate that 12 ± 4% of the Mn$^{4+}$ layer cation sites in SP6-MnO$_x$ are vacant, whereas the analysis of the XANES suggests that the average oxidation state of Mn is 3.8 ± 0.3. Therefore, the average chemical formula of SP6-MnO$_x$ is $\text{Mn}^{3+}_{1.12}\text{Mn}^{4+}_{0.88}\text{O}_2\cdot\text{H}_2\text{O}$, where $\text{Mn}^{3+}$ represents hydrated interlayer cations, $\text{Mn}^{4+}_{1.12}$ represents Mn$^{4+}$ cation vacancies within the layer, and Mn$^{3+}_{0.88}$ represents hydrated cations that occupy sites above/below these cation vacancies.

Introduction

Manganese oxides ($\text{MnO}_x$) with layered topologies are ubiquitous in industry and throughout the natural environment. The importance of these materials in industrial applications and natural systems stems from the variability of several key structural parameters that influence their properties. The porosity, degree of interlayer hydration, average manganese oxidation state, and presence/quantity of cation vacancies within the layer can all vary and determine specific materials properties. Layered MnO$_x$ are utilized currently in Li$^+$ ion battery cathodes,\(^1\)\textasciitilde\(^3\) taking advantage of high surface areas derived from porosity and small particle sizes.\(^5\) Other applications of MnO$_x$\(^6\)\textasciitilde\(^11\) exploit the mixed oxidation states of manganese (3$+$ and 4$+$). The cation vacancies within MnO$_x$ layers create negative charge,\(^12\)\textasciitilde\(^16\) providing a driving force for cation intercalation\(^17\)\textasciitilde\(^19\) and cation sorption.\(^20\)\textasciitilde\(^24\) Consequently, these oxides have potential applications in the environmental remediation of metals.\(^25\)\textasciitilde\(^30\)

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variability in structural and electronic parameters of MnO plays a determining role in the transport, speciation, and ultimate fate of metals and natural organic matter in the natural environment.31

Most natural manganese oxides formed at low temperature result from microbiologically mediated oxidation of aqueous Mn2+ to Mn3+–Mn4+. In natural systems, Mn3+–Mn4+ and Mn4+ are the most favored oxidation states,35 of which only Mn2+ and complexed Mn3+ are soluble in water.36,37 Therefore, the formation of Mn3+/Mn4+ oxides proceeds by oxidation of aqueous Mn2+ ions, a thermodynamically favorable but kinetically slow reaction.38,39 Consequently, the oxidation of aqueous Mn2+ must be catalyzed owing to the scarcity of sufficiently strong oxidants in natural systems. In the environment, this catalytic role is filled by a class of microorganisms collectively referred to as “manganese oxidizers.”2,40

Detailed knowledge of MnOx structures formed by microorganisms living in a variety of environmental conditions can provide insight into their biological importance and how they ultimately affect the speciation of otherwise mobile organic compounds and metals. Within the past several years, the structures of the MnOx formed by the spore-forming marine Bacillus sp. strain SG-141,42 and Pseudomonas putida strain MnB143 bacteria have been investigated in great detail and successfully identified. These distinct bacteria species produce similar mixed-valent, layered Mn3+/4+/5+Ox. Additionally, the structure of MnOx formed by the freshwater bacterium Leptothrix discophora SP6 (SP6-MnOx) has been reported by Kim et al.44,45 and Juergensen et al.46 Disagreement exists on the basic atomic structure of SP6-MnOx. Kim et al.44,45 reported that the SP6-MnOx UV Raman spectrum closely resembles a todorokite-like tunnel structure, whereas Juergensen and co-workers46 concluded that SP6-MnOx possesses a layered topology.

In this work, we report a detailed investigation of the structure of biogenic MnOx as well as abiotic counterparts. In addition, we propose a quantitative analysis of metal cation vacancies. The detailed structure resolution was performed by a combination of X-ray absorption spectroscopy (XAS), including full multiple-scattering analysis and powder X-ray diffraction (XRD). Particle morphology was investigated by transmission electron microscopic (TEM) imaging. The structure of biogenic MnOx was elucidated by spectroscopic comparison to a variety of synthetic MnOx phases for which detailed structures are known. Moreover, this biogenic MnOx was characterized in its near-natural, hydrated state, not in a dried form that may alter the structure of metastable phases.47 The existence and quantity of cation vacancies in abiotic layered MnOx have been matters of dispute within the literature,48 and their investigation previously has been limited to cases in which highly crystalline materials can be formed. In this work, we propose an EXAFS-based method that uses local structure information to quantify the number of metal cation vacancies in poorly ordered MnOx, precipitates by comparison to crystalline, abiotic reference materials.

**Experimental Section**

**Growth of L. discophora SP6 and Biogenic MnOx Production.** A frozen stock culture of *L. discophora* SP-6 (ATCC 51168) was obtained from American Type Culture Collection (ATCC).49,50 Liquid cultures of SP-6 were grown in mineral salts, vitamins, and pyruvate (MSVP/noFe), a modified version of the MSVP medium (ATCC Culture Medium 1917) used by Emerson and Ghiorse.49,50 The difference between MSVP/noFe and MSVP was that FeSO4 was not added to MSVP/noFe. Iron was limited to ensure slow bacterial growth conditions that promote sheath formation and consequent Mn2+/3+/4+ oxidizing capability.49,50 In addition, the presence of Fe2+ in the medium can lead to the incorporation of iron within the MnOx structure and can complicate matters.29 All cultures were maintained aerobically at room temperature (~25 °C), except for the SP-6 stock cultures (frozen stocks were maintained at ~85 °C). Liquid cultures were stirred slowly (~100 rpm) on a shaker table. Biomass in liquid cultures was measured by absorbance at 600 nm, according to the spectrophotometric method used by Emerson.49 Light microscopy was used to confirm the presence of sheaths and the characteristic morphological traits of SP-6. The SP6-MnOx was produced by growing a liquid culture of SP-6 in MSVP/noFe to stationary phase. Under these conditions, Fe is considered the limiting growth factor. A total of 62.5 mL of a 20 mM Mn2+/4+ stock solution was added to the 5 L SP-6 culture to obtain a final concentration of [Mn2+] ~ 250 μM.51

**Binary MnOx Reference Materials.** MnOx, Mn2O3, and β-MnO2 (pyrolusite) were purchased from Aldrich, and phase purities were confirmed by powder X-ray diffraction.

**Synthesis of c-Ordered Hexagonal K+-Birnessite.** This compound was synthesized by the thermal decomposition of KMnO4 at 800 °C for 16 h. Five grams of KMnO4 was heated at 2 °C/min to 800 °C, (47) Fritsch, S.; Post, J. E.; Suib, S. L.; Navrotsky, A. J. Chem. Mater. 1998, 10, 474–479.


maintained at 800 °C for 16 h, and cooled at 1 °C/min to room temperature to promote crystallization. The product was washed 7–10 times with 18 MΩ Milli-Q (MQ) water, until the filtrate became clear, to remove soluble manganese species with oxidation states >4.

Synthesis of ε-Disordered Hexagonal H⁺-Birnessite. This synthesis was adapted from that of McKeen and McMurdo.52–54 KMnO₄ (1.5803 g) was dissolved in 100 mL of MQ water; 1.64 mL of concentrated HCl was added dropwise to the 0.10 M KMnO₄ solution. Note: This reaction should be performed in a fume hood as copious amounts of Cl₂gas are released. The precipitate was collected by vacuum filtration through a fine-pored glass frit and divided into two dialysis tubes. Each dialysis tube was submerged in ~5 L of MQ water and stirred for 24 h. The water was changed four times to ensure complete dialysis of free ions. The dialyzed precipitate was dried for 12 h at 40 °C.

Synthesis of Todorokite-like Mg²⁺-OMS-1. Mg²⁺-OMS-1 was synthesized using a two-step procedure adapted from that of Malinger and Suib.55 First, a Na⁺-birnessite precursor (Na⁺-OL-1) was synthesized by the oxidation of Mn(II)₉aq by KMnO₄ in sodium hydroxide solution, and 0.5058 g of KMnO₄ (3.2 mmol) was added to a 3 M NaOH (~35 mL) solution. The solution was stirred and heated at 50 °C for 1 h. A solution of 0.3200 g of MgCl₂ (3.4 mmol) and 1.3610 g of MnSO₄ (9 mmol) dissolved in 35 mL of MQ H₂O was added dropwise to the KMnO₄/Mg(OH)₂ solution. The resultant brown suspension was sealed in an amber bottle and aged at 50 °C for 24 h in a convection oven, resulting in the formation of Na⁺-OL-1. Na⁺-OL-1 was then washed 10 times with MQ H₂O and transferred into a 125 mL Teflon-lined Parr autoclave with ~83 mL of 1 M MgCl₂ solution and treated hydrothermally at 125 °C for 48 h. After slow cooling (0.1 °C/min) to room temperature, the resulting suspension was filtered and washed several times with 150 mL of 100 °C MQ water and dried at 40 °C.

Synthesis of CaMnO₄ and Ca₂Mn₃O₈. The carbonate precursor technique for the syntheses of CaMnO₄ and Ca₂Mn₃O₈ was adapted from that of Horowitz and Longo.56 Polycrystalline CaMnO₄ was identified on the basis of the crystal structure determined by Poppelmeier and co-workers.57 Stoichiometric quantities of CaCO₃ and MnCO₃ were dissolved in dilute nitric acid. MnCO₃ should be freshly prepared and stored under vacuum until needed. Commercially available MnCO₃ was found to be oxidized, as evidenced by brown color, and was not used. MnCO₃ was prepared by dissolution of Mn(NO₃)₂ in MQ H₂O followed by precipitation with excess (NH₄)₂CO₃. In a typical synthesis of CaMn₂O₄, 0.4244 g of CaCO₃ (4.42 mmol) and 0.7616 g of MnCO₃ (6.63 mmol) were dissolved in 250 mL of acidified MQ H₂O. A 10-fold excess of dissolved (NH₄)₂CO₃ was added to precipitate the CaMn₂O₄ soluble solid solution. The CaMn₂O₄ solid solution was dried at 100 °C under vacuum. CaMn₂O₄ was formed by calcination and oxidation of CaMn₂O₄ for 5 h at 800 °C under flowing O₂ with one intermediate regrind. CaMnO₄ synthesis follows the same reaction scheme with a 1:1 molar ratio of Ca to Mn.

Synthesis of ZnMn₉O₈·3H₂O (Chalcophanite). The preparation of synthetic Zn-chalcophanite was adapted from that of Stein et al.58 The synthesis proceeds by preparation of Na⁺-birnessite followed by cation exchange of Na⁺ for Zn²⁺. Eleven grams of Mn(II)(CH₃COO)₂ (0.0636 mol) and 5.4056 g of NaNO₃ (0.0636 mol) were dissolved in deoxygenated MQ H₂O. The solution was stirred vigorously and heated at 100 °C until dry. A tan/brown powder resulted. The resultant powder was calcined at 400 °C in air for 7 h, resulting in Na⁺-birnessite. Na⁺-birnessite was resuspended in a 0.66 M Zn(NO₃)₂ solution and stirred vigorously overnight and washed several times to ensure complete cation exchange. The suspension was vacuum filtered and dried at 40 °C for 5 h.

Transmission Electron Microscopy. Bacterial specimens were fixed, stained, dehydrated, embedded in resin, microtomed, and poststained. Two types of embedding were used to identify/reduce artifacts: hydrophobic epoxy embedding59 and hydrophilic melanin embedding.60 Epoxy embedding was achieved by glutaraldehyde fixation followed by staining with uranyl acetate, dehydration with ethanol and propylene oxide, embedding in resin, microtomy, and poststaining. Thin sections used for elemental X-ray microanalysis omitted staining for purposes of accurate determination of the elemental composition.

A modification of the method of Leppard et al.61 was used for melanin embedding. The procedure consists of directly infiltrating L. discophora SP6 and associated MnO₃ with the melanin resin and polymerization/curing for 2 days at 40 °C in a desiccator and for 2 days at 60 °C. Owing to the brittleness of the melanin-embedded sample, an epoxy resin was used to form a composite section. A thin strip of the sample already embedded in hardened melanin resin was embedded in epoxy resin, making possible microtomy of a thin section. This method omits fixation, dehydration, and staining compared to epoxy embedding, which are deemed to be appropriate for structure imaging, but potentially interfere with MnO₃ microstructure visualization. Thin sections of embedded samples were collected and supported on 200 mesh Formvar-coated copper grids, obtained from SPI, Structure Probe Inc.

Transmission electron microscopy (TEM) micrographs (Figure 1a) of L. discophora SP6 and associated MnO₃ were collected on a Hitachi HF-2000 microscope equipped with a Princeton Gamma Tech X-ray detector, used for energy dispersive spectrometry (EDS). A tilt series, in which the tilt was varied between 0° and 30°, was taken using Philips Tecnai BioTwin TEM to investigate the morphology of SP6-MnO₃ particles (Figure 1b). Collection of a series of TEM images with varied stage tilt angles affords information on particle morphology in three dimensions.

X-ray Absorption Spectroscopy. Biogenic MnO₃, samples were separated from the growth medium by centrifugation and resuspension in MQ water. The sample was washed, centrifuged, and resuspended several times to ensure removal of unoxidized, loosely bound Mn(II)₉aq remaining from the oxidation procedure. Wet samples containing bacteria and biogenic MnO₃ were vacuum filtered, and samples were prepared by mounting the filter paper containing the wet solid (bacteria and MnO₃) between pieces of Kapton tape. MnO₃ reference standards were prepared for transmission measurements by spreading finely ground powders between pieces of Kapton tape. Typically, four layers of powder on Kapton tape were combined to obtain a μ ~ 2 and Δμ ~ 1 and to ensure the absence of pinholes. Transmission spectra were collected at several different locations on each sample, and reproducibility ensured sample homogeneity.

X-ray absorption spectra were collected at DND-CAT (Sector 5 beamline 5BMD) of the Advanced Photon Source at the Argonne National Laboratory in transmission and fluorescence modes. A Si(111) double-crystal monochromator was used to select wavelength. Harmonics were eliminated with a flat, Rh-coated mirror with a cutoff energy of 20 keV at 3 mrad and slight detuning (90% of Iλ_0,0). For edge energy calibration, the X-ray absorption spectrum of a reference Mn foil was collected with each sample. The metal foil edge energy was set to the first inflection point of the absorption edge, determined by the first derivative, and was set to 6539 eV.62 The average oxidation states of MnO₃ compounds was 5+.

were determined by calibration of edge energies with known oxidation states. MnO, Mn$_6$O$_8$, and MnO$_2$ were used as reference compounds for average oxidation state determination in biogenic MnO$_x$ (see Figure S1, Supporting Information). Spectra of synthetic standards were collected in transmission geometry using Oxford ionization chambers with a path length of 29.6 cm. SP6-MnO$_x$ samples were measured in fluorescence yield mode. Self-absorption corrections were not necessary given the small size and dilute nature of the biogenic nanoparticles. Ionization chambers were filled with gas mixtures to obtain 10% absorption (25% N$_2$/75% He) of the incident beam in I$_0$, 30% absorption (75% N$_2$/25% He) in I$_1$ (sample), and 60% absorption (95% N$_2$/5% He) in I$_2$ (reference foil). Spectra of biogenic MnO$_x$ were collected in fluorescence geometry using a Stern–Heald “lytle” cell detector equipped with soller slits and a Z-1 filter. The Lytle cell was continuously flushed with argon. For each reference MnO$_x$ and sample, nine replicate XAS spectra were collected to improve counting statistics. A smooth atomic background was removed from each XAS spectrum, and the data were normalized to a step height of one. This is done so that $E_0$, the Fermi energy, can be consistently chosen as the first inflection point at the absorption edge step. $E_0$ is obtained by AutoB$^K$ to determine the photoelectron wavenumber: $k = (2m(E - E_0)\hbar)^{1/2}$, where $m$ is the electron mass, $\hbar$ is Planck’s constant, and $E$ is the incident X-ray energy. This equation was used to convert the data from the measured EXAFS $\chi(E)$ to $\chi(k)$. The background function generated by the AutoB$^K$ algorithm is made from third-order polynomial spline functions that are connected by knots. Similar background polynomial spline parameters were used ($R_{kg} = 1.0$) to obtain the same background functions for all MnO$_x$ spectra. The background was removed from each data set, and then the resulting $\chi(k)$ data (nine replicates) were averaged. Processing of all XAS data was performed using SixPack, $^{66}$ Athena, $^{67}$ and Artemis $^{67}$ graphical user interfaces for XAS processing built on IFEFFIT. $^{68}$ Data reduction and analysis were performed using the methods of Athena and Artemis. $^{68}$ The data were fit in $R$-space with theoretical amplitudes and phases for single- and multiple-scattering paths that were calculated from crystal structures using the ab initio FEFF 8.10 code. $^{70}$

Figure 1. Morphology of SP6-MnO$_x$: (a) transmission electron micrograph of $L$. discophora SP6 that illustrates the location of the MnO$_x$ fibrils; (b) tilt series that focuses on a typical bundle of MnO$_x$ fibrils that surround $L$. discophora SP6 and illustrates twisted-sheet particle morphology.

| Table 1. Abiotic Model Compounds for Spectroscopic Comparison to SP6-MnO$_x$. |
|-----------------------------|-----------------|--------|---------|------|
| model compound              | topology        | vacancy (%) | stacking | ref  |
| $^{4+}$-homeite              | layered         | ~12     | ordered | 73, 87|
| $^{4+}$-biennseite           | layered         | ~16.7   | disordered | 73, 90|
| Zn-chalcopyhanite           | layered         | 14.3    | ordered | 58, 81|
| Ca$_3$Mn$_6$O$_8$            | layered         | 25      | ordered | 57    |
| Mg$_2$-OMS1 (todorokite)    | (3 x 3) tunnel  | n/a     | n/a     | 11, 92, 95 |

were taken over similar photoelectron wavenumber ranges with the endpoints terminating at nodes, typically between 2.95 Å$^{-1}$ ≤ $k$ ≤ 13.35 Å$^{-1}$. A Kaiser–Bessel window function was used to dampen the EXAFS oscillations at endpoints. Least-squares fitting of theoretical phases and amplitudes to MnO$_x$ model compounds and biogenic MnO$_x$ were performed in Artemis. $^{57}$ Continuous scanning XAS experiments were conducted on SP6-MnO$_x$ to ensure that beam damage to the sample did not occur. $^{51}$ The complete energy range around the Mn K edge was scanned in approximately 2 min, and replicates were collected over several hours, exhibiting no spectral change. $^{51, 72}$ Therefore, it is concluded that no significant beam damage occurs to SP6-MnO$_x$. Additionally, SP6-MnO$_x$ sample spectra were collected 6 months apart to investigate the effects of aging. No changes in the SP6-MnO$_x$ spectra were witnessed after a period of 6 months.

Analysis of EXAFS: Model. The crystal structure of $^{4+}$-biennseite was used as a model to fit the biogenic MnO$_x$ owing to their spectral similarity. The $^{4+}$-biennseite structure was fit using a symmetrical, hexagonal layer model derived from the reported crystal structures and is illustrated in Figure 8. $^{30}$ Scattering paths up to 6 Å were generated with TkAtoms$^5$ using a hexagonal unit cell ($P 6/mmm$, a = 2.840 Å, c = 14.50 Å) reported by Gaillot et al.$^{73}$ FEFF 8.10 was used to calculate single- and multiple-scattering path phases and amplitudes used in the fits. The number of variables for the $k = 2.95$–13.55 Å$^{-1}$ and $R = 0$–5.8 Å fitting range is 22, with 38 independent points, and meets the Nyquist criterion. The fit converged with goodness-of-fit parameters $R = 0.0177$ and $\chi^2 = 100.79$ (Table 2 and Figure 6). The average local environment around manganese within layered structures built up from edge-shared MnO$_6$ octahedra, up to 6 Å, was fit using a total of four Mn–O single-scattering paths, two Mn–Mn single-scattering paths, and one Mn–Mn multiple-scattering (MS) path. Nearest-neighbor coordination numbers for manganese and oxygen shells were constrained according to ideal crystallographic values with the exception of Mn$^{4+}$–Mn$^{3+}$ corner-sharing octahedra at ~3.85 Å, which was allowed to float freely. $S_2^2$ was constrained to 0.83 derived

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*Further details on data and methods, crystal structures, and additional references are provided in the supporting information.*
from temperature variable fits of Table 2.

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and Debye distances were constrained to integral multiples of Mn excess surface charge. In layered MnO hierarchical structure analysis of layered biogenic MnO can be created by the absence of layer cations (i.e., vacancies) several length scales is a multitechnique approach that combines curve-fit results in coordination numbers (CN) to understand their environmental role and potential application. In general, the energy of the X-ray absorption edge increases with increasing oxidation states as the successive removal of electrons from the absorbing atom raises the electron binding energy. On the basis of calibration with MnO standards with known oxidation states, the average oxidation state of manganese in SP6-MnO was found to be 3.8 ± 0.3 (see Figure S1, Supporting Information), and manganese cations were found to be octahedrally coordinated. Local manganese coordination geometry and average oxidation state were ascertained by comparison of the SP6-MnO XANES spectrum to abiotic reference standards with known oxidation states and crystal structures. An average oxidation state of 3.8 ± 0.3 indicates that SP6-MnO consists primarily of MnIII with a fraction of manganese cations in a lower oxidation state.14,71 The XANES spectrum of SP6-MnO exhibits a double-hump feature in the pre-edge region, centered around 6540 and 6542 eV, that arises from bound state, quadrupole-allowed transitions (Figure 2). A double-hump pre-edge resonance below the Mn K edge is characteristic of the octahedral crystal field splitting between eg and t2g orbitals.71 As a result, MnIV+O4− with structures built from MnO6 octahedra were synthesized as references for structure comparison with SP6-MnO by EXAFS (Figure 3).

XANES was used as the principal means to characterize the average oxidation state and coordination geometry of manganese.

Table 2. EXAFS Best Fit Results of Hexagonal K+-Birnessite

<table>
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<tr>
<th>R</th>
<th>χ²</th>
<th>tocc</th>
<th>shell</th>
<th>CN</th>
<th>distance (Å)</th>
<th>σ² (Å²)</th>
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<tr>
<td>0.0177 100.79 1.0 Mn5+—O</td>
<td>6</td>
<td>1.90 (1)</td>
<td>0.001 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 Mn5+—O</td>
<td>6</td>
<td>2.19 (2)</td>
<td>0.010 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.89 (3) Mn5+—Mn—O</td>
<td>6</td>
<td>2.84 (3)</td>
<td>0.003 (1)</td>
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</tr>
<tr>
<td>1.0 Mn5+—Mn—corner</td>
<td>0.8 (6)</td>
<td>3.43 (1)</td>
<td>0.017 (6)</td>
<td></td>
<td></td>
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<tr>
<td>1.0 Mn5+—O</td>
<td>6</td>
<td>3.40 (3)</td>
<td>0.002 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 Mn5+—O</td>
<td>12</td>
<td>4.60 (2)</td>
<td>0.005 (2)</td>
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<tr>
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<td>5.69 (2)</td>
<td>0.012 (2)</td>
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TEM images of hydrophilic resin embedded *L. discophora* SP6 and associated SP6-MnO are shown in Figure 1a. Uranyl acetate stained samples illustrate that MnO nucleation occurs on the exopolysacharride sheath which surrounds the bacterium, which is consistent with the observations of Ghiorse.49,50 Nucleation of MnO on the exopolysacharride sheath (EPS) may indicate that a catalytically active functionality is present on the surface. The mechanism of bacterial MnIV+ (aq) oxidation remains an active area of research with important questions unanswered.34,75 Structure determination of biogenic MnO is one key parameter toward a comprehensive understanding of their formation as well as their environmental role.

X-ray Absorption Near Edge Spectroscopy (XANES). Given the existence of nearly 30 known MnO mineral structures of different oxidation states, targeted syntheses of representative model compounds for comparison to the biogenic MnO are challenging without a priori knowledge of the average oxidation state. Therefore, the first step taken toward structure elucidation was the determination of the average oxidation state and local coordination geometry of metal ions.

The XANES region of the X-ray absorption spectrum, approximately 100 eV below the ionization edge (Fermi energy), is particularly sensitive to average oxidation state, local coordination geometry, and resultant crystal field splitting.76 In general, the energy of the X-ray absorption edge increases with increasing oxidation states as the successive removal of electrons from the absorbing atom raises the electron binding energy.76 On the basis of calibration with MnO standards with known oxidation states, the average oxidation state of manganese in SP6-MnO was found to be 3.8 ± 0.3 (see Figure S1, Supporting Information), and manganese cations were found to be octahedrally coordinated. Local manganese coordination geometry and average oxidation state were ascertained by comparison of the SP6-MnO XANES spectrum to abiotic reference standards with known oxidation states and crystal structures. An average oxidation state of 3.8 ± 0.3 indicates that SP6-MnO consists primarily of MnIII with a fraction of manganese cations in a lower oxidation state.14,71 The XANES spectrum of SP6-MnO exhibits a double-hump feature in the pre-edge region, centered around 6540 and 6542 eV, that arises from bound state, quadrupole-allowed 1s to 3d transitions (Figure 2). A double-hump pre-edge resonance below the Mn K edge is characteristic of the octahedral crystal field splitting between eg and t2g orbitals.71 As a result, MnIV+O4− with structures built from MnO6 octahedra were synthesized as references for structure comparison with SP6-MnO by EXAFS (Figure 3).

XANES was used as the principal means to characterize the average oxidation state and coordination geometry of manganese.


Redox titrations of environmental and biogenic samples are complicated by the presence of biological, organic, and inorganic substances, and it is often unclear whether the titrant solely occupies trigonal prismatic sites above and below layer vacancies to compensate for negative layer charge. This structural motif is common to anhydrous layered MnO\(_x\), and has been shown in Ca\(_2\)Mn\(_3\)O\(_8\),\(^{33,34}\) Mn\(_{2}\)O\(_{2-}\) Mn\(_{4}\)O\(_6\),\(^{83,84}\) Cu\(_2\)Mn\(_3\)O\(_8\),\(^{85}\) and K\(_2\)Mn\(_3\)(OH)(VO\(_4\))\(_2\).\(^{86}\) Similarly, ZnMn\(_2\)O\(_7\)·3H\(_2\)O is a hydrous layered Mn\(_{IV}\)O\(_2\) in which one of seven (∼14.3%) Mn\(_{IV}\) layer cations are vacant.\(^{81}\) Hydrated Zn\(_{2}\) cations occupy trigonal sites above and below layer cation vacancies.\(^{3,73,87}\)

Birnessites are a class of hydrous layered MnO\(_x\) that are common throughout the natural environment.\(^{31,88}\) Hexagonal birnessites contain variable quantities of Mn\(_{4+}\), layer cation vacancies, and hydrated Mn\(_{3+}\) occupied sites above or below the vacancies. In hexagonal birnessites, electroneutrality is achieved by the presence of additional hydrated cations between adjacent layers, usually alkali or alkali earth metals. Attempts to determine the detailed structures of various birnessites have been complicated by low crystallinity and the inability to grow large single crystals. Over the past several years tremendous progress has been made in birnessite structure characterization (87) Poepplmeier, K. R.; Leonowicz, M. E.; Scanlon, J. C.; Longo, J. M.; Yelon, W. B. J. Solid State Chem. 1982, 45, 71–79.


and classification of their numerous structure variations, 73,82,87,89,90
The work of Lanson 73,82,87,89,90 and Wang 48,91 has proven to be
seminal to the structure determination of biogenic MnO₆ and
facilitated the first complete structure determinations of biogenic
MnO₆ formed by Pseudomonas putida strain MnB 43 and
Bacillus sp. strain SGI. 41

Qualitative Comparison of Abiotic Layered MnO₆ with
Biogenic MnO₆. Figure 4 presents the EXAFS spectra of SP6-
MnO₆, several layered MnO₆ structures, and one tunnel-type
MnO₆ structure and provides a comparison of their local
structures. The local manganese environment in SP6-MnO₆ is
qualitatively similar to layered H⁺-birnessite, K⁺-birnessite, Zn-
chalcophanite, and Ca₂MnO₆ as evidenced by its EXAFS
spectrum.

Figure 4 demonstrates that the spectrum of Mg-OMS1 is
significantly different from that of SP6-MnO₆. Mg-OMS1 11 is
a synthetic analogue of todorokite 92 with a 3 × 3 tunnel-type
structure and was included in this study because Kim and co-
workers 44,45 recently reported that SP6-MnO₆ closely resembles a
todorokite-like mineral. Kim et al. used UV Raman vibrational
spectroscopy and EXAFS to compare SP6-MnO₆ to several
natural MnO₆ mineral samples. 44,45 The use of natural MnO₆
minerals as XAS reference standards is challenging owing to the
common presence of several phases within one sample. Kim et al. 44 do not provide evidence that mineral samples used as
XAS reference standards were monophasic. In addition, the poor
quality of their EXAFS data did not allow detailed spectral
analysis. We demonstrate that the EXAFS spectra of SP6-MnO₆ and
phase pure, synthetic todorokite (Mg-OMS1) differ, part-
icularly in the region 7.5 Å - 9.5 Å - 1 (Figure 3).

Figure 9 reveals that the peak positions in the XRD pattern of
Mg-OMS1 do not match that of the biogenic oxide, consistent with
different bulk structures. Consequently, we do not believe that
SP6-MnO₆ has a todorokite-like tunnel structure under
environmentally relevant conditions, and the remainder of the
structure determination focuses on comparisons with layered
structures.

![Figure 4. EXAFS comparison of biogenic MnO₆ with abiotic layered and
tunnel-type MnO₆. Mg-OMS1 is a synthetic todorokite with a 3 × 3
tunnel framework structure.](image-url)

A high degree of qualitative similarity was observed in the
EXAFS spectra of biogenic MnO₆, hexagonal birnessites, Zn-
chalcophanite, and Ca₂MnO₆ (Figure 4). Each abiotic reference
MnO₆ example represents a different fraction of cation vacan-
cies, degree of interlayer hydration, or stacking pattern of
adjacent layers as summarized in Table 1. The structures of
Zn-chalcophanite, hexagonal birnessite, and Ca₂MnO₆ are closely
related. Most significant in terms of the short-range structure
are variations in the number of layer cation vacancies between
these three structures. The percentage of cation vacancies varies
from approximately 12% in hexagonal K⁺-birnessite 73 to 14.3% in
ZnMn³O₇·3H₂O 31 to 25% in Ca₂MnO₆. 37 Given the spectral
similarities between these three layered MnO₆ structures, the
layer structure of K⁺-birnessite was chosen as our structural
model (Figure 5) as its complete structure has been determined
recently by EXAFS and XRD. 41,73

Biogenic MnO₆ Curve Fit. The model used to fit the K⁺-
birnessite EXAFS spectrum is illustrated in Figure 5, the best
fit is illustrated in Figure 6, and best fit results are summarized in
Table 2. Best fit values from EXAFS match crystallographic
interatomic distances to within ± 0.02 Å. The model used to fit the
K⁺-birnessite spectrum was validated by successful application
to the EXAFS spectra of nearly isomorphic H⁺-birnessite and
Zn-chalcophanite. The local structure of SP6-MnO₆ was determined by fitting the K⁺-birnessite structure model to the
biogenic MnO₆ EXAFS spectrum (Figure 7; Table 3).

The first Mn−O shell was fit with six oxygen backscatters at
a distances of 1.89 Å (layer Mn⁴⁺−O) and 2.14 Å (interlayer
Mn³⁺−O). The second shell was fit with six Mn−Mn scattering
paths at a distance of 2.85 Å, representative of edge-sharing
MnO₆ octahedra with hexagonal layer symmetry. The coordination
number for nearest-neighbor Mn⁴⁺ cations at 2.85 Å was
multiplied by an occupancy factor ( f occ ) to account for cation
vacancies. The third shell was fit with a Mn−Mn path at 3.45
Å, representative of corner-sharing octahedra, and six Mn−O
paths at a similar distance. The Mn⁴⁺−Mn³⁺ coordination
number was allowed to float, whereas the Mn−O coordination
number was fixed to six. The number of Mn⁴⁺−Mn³⁺ paths
should be equivalent to the number of cation vacancies (1
− f occ ), a relatively small fraction of the total number of layer

cations. Fitting the low-amplitude, broad shell at 3.45 Å with two different scattering atoms at similar distances (six Mn–O and Mn$^{4+}$–Mn$^{3+}$) is difficult and results in a Mn$^{3+}$–Mn$^{4+}$ fit coordination number value of less than one and a Debye–Waller factor ($\sigma^2$) that is an order of magnitude larger than edge-sharing Mn$^{4+}$–Mn$^{4+}$ paths. MS paths centered around 5 Å were best fit by constraining path distances to integral multiples of Mn–Mn single-scattering paths with scattering angles of 180°, indicating linear layers along [100] and [010] crystallographic directions (Figure 5). The MS path coordination numbers were constrained by multiplication of six backscattering atoms with $f_{oc}$ to account for cation vacancies. Best fit results are shown in Figure 6 and summarized in Table 2 and illustrate that physical parameters coincide with the crystallographic interatomic distances.

In hexagonal K$^+$-birnessite, approximately 12% of Mn$^{4+}$ cations are absent within the layers.$^{73,87}$ The absence of layer cations causes an amplitude reduction in the EXAFS spectrum for all Mn–Mn single- and multiple-scattering paths at or integer multiples of distances representative of edge-sharing MnO$_6$ octahedra, at approximately 2.85 Å. Accounting for cation vacancies is accomplished with the use of a few physically realistic approximations, as demonstrated by Ressler et al.$^{71}$ and Webb et al.$^{41}$ The EXAFS equation two multiplicative terms describe the spectral amplitude, the amplitude reduction factor, $S_0^2$, and coordination number, $N$, at distance $R$ from the absorbing atom. $S_0^2$ is the amplitude reduction factor and is a constant characteristic of the absorbing atom. Therefore, cation vacancies will reduce the observed coordination number ($N$) at distance $R$ from the absorbing atom. The cation vacancies in biogenic MnO$_x$ and abiotic model compounds were accounted for by constraining $S_0^2$ to 0.83, $\Delta E_0 = 0$, and coordination numbers ($N$) to their crystallographic values, while allowing a multiplicative fractional occupancy parameter ($f_{oc}$) to vary freely.$^{41}$ Our model was based on the conceptual framework of Ressler et al.$^{71}$ Its importance has been demonstrated elegantly in the identification of the biogenic MnO$_x$ phase formed by the marine Bacillus sp. strain SG-1 by Webb and co-workers.$^{41}$

**Quantitative Analysis of Cation Vacancies by EXAFS.** Quantitative analysis of cation vacancies in poorly crystalline,
Figure 7. Best fit of the SP6-MnOₙ EXAFS spectrum: (a) EXAFS of biogenic MnOₓ (solid line) with best fit overlay (dotted line); (b) Fourier transformed EXAFS spectrum of SP6-MnOₓ (solid line) with best fit overlaid (dotted line).

Table 3. EXAFS Best Fit Results of the SP6-MnOₓ Local Structure

<table>
<thead>
<tr>
<th>R</th>
<th>χ²</th>
<th>focc</th>
<th>shell</th>
<th>CN</th>
<th>distance (Å)</th>
<th>σ² (Å²)</th>
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<tbody>
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<td>0.0156</td>
<td>296.25</td>
<td>1.0 Mn⁴⁺−O</td>
<td>6</td>
<td>1.89 (1)</td>
<td>0.001 (2)</td>
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</tr>
<tr>
<td>1.0</td>
<td>2.14 (3)</td>
<td>0.002 (2)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.88 (3)</td>
<td>2.86 (1)</td>
<td>0.003 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>Mn⁴⁺−Mn⁴⁺ corner</td>
<td>8.6</td>
<td>3.43 (2)</td>
<td>0.016 (6)</td>
<td></td>
<td></td>
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<tr>
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<td>Mn⁴⁺−O</td>
<td>6</td>
<td>3.41 (3)</td>
<td>0.003 (2)</td>
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</tr>
<tr>
<td>1.0</td>
<td>Mn⁴⁺−O</td>
<td>12</td>
<td>4.61 (6)</td>
<td>0.005 (2)</td>
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<td></td>
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<tr>
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<td>Mn⁴⁺−Mn⁴⁺ (MS)</td>
<td>6</td>
<td>5.72 (2)</td>
<td>0.012 (2)</td>
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<td></td>
</tr>
</tbody>
</table>

Figure 8. Quantification of layer cation occupancy (focc). Peak intensities of Mn−Mn single-scattering and Mn−Mn (MS) multiple-scattering paths were normalized by the intensity of the Mn−O peak. The SP6-MnOₙ layer has a Mn⁴⁺ cation occupancy of 0.88 ± 0.04.

layered MnOₓ by EXAFS is feasible and demonstrated below (Figure 8). ZnMn₃O₇·3H₂O and Ca₂MnO₆ contain ordered and stoichiometric quantities of layer cation vacancies and are excellent standards for the quantification of cation vacancies in environmental and nanocrystalline materials.

In accordance with the Beer−Lambert law, absorbance increases (decreases) proportionally with concentration (vacancies). Therefore, it is expected that the RDF peak amplitudes of single- and multiple-scattering paths associated with Mn−Mn edge-sharing octahedra decrease with increasing layer cation vacancies. Theoretical EXAFS calculations made on a K⁺-birnessite model with various numbers of vacancies demonstrate the inverse proportionality between the number of cation vacancies and RDF peak amplitudes (see Figure S5, Supporting Information). This linear relationship between RDF peak amplitude and focc provides a basis for calibration of cation vacancies with the use of well-characterized, layered MnOₓ reference materials. Analysis of cation vacancies based on Fourier transform (FT) moduli is possible only among materials that are structurally similar on the scale probed by EXAFS and assumes that the σ² and S₀² are the same and ΔE₀ = 0. Therefore, changes in the second shell (Mn⁴⁺−Mn⁴⁺) and MS shell RDF peak amplitudes result from changes in the number of atoms that give rise to those peaks. This affords a direct comparison of cation vacancies in layered MnOₓ compounds.

Figure 8 illustrates cation vacancy calibration curves using single- and multiple-scattering FT moduli amplitudes from the EXAFS spectra of ZnMn₃O₇·3H₂O (focc = 0.857) and Ca₂MnO₆ (focc = 0.75). Owing to small variations in Mn−Mn single- (2.85 Å) and multiple-scattering (5.7 Å) peak intensities between the RDF spectra of each reference compound, their intensities were normalized with respect to the intensity of the Mn−O peak (∼1.92 Å) in each spectrum. Normalization of Mn−Mn peaks, by division of the Mn−O peak intensity, affords interspectral comparison. Therefore, the intensity ratios of I_Mn−Mn(2.85 Å)/I_Mn−O and I_MS(5.7 Å)/I_Mn−O are plotted in Figure 8. On the basis of the calibration of cation vacancies in layered MnOₓ, SP6-MnOₙ contains 12 ± 4% layer cation vacancies and is similar to hexagonal birnessite and ZnMn₃O₇·3H₂O (Zn-chalchophanite). The layer structures of c-ordered hexagonal birnessite and Zn-chalchophanite are structurally similar, containing ∼12% and ∼14% layer cation vacancies, respectively. Hydrated interlayer Mn⁴⁺ and metal cations compensate for the negative layer charge in hexagonal birnessites, whereas in ZnMn₃O₇·3H₂O hydrated Zr²⁺ cations compensate for vacant Mn⁴⁺ layer cations. Therefore, on the basis of the number of cation vacancies, the biogenic MnOₓ possesses a structure similar to hexagonal birnessite and Zn-chalchophanite. Because biogenic MnOₓ contains Mn⁴⁺ cations above/below layer cation vacancies, it belongs to the hexagonal birnessite family of compounds.

Similar analyses of EXAFS peak intensities have been demonstrated in site occupancy determination of cations in...
spinel ferrite nanoparticles. The calibration of cation vacancies in layered oxides has never been directly demonstrated previously. The quantification of cation vacancies in noncrystalline materials is extremely desirable as they play an important role in the determination of catalytic and cation-exchange properties.

**Local Structure of Biogenic MnOx.** The best fit of the biogenic MnOx spectrum (Figure 7; Table 3) indicates that its structure consists of symmetrical edge-sharing MnO6 octahedra which form two-dimensional layers that closely resemble hexagonal birnessite. The best fit of the SP6-MnOx EXAFS spectrum converged with goodness-of-fit parameters $R = 0.0156$ and $\chi^2 = 296.25$. Detailed analysis of the EXAFS spectrum indicates that 12 ± 4% of the Mn4+ layer cation sites in SP6-MnOx are vacant, whereas the analysis of the XANES suggests that the average oxidation state of Mn is 3.8 ± 0.3. Therefore, the average chemical formula of SP6-MnOx is $\text{Mn}^{3+}_{0.12}\text{Mn}^{4+}_{0.88}\text{O}_2\text{H}_2\text{O}$, where $\text{Mn}^{4+}$ and $\text{Mn}^{3+}_{0.12}$ represent hydrated interlayer cations and $\text{Mn}^{3+}_{0.12}$ represents the number of cation vacancies within the layer. Adjacent layers in hydrous layered MnOx are typically at least 7 Å apart and cannot be interrogated by EXAFS. Consequently, powder XRD was used to investigate the long-range stacking relationship between adjacent layers.

**Powder XRD: Long-Range, Bulk Structure.** The long-range structure of hexagonal birnessites is delineated according to the degree of ordering along the layer stacking axis (usually the $c$-axis). Hexagonal birnessites are either ordered or disordered along the $c$-axis and are referred to as "c-ordered" or "c-disordered" hexagonal birnessite, respectively. $\delta$-MnO2 represents the extreme case of c-disordering and is essentially single layered with hexagonal symmetry. Polymorphs of hexagonal birnessite are readily distinguishable by the relative intensities of 00$l$ reflections measured by powder X-ray. The relative intensities of 00$l$ reflections vary with the degree of stacking order/disorder along the $c$-axis.

$L. \text{discophora}$ SP6 produces a MnOx that either is composed of single layers similar to $\delta$-MnO2 or has poorly ordered stacking of adjacent layers similar to H$^+$-birnessite. To isolate Bragg diffraction peaks that arise from the biogenic MnOx, XRD patterns of SP6 cells with and without MnOx were collected (see Figure S6, Supporting Information). Scattering from SP6-MnOx gives rise to two asymmetric peaks centered around $d \sim 2.5$ Å and $d \sim 1.45$ Å (Figure 9). A comparison of the powder XRD patterns of Mg-OMS1 with SP6-MnOx clearly demonstrates that their long-range structures are different from that of Mg-OMS1 and confirms local differences seen by EXAFS. The hexagonal birnessite structure was used to index the two asymmetric XRD peaks in SP6-MnOx because their local structures are similar according to EXAFS.

The powder XRD patterns of K$^+$-birnessite and H$^+$-birnessite (Figure 9) are indexed with a hexagonal unit cell ($P 6_3/mmc$, $a = 2.840$ Å, $c = 14.50$ Å). The XRD pattern of K$^+$-birnessite exhibits sharp 00$l$ reflections, indicative of relatively ordered layers. In comparison, the XRD patterns of H$^+$-birnessite and SP6-MnOx demonstrate relatively disordered stacking of adjacent layers, as illustrated by weak or absent 00$l$ reflections.

**Figure 9.** Comparison of biogenic MnOx with model compounds by powder XRD. SP6-MnOx most closely resembles c-disordered hexagonal birnessite, indicating a layered MnOx with poorly crystalline stacking along the $c$-axis. The diffraction patterns of hexagonal birnessites are indexed with a hexagonal cell ($P 6_3/mmc$, $a = 2.840$ Å, $c = 14.50$ Å, $\alpha = 90^\circ \beta = 90^\circ \gamma = 120^\circ$). Note the similarity between the powder XRD patterns of SP6-MnOx and c-disordered hexagonal birnessite.

However, the XRD patterns of H$^+$-birnessite and biogenic MnOx exhibit $h00$ and $hk0$ peaks that arise from layers. The two asymmetric peaks in the powder XRD pattern of SP6-MnOx centered around $d \sim 2.5$ Å and $d \sim 1.45$ Å correspond to the (100) and (110) reflections that result from the $ab$-layer plane. Owing to background scatter from biological and organic media, weak 00$l$ reflections may be hidden, and it is not possible to determine whether biogenic MnOx consists of discrete layers, as in the case of $\delta$-MnO2, or poorly ordered stacking of adjacent layers, as in H$^+$-birnessite.

**Conclusions**

Microorganisms accelerate aqueous Mn$^{2+}$ oxidation up to five orders of magnitude relative to abiotic processes. The mechanism of aqueous Mn$^{2+}$ oxidation and extracellular MnOx formation by microorganisms is not yet understood, although several studies are ongoing. Structure determination of biogenic MnOx is an important step toward a comprehensive understanding of their role in natural systems. In this study, a combination of TEM, XAS, and XRD was used to determine the morphology and local and long-range structure of the biogenic MnOx precipitate formed by the freshwater bacterium $L. \text{discophora}$ SP6. The bacteria form a mixed-valent, layered MnOx with an average chemical formula of $\text{Mn}^{3+}_{0.12}\text{Mn}^{4+}_{1-0.12}\text{O}_2\text{H}_2\text{O}$. This work demonstrates that the biogenic MnOx exhibits a unique structure that is different from model compounds.
MnO$_x$ layer structure is very similar to hexagonal birnessites, Zn-chalcophanite, and Ca$_3$Mn$_3$O$_8$. Nearly identical structures have recently been reported as the oxide products produced by other phylogenetically diverse manganese oxidizing bacteria, such as the spore-forming marine Bacillus sp. strain SG1 and P. putida strain MnB1. The ability of microorganisms to catalytically oxidize Mn$^{2+}$ is linked to the presence of genes that express extracellular multicopper oxidases. The formation of isomorphic MnO$_x$ products formed by otherwise phylogenetically distinct bacteria that live in diverse environments provides important insight into their formation. Specifically, biogenic formation of poorly crystalline, layered manganese oxides likely proceeds without biological templating or direct control by the microorganism.

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Supporting Information Available: The XANES spectrum of SP6-MnO$_x$, average manganese oxidation state calibration curve, X-ray absorption spectra and fits of ZnMn$_3$O$_7$·3H$_2$O and Ca$_3$Mn$_3$O$_8$, EXAFS model validation, theoretical calculation of EXAFS as a function of cation vacancies, and powder XRD patterns of Leptothrix discophora SP6. This material is available free of charge via the Internet at http://pubs.acs.org.